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Differential responses of a mine tailings *Pseudomonas* isolate to cadmium and lead exposures

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Abstract

We examined cadmium and lead resistance in *Pseudomonas* sp. S8A, an isolate obtained from mine tailings-contaminated soil. Resistant to soluble metal concentrations up to 200 mg l^{-1} cadmium and 300 mg l^{-1} lead, S8A produced both exopolymer and biosurfactant. Upon growth, this pseudomonad diverged into two morphologically distinct colony subtypes; small and round or large and flat. In the presence of lead and in the no metal control the large morphotype appeared only in late stationary phase. With cadmium the large morphotype appeared immediately following exposure. Results show that the large morphotype produced greater amounts of surfactant than the small morphotype, suggesting a unique subpopulation response to cadmium toxicity. Results also indicate that an unidentified 28 kDa protein was expressed following exposure to >10 mg l^{-1} cadmium. This study demonstrates new links between surfactant production, differential subpopulation response and metal exposure.

Introduction

Microbial resistance to metals, such as arsenic (As), copper (Cu), cadmium (Cd), mercury (Hg), lead (Pb) and zinc (Zn), is thought to be well understood (Nies 1999, 2003). However, given the metabolic flexibility of microorganisms we are only now recognizing the ability of some microorganisms to use multiple concurrent mechanisms of resistance, which can dramatically influence the outcome of a toxicity response. For example, given a microorganism that produces both exopolymer and biosurfactant (Whyte et al. 1999), it is not yet clear how the increase in metal solubility caused by the surfactant and the decrease in metal solubility due to exopolymer binding each contribute to metal toxicity/resistance. Also at issue is whether, during metal exposure, all the cells within a single population use the same resistance mechanism or whether there can be differential subpopulation responses to the exposure, where physiological variations can occur among cells within a population.

Involvement of subpopulation responses resulting in colony morphology changes are now being recognized in biofilm formation (Monk et al. 2004), phase variation (van den Broek et al. 2005), oil tolerance (Iwabuchi et al. 2000), metabolite production (Monk et al. 2004; Silby et al. 2005), starvation (Finkel et al. 2000; Outten et al. 2000) and antibiotic resistance (Drenkard & Ausubel 2002; Massey et al. 2001). Morphological variations in colony structure can result from changes in cell surface properties (Dybvig 1993; Henderson et al. 1999; Rosengarten & Wise 1990). For example, in oil tolerance, mucoidal smooth colonies of *Rhodococcus rhodochrous* were resistant to 10% (vol/vol) *n*-hexadecane, while rough versions

were sensitive (Iwabuchi et al. 2000). Iwabuchi et al. (2000) also found that 25% of the isolates showed spontaneous rough-smooth colony morphotype switching when grown in the presence of crude oil. The study presented here addresses subpopulation response to metal toxicity.

For cadmium and lead, many highly resistant microorganisms can be isolated from metal-contaminated sites (Mergeay 2000; Roane 1999; Roane & Pepper 2000). In these organisms, mechanisms for cadmium and lead resistance include exopolymer binding (Loaëc et al. 1998; Mohammad 2001; Teitzel & Parsek 2003), intracellular accumulation (Cavet et al. 2003; Daniels et al. 1998; Ybarra & Webb 1999), cadmium efflux via the *cad*, *czc* and *czr* operons (Lee et al. 2001; Legatzki et al. 2003; Nies 2003; Oger et al. 2003; Rensing et al. 1997), and biosurfactant production (Al-Tahhan et al. 2000; Mulligan et al. 2001; Sandrin et al. 2000). In this study we examine a single bacterial isolate from a metal-contaminated soil that displays two morphotypes while growing in the presence of high soluble cadmium and lead concentrations (>100 mg l^{-1}). The objective of this study is to examine the dichotomous growth response of the isolate to high concentrations of cadmium and lead.

Materials and methods

Field site

A metal-contaminated soil was collected from the Silver Valley mining region (ca. 116°07′30″ W, 47°32′30″ N; altitude 878 m) in Northern Idaho. The pH 4.3 sandy loam soil contained 4.9 mg kg^{-1} Cd and 385 mg kg^{-1} Pb as determined by the Sunshine Mining Company, Idaho. Soil texture and pH were determined using protocols from Methods of Soil Analysis (1986). Soil was collected from the top 10 cm of the surface horizon and shipped overnight the same day to the University of Colorado, Denver. Culturable and total counts were immediately performed upon receipt. Soils were stored at 4 °C.

