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Aerobic sulfide production and cadmium precipitation by *Escherichia coli* expressing the *Treponema denticola* cysteine desulfhydrase gene

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Abstract The cysteine desulfhydrase gene of *Treponema denticola* was over-expressed in *Escherichia coli* to produce sulfide under aerobic conditions and to precipitate metal sulfide complexes on the cell wall. When grown in a defined salts medium supplemented with cadmium and cysteine, *E. coli* producing cysteine desulfhydrase secreted sulfide and removed nearly all of the cadmium from solution after 48 h. A control strain produced significantly less sulfide and removed significantly less cadmium. Measurement of acid-labile sulfide and energy dispersive X-ray spectroscopy indicated that cadmium was precipitated as cadmium sulfide. Without supplemental cysteine, both the *E. coli* producing cysteine desulfhydrase and the control *E. coli* demonstrated minimal cadmium removal.

Introduction

The biological precipitation of cadmium as cadmium sulfide has several potential applications. As a remediation strategy, precipitation is extremely effective, because cadmium sulfide is extremely stable and insoluble (solubility product constant, $K_{sp}=1.4\times10^{-28}$). Sulfide-producing organisms may be used to treat cadmium (as well as other heavy metal)-containing waste streams in a reactor (White and Gadd 1996), or the organisms could be utilized at a waste site to detoxify and immobilize heavy metals in situ (Czupyrna et al. 1989). Microbial formation of cadmium sulfide has also been proposed as a means to generate photoactive "quantum particles" for semiconductor applications (Holmes et al. 1997).

Although sulfate-reducing bacteria can produce hydrogen sulfide and precipitate cadmium (White and Gadd 1996, 1998), they generate sulfide only under strict anaerobic conditions and therefore cannot be directly applied to aerobic systems. *Clostridium thermoaceticum*, an anaerobe, can produce sulfides and precipitate cadmium. Its production of sulfides has been linked to the expression of cysteine desulfhydrase, an aminotransferase that converts cysteine into pyruvate, ammonia, and hydrogen sulfide (Cunningham and Lundie 1993). Because cysteine desulfhydrase activity is not restricted to anaerobic conditions, expression of cysteine desulfhydrase in a suitable host could result in aerobic precipitation of cadmium as cadmium sulfide.

Chu et al. (1995) cloned the gene for an especially active cysteine desulfhydrase from *Treponema denticola*, a bacterium isolated from a dental patient. The production of cysteine desulfhydrase by *T. denticola* resulted in hemolysis (i.e., lysing of red blood cells); and *T. denticola* was postulated to be a cause of gingivitis (Chu et al. 1997). In this work, *Escherichia coli* was engineered to over-express the *T. denticola* cysteine desulfhydrase gene. An evaluation of cysteine desulfhydrase production, sulfide generation, and cadmium precipitation under aerobic conditions is presented.

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Materials and methods

Construction of plasmid vectors

Plasmid pMEX7 (Amersham Life Science) was digested with *Bam*HI and end-filled with the Klenow fragment of *E. coli* DNA polymerase 1 (Klenow); and pMMB206 (Morales et al. 1991) was digested with *Ssp*I. The 1.5-kb *Ssp*I fragment from pMMB206 containing the *lacI*^{Q} gene was ligated with the pMEX7 DNA to form pKLJ01. pKLJ01 was digested with *Alw*NI and *Nae*I. pBR322 was digested with *Sty*I, end-filled with Klenow, and then redigested with *Alw*NI. The 1.5-kb pBR322 fragment containing the ColE1 origin of replication and the *ROP* gene was ligated with the 3.7-kb pKLJ01 fragment to form pKLJ07. Plasmid pKG1022 (Gerdes 1988) was digested with *Sal*I and end-filled with Klenow. The 1.6-kb fragment from pKG1022 containing the *hok*, *sok*, and kanamycin resistance genes was ligated with *Msc*I-digested pKLJ07 to form pRock. The cysteine desulfhydrase gene was amplified by polymerase chain reaction (PCR) from pLC67, using an annealing temperature of 48 °C. Primers used were 5′-CTATGCATGCAGGAGGTTTTTATTATGATTTACGATTTTACA-

