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A deep-sea hydrothermal vent isolate, *Pseudomonas aeruginosa* CW961, requires thiosulfate for Cd²⁺ tolerance and precipitation

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Abstract

Pseudomonas aeruginosa CW961, an isolate from the vicinity of a deep-sea hydrothermal vent, grew in the presence of 5 mM Cd²⁺ and removed Cd²⁺ from solution. Sulfate was sufficient for growth when Cd²⁺ was not present in the culture medium; however, thiosulfate was necessary for Cd²⁺ precipitation and cell survival in the presence of Cd²⁺.

Keywords

cadmium sulfide; deep-sea hydrothermal vent; precipitation; thiosulfate

Introduction

Heavy metals, in their aqueous and cationic forms, are extremely toxic pollutants. For a microorganism to be applied to the remediation of heavy metal wastes, it must survive the toxic effects of the metals and remove them from solution. Deep-sea hydrothermal vents lie deep below the ocean and spew waters enriched with metals such as cadmium, zinc, lead, iron, and mercury (Von Damm 1990, Whitfield 1999). These metal enriched waters provide a constant selection pressure making the deep-sea hydrothermal vent environment a likely place to find heavy metal resistant microorganisms (Jeanthon & Prieur 1990).

In an earlier study, we described a bacterium isolated from the waters of a deep-sea hydrothermal vent located in the Juan de Fuca Ridge (Wang *et al.* 1997). This bacterium removed Cd²⁺ under aerobic conditions by precipitating it as CdS. We identified the microorganism as a new strain of *Pseudomonas aeruginosa* and designated it CW961. In this study we further characterize *P. aeruginosa* CW961 and demonstrate that thiosulfate is essential for Cd²⁺ precipitation and resistance.

Materials and methods

Culture conditions

Cultures were grown in a basic salts medium buffered with 40 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5] and that contained 9.5 mM NH₄Cl, 3.2 mM KCl, 50 μM CaCl₂, 50 mM NaCl, 0.01 mM FeSO₄, 2 mM glycerol phosphate (a mixture of glycerol 1- and 2-phosphates), and micronutrients as described by Neidhardt *et al.* (1974). Varying amounts of Na₂SO₄ (0 or 30 mM) and NaS₂O₃ (0–30 mM) were added

as sulfur sources. Glucose (30 mM) served as the carbon source. The medium was adjusted to pH 7.5 with 10 M NaOH and filter sterilized prior to use.

P. aeruginosa CW961 was grown aerobically in 125- or 250-ml culture flasks (with a volume of medium that never exceeded $\frac{1}{10}$ th of the volume of the flask) in a shaker at 200 rpm and 30 °C. Cultures were inoculated using cells in an exponential growth phase at an optical density at 600 nm (OD₆₀₀) of 0.01. For the *E. coli* strain used here, 1 OD₆₀₀ is approximately 0.4 mg dry wt ml⁻¹. Growth of the cultures was monitored by measuring the optical density and by viable cell plating on LB agar (Sigma). The pH of the culture medium during growth was also monitored. Cd²⁺ was added from a 1 M CdCl₂ stock solution as needed.

Cd²⁺ analysis

To analyze the medium for Cd²⁺ in solution, samples of cultures were transferred to microfuge tubes and centrifuged at $> 17\,500 \times g$ for 3 min. Supernatant was drawn, passed through a 0.2 µm syringe filter, diluted $\frac{1}{100}$ in 10% HNO₃, and then analyzed on a Perkin Elmer 3000 Optima inductively coupled plasma spectrometer.

Results

Growth and Cd²⁺ removal in HEPES medium

Growth and Cd²⁺ removal by *P. aeruginosa* CW961 were evaluated using HEPES medium containing 30 mM sulfate and 30 mM thiosulfate. Cultures began growing without a significant lag phase in medium containing 0 and 1 mM Cd²⁺, though with 5 mM Cd²⁺ growth was not observed until one day after inoculation (Figure 1A). Approximate growth rates determined by OD₆₀₀ measurements for cultures containing 0 and 1 mM Cd²⁺ were 0.72 h⁻¹ ($t_{\text{double}} = 0.96$ h) and 0.4 h⁻¹ ($t_{\text{double}} = 1.75$ h), respectively, demonstrating that Cd²⁺ did show growth. The growth rate for cultures in 1 mM Cd²⁺ was also measured by viable cell plating and was 0.38 h⁻¹ ($t_{\text{double}} = 1.85$ h). Because the cell plating and optical density measurements yielded similar results, the effect of Cd²⁺ sulfide precipitation on growth rate calculations from optical density measurements can be considered negligible for this system. The pH of cultures increased to approximately 8.5 for the cultures containing 0 and 1 mM Cd²⁺ and to approximately 7.8 for those containing 5 mM Cd²⁺. Near complete removal of 1 mM Cd²⁺ was observed after 40 h and 92% removal of 5 mM Cd²⁺ was observed after 90 h (Figure 1B). However, the cultures initially containing 5 mM Cd²⁺ still contained 0.37 mM Cd²⁺ after 150 h.