Total bacterial numbers were determined using acridine orange direct counts with epifluorescence microscopy (Hobbie et al. 1977) on diluted soil (between 10 and 100 cells per field) slurries (5 g soil dry weight in 20 ml sterile 0.1% sodium glycerophosphate, $C_3H_7O_6PNa_2$). Conventional plate counts were performed from the soil slurries above onto a minimal salts medium (MSM) for heterotrophic organisms (Roane 1999). The MSM contained the following: 1.0 g glucose ($C_6H_{12}O_6$), 0.1 g magnesium sulfate ($MgSO_4{\cdot}7H_2O$), 1.0 g ammonium sulfate [$(NH_4)_2SO_4$], 0.5 g sodium citrate ($C_6H_5Na_3O_7$), and 0.1 g sodium pyrophosphate [$Na_4P_2O_7(H_2O)_{10}$], buffered to pH 6.0 with 2-[*N*-morpholino]ethanesulfonic acid ($C_6H_{13}NO_4S$). Plates were incubated for 1 week at 28 °C prior to enumeration.

Maximum metal resistance level

Culturable isolates from above were randomly screened for metal resistance by growing individual isolates in MSM broth amended with cadmium chloride ($CdCl_2$) or lead acetate ($Pb(C_2H_3O_2)_2{\cdot}3H_2O$) from 0 to 300 mg l^{-1}. Beyond 300 mg l^{-1}, maintaining metal solubility was a concern. The maximum metal resistance level (MRL) was defined as the highest concentration of cadmium and lead at which at least 10^4 cells ml^{-1} consistently remained viable after 72 h from an initial inoculum of 10^5 cells ml^{-1}. An unusually resistant isolate capable of growth in up to 200 mg l^{-1} soluble Cd and 300 mg l^{-1} soluble Pb was chosen for further study.

Growth in the presence of metal

Cell growth from an initial inoculum of 10^5 CFU ml^{-1} was assessed every 12 h via dilution and plating onto MSM agar. To work with optimally growing cultures, the following metal concentrations were analyzed in triplicate: 0, 20, 40, and 60 mg l^{-1} soluble Cd, and 0, 20, 40, and 60 mg l^{-1} soluble Pb. The 50 ml MSM broth cultures were incubated at 28 °C at 130 rpm for 144 h.

Soluble or aqueous phase cadmium and lead concentrations were measured as a function of time during growth experiments. Samples were periodically removed and soluble metal was determined by centrifugation at $2500 \times g$ for 10 min. Metal remaining in solution (soluble metal) was measured with flame atomic absorption (PerkinElmer 5000, Boston, MA).

Isolate identification

The bacterial isolate used in this study was identified using 16S rRNA gene sequencing using the 63F (5′cag gcc taa cac atg caa gtc 3′) and 1387R (5′ ggg cgg wgt gta caa ggc 3′) 16S primers to yield a 1300 bp product (Lane 1991; Marchesi et al. 1998). The primer concentration used was 40 μM per reaction. The 30 PCR cycles on a PerkinElmer GeneAmp 2400 consisted of 95 °C (1 min.), 55 °C (1 min.), 72 °C (1 min 30 sec), followed by a final extension at 72 °C for 5 min. PCR products were purified using the Promega Wizard PCR Purification Kit (Madison, WI), and sequenced at the University of Colorado Health Sciences Center, Cancer Center DNA Sequencing Core. Sequences were identified using the NCBI GenBank Bacterial Identification Database (www.ncbi.nlm.nih.gov).

Biosurfactant production

Determination of biosurfactant production was done in accordance with Bodour & Miller-Maier (1998). Biosurfactant production was determined by the reduction in surface tension in comparison to an uninoculated control.

Exopolymer production

Exopolymer production was determined using Sudan Black B staining. Metal-exposed 40 h old colonies on tryptic soy agar (Difco) and MSM agar were flooded with Sudan Black for 10 min. Excess stain was removed and the colonies were observed. Black-colored colonies indicating stain uptake reflected no exopolymer production (Liu et al. 1998).