ACAAAAATTTA-3′ and 5′-ATATTCTAGAAAGCTTGAGGAA-TTTTACCGGCAGTAA-3′. In addition to restriction endonuclease sites, this PCR reaction added a strong consensus ribosomal binding site and an 8-bp spacer (AGGAGGTTTTTATT) to the 5′-end of the cysteine desulfhydrase gene (Gerngross et al. 1994). Both the PCR product and pKLJ07 were digested with *Sph*I and *Hin*dIII. The digested PCR product and 5.2-kb fragment from pKLJ07 were ligated to form pCysDesulf/LacI2. Both pCysDesulf/LacI2 and pRock were digested with *Sph*I and *Nde*I. The 3.6-kb fragment from pCysDesulf/LacI2 was ligated to the 4.6-kb fragment from pRock to form pCysDesulf/LacI2/Rock. *E. coli* DH10B (F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara*,*leu*)7697 *gal*U *gal*K λ⁻ *rps*L *nup*G), purchased from Gibco, was transformed with pCysDesulf/LacI2/Rock and pRock by electroporation.

Culture medium

E. coli cultures were grown in a defined medium buffered with 3-(*N*-morpholino)propanesulfonic acid (MOPS) at pH 7.3. The culture medium was prepared as described by Neidhardt et al. (1974) with the following exceptions: 2 mM glycerol phosphate was substituted for inorganic phosphate to avoid precipitation of cadmium phosphate, and 2 mM K_2SO_4 was used as the sulfate source. In addition, the medium was always supplemented with 17 amino acids (all except tyrosine, methionine, and cysteine) at concentrations recommended by Wanner (1977).

When necessary, 1 mM cysteine was also added. Cysteine was not included in the culture medium when cells were harvested for measurement of cysteine desulfhydrase activity. Also, 30 mM glucose and 100 µg ampicillin/ml were included in the culture medium. Isopropyl β-D-thiogalactoside (IPTG; 100 µM) was added to induce gene expression. $CdCl_2$ was added to a final concentration of 0–0.4 mM.

Growth conditions

E. coli cultures were inoculated from an exponentially growing culture into 5 ml of medium and grown aerobically in culture tubes in an incubating shaker (37 °C, 200 rpm). The optical density of the cultures was monitored at a wavelength of 600 nm (OD_{600}) with a spectrophotometer.

Monitoring plasmid maintenance

E. coli cultures were diluted and plated on LB agar plates with and without 100 µg ampicillin/ml. Cells not viable on ampicillin-containing media were considered to have lost their plasmids.

Preparation of cell lysates

Late-exponential phase cells (5–10 ml; $0.6<OD_{600}<1.2$) were centrifuged at 17,000 *g* for 10 min and then suspended in 60 µl of a 10% sucrose/50 mM Tris solution (pH 7.5). To this suspension, 75 µl of lysis buffer (10% sucrose, 300 mM NaCl, 90 mM EDTA, 3 mg lysozyme/ml, 50 mM Tris-HCL; pH 7.5) were added. Next, the suspension was mixed and incubated on ice for 2 h. The cell suspension was then frozen and thawed five times by cycling between 37 °C incubation in a water bath and freezing in liquid nitrogen. Finally, the suspension was sonicated (40% power) for 5 s, using a Branson Sonifier.

Cysteine desulfhydrase activity assay

Cysteine desulfhydrase activity of the cell lysate was measured using a colorimetric assay adapted from Chu et al. (1997).

Analysis of cadmium removal

To determine the effect of cysteine desulfhydrase on cadmium removal, cultures were inoculated at an OD_{600} of 0.01 into medium containing 0.1–0.4 mM cadmium. Cadmium concentrations were analyzed after 48 h. Cell cultures were centrifuged at 17,000 *g* for 2 min. The culture supernatant was filtered through 0.22-µm pore diameter syringe filters, diluted in 10% HNO_3, and analyzed for cadmium content on a Perkin-Elmer Optima 3000DV inductively coupled plasma spectrometer.