Effect of sulfate and thiosulfate concentrations

Previously we demonstrated that Cd²⁺ is precipitated as Cd²⁺ sulfide by using energy dispersive X-ray spectroscopy, Kliglar's agar plating (Wang *et al.* 1997), and an acid-labile sulfide assay (data not shown). The culture medium originally used to culture the vent microbe contained two components that could be converted to sulfide: thiosulfate and sulfate. To investigate which sulfur source was converted to sulfide, *P. aeruginosa* CW961 cultures were grown for 72 h in medium initially containing 1 mM Cd²⁺, 30 mM sulfate, and 0 to 30 mM thiosulfate. Also, one culture was grown in medium containing 1 mM Cd²⁺, 0 mM sulfate, and 30 mM thiosulfate. Cultures grown with only sulfate did not remove Cd²⁺ from solution, while cultures grown with only thiosulfate removed 86.9% of the initial Cd²⁺. With increasing thiosulfate, the removal efficiency improved, and 99.8% removal was achieved with both 30 mM thiosulfate and 30 mM sulfate in the medium (Figure 2A).

To investigate the effect of the sulfur compounds on Cd^{2+} toxicity, cultures were inoculated at an OD_{600} of 0.01, or approximately 10^7 cells ml^{-1} , in medium containing various amounts of thiosulfate and sulfate and 1 mM Cd^{2+} . Seventy-two hours after inoculation, the number of viable cells ml^{-1} was determined by plating on LB agar medium (Figure 2B). Thiosulfate strongly reduced the toxicity of Cd^{2+} to *P. aeruginosa* CW961. Although *P. aeruginosa* CW961 grew extremely well in Cd^{2+} -free medium with sulfate as the sole sulfur source, when 1 mM Cd^{2+} was added, approximately 98% of the cells died. With 5 mM thiosulfate in the culture medium, there was no change in the viable cell number indicating that cells did not proliferate significantly. Upon addition of 10 mM or greater thiosulfate, cells grew to high densities (4×10^9 cells ml^{-1}).

Discussion

Formulating the HEPES medium

In our first study (Wang *et al.* 1997), a seawater culture medium designed for hydrothermal vent organisms was used. However, as a culture medium for experiments involving Cd^{2+} , it had several disadvantages that could affect the interactions of *P. aeruginosa* CW961 with Cd^{2+} . Because the medium contained only 3.3 mM PIPES and thus was poorly buffered, large fluctuations in pH were possible during growth. Also, the bioavailability of Cd^{2+} could be affected by the presence of chelating agents in the medium. Citrate and yeast extract both have an affinity for Cd^{2+} , and inorganic phosphate spontaneously precipitates Cd^{2+} as Cd^{2+} phosphate.

To circumvent these disadvantages, a defined medium was formulated for this study. The composition of the medium was based on that prescribed by Neidhardt *et al.* (1974). To avoid precipitation of Cd^{2+} phosphate, glycerol phosphate was substituted for inorganic phosphate. To reduce possible chelation by media components, glucose was used as a carbon source, yeast extract was omitted, and unlike the Neidhardt medium tricine was not used as a buffering agent. To avoid large swings in pH, the medium was strongly buffered with 40 mM HEPES. The medium was adjusted to pH 7.5 as preliminary studies (data not shown) indicated that Cd^{2+} precipitation as CdS occurred most efficiently at this initial pH.

Mechanism of sulfide generation

Previously, we showed that *P. aeruginosa* CW961 removed Cd^{2+} by precipitation as Cd^{2+} sulfide (Wang *et al.* 1997). Here, we have shown that cultures grown with only thiosulfate removed a large amount of Cd^{2+} while cultures grown with only sulfate removed little or no Cd^{2+} . Thus, thiosulfate is likely the source of sulfur for sulfide precipitation. Recently, we cloned the genes for thiosulfate reductase and expressed them in *E. coli*; the engineered *E. coli* was able to reduce thiosulfate to sulfide and precipitate heavy metals on the cell wall (Bang *et al.* 2000a, b). However, in this case, complete Cd^{2+} removal occurred only with both sulfate and thiosulfate present in the culture medium and, in this case, sulfate may be preferably metabolized for biomass synthesis while thiosulfate is used for Cd^{2+} sulfide formation.