Twitching motility

A modified method of Semmler et al. (1999) was used to identify twitching motility. MSM agar plates amended with 0.05% TTC (triphenyltetrazolium chloride) were stabbed with the isolate and incubated at 30 °C and 37 °C for 18 h. Stabs were visualized using an Olympus Phase Contrast inverted microscope (model (UCWCD 0.30) for halo formation and satellite colonies in the interstitial layer indicative of twitching motility.

Plasmid extraction

The alkaline lysis procedure of Kado & Liu (1981) was used to isolate plasmid DNA. Cultures were grown in the presence and absence of metal to an absorbance of 0.8 at 600 nm, pelleted and resuspended. Following cell lysis, phenol/chloroform extractions were used to purify the DNA. Plasmids were isolated on a 0.7% agarose gel at 120 V for 1 h.

One-dimensional protein analysis

Cells were harvested during exponential and stationary phase growth, concentrated to an OD_{600nm} of 0.1, and lysed at 95 °C for 10 min. Analysis of the protein profiles using 16% SDS-PAGE was used to monitor possible changes in protein expression with metal exposure. Staining with coomassie Blue R-250 allowed gel visualization. Edman degradation and HPLC-Mass Spectroscopy were used for protein sequencing (Beckman Center, Protein and Nucleic Acid Facility, Stanford University). Protein sequences were analyzed using the NCBI database.

Results

Isolation and identification of Pseudomonas *sp. S8A*

Comparison between the total microscopic counts and the total culturable counts from the metal-contaminated soil, $6.3\times10^{11}\pm2.7\times10^{11}$ cells g^{-1} versus $7.6\times10^{5}\pm1.6\times10^{5}$ CFU g^{-1}, respectively, showed a 6-log decrease reflecting metal toxicity. Random colonies were chosen from the culturable count plates for further characterization of cadmium and lead tolerance. While several isolates showed metal tolerance to varying degrees, one isolate in particular was able to grow in the presence of unusually high soluble metal concentrations, up to 200 mg l^{-1} Cd and 300 mg l^{-1} Pb (data not shown). The organism was identified using 16S rRNA sequencing as a *Pseudomonas* sp. (99% similarity) and designated as *Pseudomonas* sp. S8A (GenBank accession no. AY972537). S8A is closely related to *Pseudomonas putida* (98%) and *Pseudomonas fluorescens* (98%).

Effects of metals on the growth of Pseudomonas *sp. S8A*

Growth of *Pseudomonas* sp. S8A in the absence of metal occurred with no measurable lag phase (the first sample point was 12 h), a 1.5-log increase in cell number, and an onset of stationary phase at 36 h corresponding to culturable counts of approximately 1.0×10^8 CFU ml^{-1} (Figure 1). The addition of lead impacted growth in two ways. First, an apparent lag phase of 12 h was observed which increased to 24 h as the amount of lead added was increased to 60 mg l^{-1}. Second, during the lag phase up to a one-log loss of culturable counts was observed indicating some cell mortality due to lead exposure. Despite metal toxicity, the final culturable counts achieved were similar to those measured for growth in the absence of metal ($\sim1.0\times10^8$ CFU ml^{-1}). It is important to note that lead bioavailability did not change significantly during the experiment. In uninoculated controls, greater than 98% of the lead also remained soluble (data not shown).

Similar results were obtained when *Pseudomonas* sp. S8A was grown in the presence of cadmium except the response to cadmium was more pronounced and was not directly correlated to metal concentration (Figure 2). For example, the lag phase increased to 12 h for 20 mg l^{-1} Cd, then peaked at 60 h for 40 mg l^{-1} Cd, and finally decreased to 36 h for 60 mg l^{-1} Cd. This pattern was repeated for cell mortality where a loss of 1.5–3-logs in culturable counts was observed with the greatest consistent loss occurring for 40 mg l^{-1} Cd treatment. Finally, the pattern was also repeated for growth rate, with S8A exhibiting a faster growth