Does *P. aeruginosa* CW961 convert thiosulfate to sulfide? The organism does act to induce precipitation of Cd^{2+} , because sterile medium containing thiosulfate and Cd^{2+} did not spontaneously form Cd^{2+} sulfide after 5 days (data not shown). *P. aeruginosa* CW961 could possibly convert thiosulfate to sulfide by action of thiosulfate reductase, however Southern blotting with probes based on known thiosulfate reductase sequences did not reveal the presence of a thiosulfate reductase gene (data not shown). While thiosulfate reductase cannot be disqualified as a possible mechanism, it is possible that *P. aeruginosa* CW961

creates a local environment conducive to thiosulfate hydrolysis. Kitaev & Uritskaya (1999) have determined that Cd^{2+} sulfide precipitation,

occurs abiotically at unique conditions with 150 mM thiosulfate.

Cd^{2+} resistance

Is *P. aeruginosa* CW961 uniquely resistant to Cd^{2+} ? In growth experiments, *P. aeruginosa* CW961 grew in the presence of Cd^{2+} only when thiosulfate was present in the culture medium. The removal mechanism, which was found to depend on thiosulfate, could also be the resistance mechanism. That is, by precipitating the Cd^{2+} as Cd^{2+} sulfide the bacterium is able to detoxify its environment and proliferate. In comparison to other studies, *Escherichia coli* growth was inhibited by 30 μM Cd^{2+} (Mitra *et al.* 1975), and other resistant organisms including *Ralstonia* harboring pMOL28 (Nies & Silver 1989) and hydrothermal vent isolates (Jeanthon & Prieur 1990) were capable of growth with approx. 1 mM Cd^{2+} . However, the comparison of reported toxic concentrations is difficult because most studies utilized different culture media for growth. Commonly used media components such as yeast extract or tricine could chelate Cd^{2+} , and components such as phosphate could precipitate Cd^{2+} . These media components reduce the bioavailable concentration of Cd^{2+} and can allow the cultured microorganism to grow in the presence of Cd^{2+} . Although we omitted many of such media components when formulating the culture medium, we do not rule out that thiosulfate interactions could contribute to Cd^{2+} resistance. The thiol group of the thiosulfate anion could chelate aqueous Cd^{2+} cations and reduce the bioavailability of Cd^{2+} . Cd^{2+} forms complexes with thiosulfate that are stronger than those with sulfate or chloride but considerably weaker than that with EDTA (Table 1).

Summary

P. aeruginosa CW961 was able to reduce aqueous Cd^{2+} to extremely low concentrations because it effectively precipitated Cd^{2+} as Cd^{2+} sulfide, which is extremely stable and insoluble. While most bacteria that precipitate metals as metal sulfides are anaerobic, *P. aeruginosa* CW961 is interesting because it was able to precipitate Cd^{2+} under aerobic conditions. To our knowledge this is one of the first reports of a naturally occurring organism capable of reducing thiosulfate to sulfide and precipitating Cd^{2+} as Cd^{2+} sulfide.

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Fig. 1.

Growth (A) and Cd²⁺ removal (B) by *P. aeruginosa* CW961 in HEPES culture medium initially containing 0 (•), 1 (■), and 5 mM (▲) Cd²⁺. Exponentially-growing *P. aeruginosa* CW961 was inoculated into medium (to an OD₆₀₀ of 0.01) and shaken at 200 rpm and 30 °C. Cd²⁺ was added from a 1 M CdCl₂ stock solution as needed. Growth of the cultures was monitored by measuring the optical density and by viable cell plating on LB agar (Sigma). The pH of the culture medium during growth was also monitored. These data were taken from a single experiment for each Cd²⁺ concentration; the growth and Cd²⁺ removal experiments were repeated several times with similar results each time.

Fig. 2.

Effect of sulfate and thiosulfate on Cd^{2+} removal (A) and cell viability (B) in the presence of 1 mM Cd^{2+} . *P. aeruginosa* CW961 cultures were grown for 72 h in medium initially containing 1 mM Cd^{2+} , 30 mM sulfate, and 0 to 30 mM thiosulfate. In addition, one culture was grown in medium containing 1 mM Cd^{2+} , 0 mM sulfate, and 30 mM thiosulfate. Measurements were taken after 72 h. The culture grown with 0 mM sulfate and 30 mM thiosulfate is represented by the lone point ($\diamond$). All other cultures were grown with 30 mM sulfate. Data and error bars represent the mean and standard error of duplicate experiments.

Table 1Stability constants for Cd^{2+} complexes.

Ligand ^a	Log K ₁ at 25 °C ^b
Cl ⁻	1.54
SO ₄ ²⁻	2.29
S ₂ O ₃ ²⁻	3.92
EDTA	16.59

^aData taken from Snell & Ettre (1969).^bK₁ = [CdL]/[Cd²⁺] (Yeh *et al.* 1992).