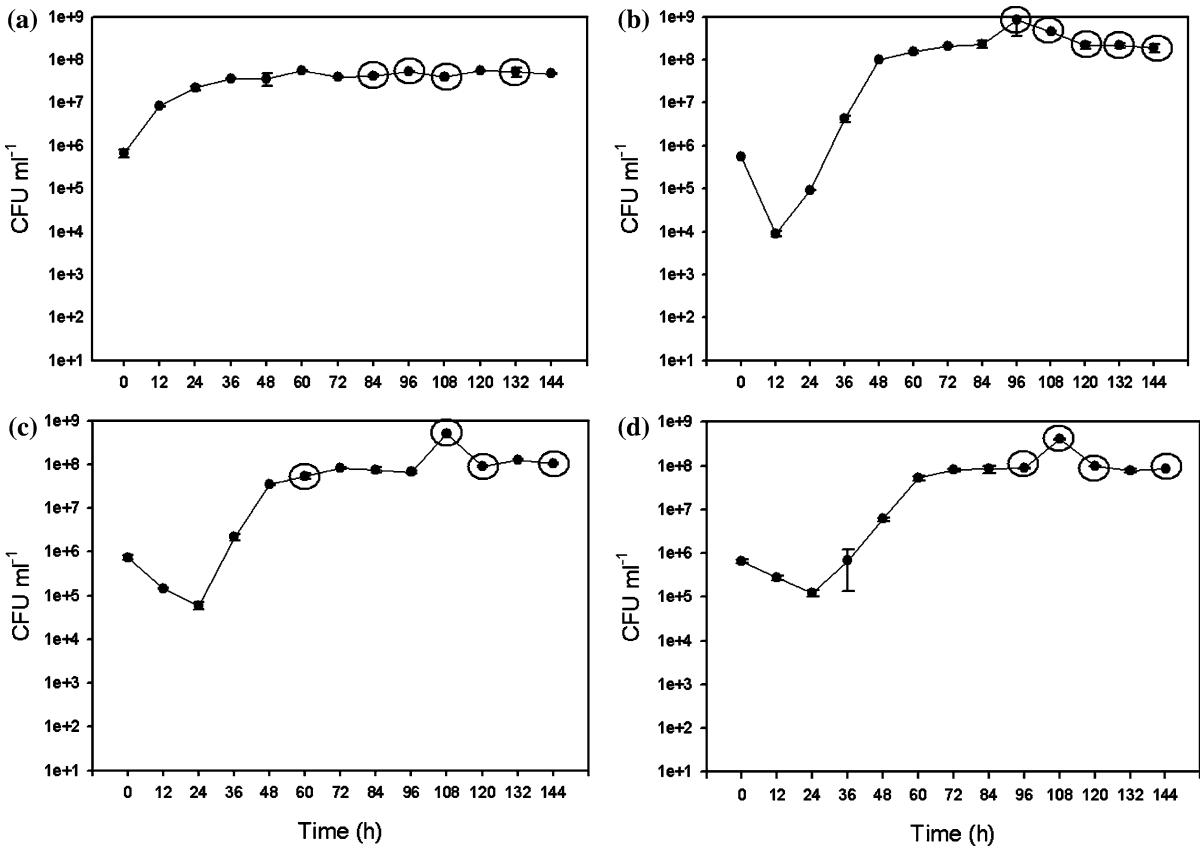

Figure 1. Growth of *Pseudomonas* sp. S8A in the presence of lead. Changes in lead solubility from the initial concentrations of 20, 40 or 60 mg l^{-1} Pb were not observed (data not shown). Line graph represents cell concentration (CFU ml^{-1}). Circled growth points represent the appearance of the large colony morphotype at >50% of the total colonies. The CFU values are means based on triplicate determinations. Error bars represent standard deviations. (a) growth in no metal. (b) growth in 20 mg l^{-1} Pb. (c) growth in 40 mg l^{-1} Pb. (d) growth in 60 mg l^{-1} Pb.

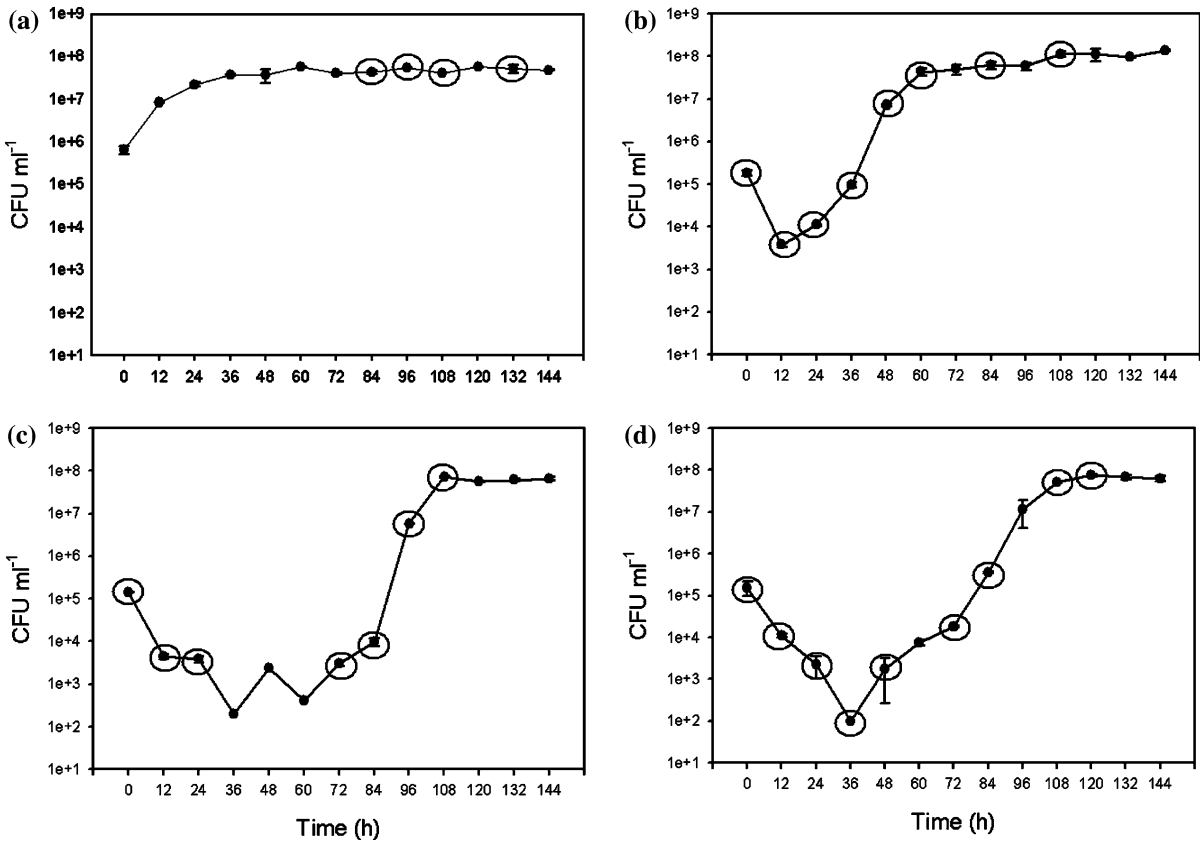

Figure 2. Growth of *Pseudomonas* sp. S8A in the presence of cadmium. Changes in cadmium solubility from the initial concentrations of 20, 40 or 60 mg l^{-1} Cd were not observed (data not shown). Line graph represents cell concentration (CFU ml^{-1}). Circled growth points represent the appearance of the large colony morphotype at >50% of the total colonies. The CFU values are means based on triplicate determinations. Error bars represent standard deviations. (a) growth in no metal. (b) growth in 20 mg l^{-1} Cd. (c) growth in 40 mg l^{-1} Cd. (d) growth in 60 mg l^{-1} Cd.

rate (0.76 generations h^{-1}) at 40 mg l^{-1} Cd than at either 20 or 60 mg l^{-1} (0.52 generations h^{-1} and 0.36 generations h^{-1}, respectively). These results certainly suggest toxicity but again the toxicity is not directly correlated with cadmium concentration. S8A growth did not change the soluble concentration of cadmium when compared to uninoculated controls (data not shown).

Effect of metals on morphotypes expressed by Pseudomonas *sp. S8A*

While conducting the growth experiments in the presence of cadmium and lead, an interesting observation was made. In assessing the cell concentrations, it became apparent that S8A had two different colony morphologies. One colony morphotype was smaller and round with a convex surface, while the second colony morphotype was larger, mucoidal with an irregular flat shape (Figure 3). The small morphotype dominated during growth in the absence of metal or in the presence of lead. In both cases the large morphotype only appeared in stationary phase and primarily in late stationary phase (Figure 1). However, when cadmium was present the large morphotype appeared immediately following exposure to cadmium and then appeared sporadically at all stages of growth thereafter (Figure 2). For either metal, the large morphotype was independent of dilution. It should be noted that when the large morphotype was put into fresh, no metal medium, the smaller, round morphology appeared within 12 h of inoculation, suggesting that the morphological change is under regulatory control.

The two morphotypes were subjected to 16S rRNA sequencing to confirm that they were indeed the same organism. Further examination of

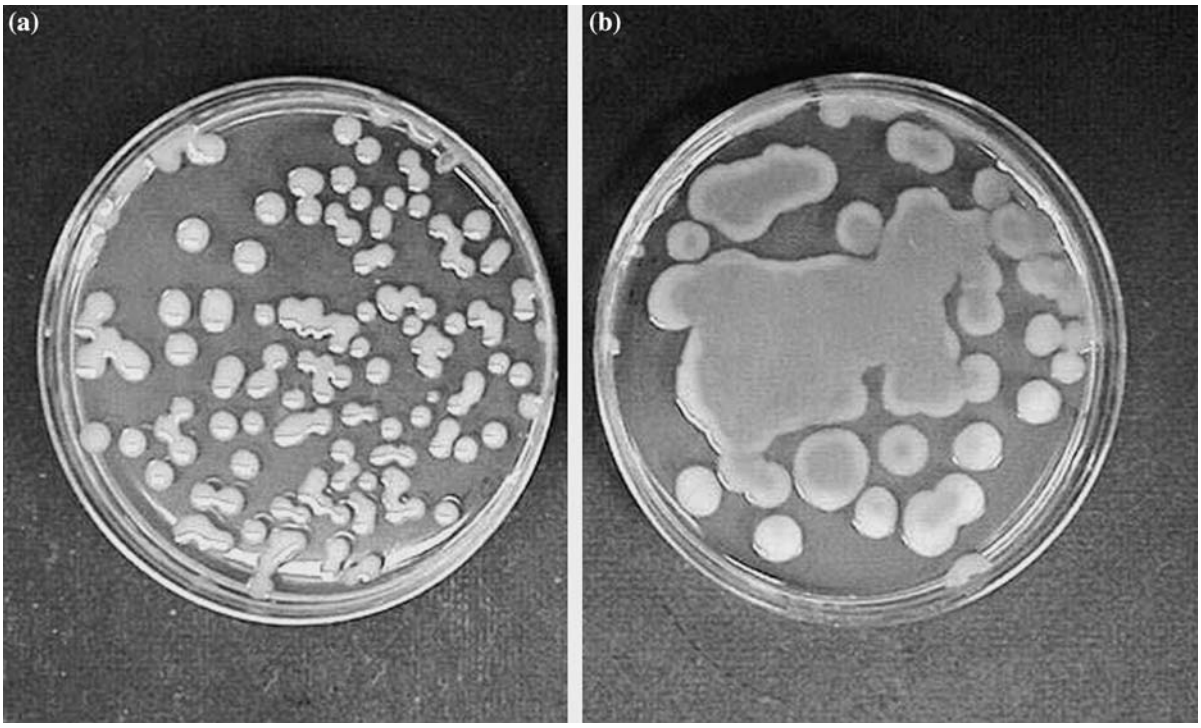

Figure 3. The two colony morphotypes of *Pseudomonas* sp. S8A. (a) normal morphotype: round and convex. (b) second, large morphotype: flat and mucoid.

selected properties of the two colony morphotypes revealed only one obvious difference. Both morphotypes produced an exopolymer, exhibited twitching motility and contained a >23 kb plasmid. However, the large morphotype produced a higher quantity of an unidentified biosurfactant than the small morphotype as measured by the ability of the supernatant to reduce the surface tension of the medium. The large morphotype reduced surface tension of the medium from 68 to 28 dyn cm^{-1}, a relatively high reduction in surface tension, while the small morphotype reduced the surface tension from 68 to 54 dyn cm^{-1}, which is considered a low to moderate reduction (Maier & Soberon-Chavez 2000).

A novel metal resistance mechanism

To further characterize the response of *Pseudomonas* sp. S8A to cadmium, one-dimensional protein expression patterns were examined (data not shown). This analysis revealed a 28 kDa protein expressed in the presence of cadmium (>10 mg l^{-1}) but not in the presence of lead or in the absence of metal. A preliminary partial amino acid sequence suggests that this protein is unrelated to known cadmium resistance proteins and that it contains a hydrophobic motif related to a membrane-associated function. Further studies are being conducted to identify the putative protein and to elucidate its role, if any, in cadmium resistance.

Discussion

This study provides evidence that there is still unexplored diversity in microbial metal resistance. Several highly metal-tolerant bacteria were isolated from severely contaminated soils. An intriguing aspect of this study was that for one isolate, *Pseudomonas* sp. S8A, two different colony morphotypes were observed. The large morphotype appeared only in late stationary phase for untreated or lead exposed cells. But for cadmium, the large morphotype was observed during all stages of growth. A study by Haldeman & Amy (1993) also has shown a link between metal resistance and colony morphology in a study of bacteria from deep subsurface rock samples, supporting the

results reported here. However, it appears that no other studies have established such a relationship.

The relationship between colony morphology and biosurfactant production has been examined previously. A study by van den Broek et al. (2003) showed that colony phase variation in *Pseudomonas* sp. strain PCL1171 reflected changes in biosurfactant activity, secondary metabolite production and exoenzyme expression. Al-Tahhan et al. (2000) report increased cell hydrophobicity due to lipopolysaccharide (LPS) loss in *Pseudomonas aeruginosa* in response to the biosurfactant rhamnolipid, and only small amounts of rhamnolipid were needed to cause a cell surface change (Maier & Soberon-Chavez 2000). *Pseudomonas* sp. S8A is closely related to *P. putida* and *P. fluorescens*, both of which are known to produce biosurfactants. *P. putida* produces an emulsifier (Bonilla et al. 2005) and the surfactant viscosin (Neu & Poralla 1990), while *P. fluorescens* produces a carbohydrate–protein–lipid polymeric surfactant (Desai et al. 1988; Persson et al. 1988). Although a direct link demonstrating biosurfactant production in response to metal exposure has not been found, studies have shown a relationship between surfactants and reduced chemical toxicity (Maslin & Maier 2000; Sandrin et al. 2000). Biosurfactants are known to bind metals thereby reducing their reactivity and toxicity (Miller 1995).

Changes in colony morphology may reflect a microbial strategy for adaptation to changing environmental conditions (Dybvig 1993). Andrews (1995) suggests that cellular altruism may occur to ensure the survival of the population as a whole. In this study, the second, large morphotype appeared immediately following exposure to > 10 mg l^{-1} Cd, which was clearly more stressful than exposure to lead (compare Figures 1 and 2). Thus, it is likely that the large morphotype subpopulation represents a stress response for isolate S8A. The morphotype also appeared both in the presence and absence of lead but only in late stationary phase. Previous work has shown that stationary phase stress can induce colony changes in response to nutrient limitations and metabolite toxicity (Finkel et al. 2000). In some cases, GASP (growth advantage in stationary phase) mutations result in colony morphology changes (Zambrano & Kolter 1996). GASP mutations resulting in increased metabolic efficiencies allow the mutants to out compete their non-GASP counterparts during stationary phase growth (Eberl et al. 1996; Finkel et al. 2000). Whether the different morphology in this study is a GASP mutant is not yet clear. However, when placed into fresh, no-metal medium, the large morphology reverted to the round, normal morphology within 12 h again suggesting that stress conditions are required for maintenance of the large morphotype.

Interestingly, cellular toxicity was not directly correlated with metal concentration as decreased metal resistance was observed at 40 mg l^{-1} Cd versus at 20 or 60 mg l^{-1}. This type of behavior has been previously reported by Sandrin & Maier (2003) and Roane & Pepper (2000). They suggest that a possible mechanism for this inverted response to metal toxicity (i.e., decreased cell toxicity with increase metal exposure) is the activation of additional metal resistance mechanisms at intermediate metal concentrations leading to increased capacity for metal resistance. As metal concentrations are then increased further, metal toxicity builds up again.

In summary, we report that exposure of a single population of cells to cadmium resulted in an inverted growth response, colony morphology changes, differential subpopulation production of surfactant and production of an unidentified 28 kDa protein – a complex array of responses to cadmium toxicity. We hypothesize that exposure to a threshold level of cadmium stress (> 10 mg l^{-1}) causes individual cells of *Pseudomonas* sp. S8A to increase production of surfactant which in turn causes changes in colony morphology. Increased levels of surfactant production may protect cells in the population by complexing cadmium and reducing the bioavailable concentration of cadmium (note that this does not affect the soluble cadmium concentration). Lead, on the other hand, while toxic did not require the early appearance of biosurfactant production or the expression of the unidentified protein. These results (i) confirm that microbial populations can respond differently depending on the metal to which they are exposed and (ii) demonstrate that a single microbial population can have subpopulations that exhibit differential responses to a single metal.

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