Acid-labile sulfide analysis

The acid-labile sulfide content of the cultures was determined by a colorimetric assay based upon the method of Aiking et al (1982). Because of heterogeneity from flocculation and precipitation, the contents of the culture tube were vigorously vortexed before analysis. Culture samples of 2 ml were centrifuged at 17,000 *g* for 2 min. The supernatant was removed and the cell pellet was resuspended in 1 ml of 0.75 M NaOH. This suspension was transferred to a 2-ml screw-cap microcentrifuge tube and incubated at 95 °C for 15 min. Next, the suspension was vortexed vigorously and 25 µl of the suspension were mixed with 375 µl of 0.75 M NaOH and 250 µl of 2.6% zinc acetate dihydrate. Then, 125 µl of 0.1% *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride in 5 M HCl (freshly prepared) were added and the solution was vortexed until clear. Next, 50 µl of 11.5 mM $FeCl_3$ in 6 M HCl were added and the solution was vortexed and incubated at room temperature for 30 min. Then, 425 µl of deionized water were added and the OD of the sample was recorded at 670 nm. Solutions of 0–0.2 mM sodium sulfide in 0.75 M NaOH served as calibration standards.

Transmission electron microscopy and energy dispersive X-ray spectroscopy

Cell samples were washed in phosphate buffer solution and fixed overnight in a solution of 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2. Samples were then rinsed with 0.1 M sodium cacodylate (pH 7.2), post-fixed in a solution of 0.5% osmium tetroxide in 0.1 M sodium cacodylate (pH 7.2), and rinsed with deionized water. The samples were then dehydrated in a graded acetone series and embedded in Epon-Araldite resin. Samples (40 nm thick) were sectioned using a Reichert Ultracut E microtome and collected on uncoated 300-mesh grids. Samples were analyzed by transmission electron microscopy (TEM) and energy dispersive X-ray spectroscopy (EDXS), using a JEOL 200CX scanning transmission electron microscope and a Kevex 8000 EDX system.

Results

Plasmid stability

In preliminary experiments, the cysteine desulfhydrase gene was expressed using pLC67 (cysteine desulfhydrase gene in pUC19) and pUC19 served as the control. However, when ampicillin was used as the antibiotic resistance marker and the culture medium was amended with cadmium, neither pLC67 nor pUC19 was stably maintained. For example, after 3 days, cells containing pLC67 and pUC19, inoculated initially at an OD_{600} of 0.01 into medium containing 0.3 mM Cd and 1 mM cysteine, produced no colonies when 100 µl of a 10^{-6}-diluted culture were spread on LB agar plates containing 100 µg ampicillin/ml. The same dilutions of identical

cultures of pLC67 and pUC19 produced 2,010 and 480 colonies on LB agar plates without ampicillin.

The *hok* and *sok* genes were inserted into the plasmids to maintain stability. pRock and pCysDesulf/LacI2/Rock both carried the stability genes. To evaluate the insertion of the *hok/sok* genes, cells harboring pCysDesulf/LacI2 or pCysDesulf/LacI2/Rock were inoculated into MOPS medium with no ampicillin at an OD of 10^{-6} and monitored for plasmid maintenance. Plasmid pCysDesulf/LacI2, which did not carry the *hok/sok* genes, was lost after 5 days of culture (approximately 100 generations) while pCysDesulf/LacI2/Rock, which did contain the *hok/sok* genes, was stably maintained after 6 days (approximately 130 generations). Also, both pRock and pCysDesulf/LacI2/Rock were stably maintained in the presence of up to 0.4 mM cadmium over 3 days.

Cysteine desulfhydrase activity

When induced with 100 µM IPTG, the *E. coli* containing pCysDesulf/LacI2/Rock exhibited a cysteine desulfhydrase activity (1.41 µmol S^{2-}/h for 10^9 cells) more than seven times that of *E. coli* containing pRock (0.19 µmol S^{2-}/h for 10^9 cells)). Throughout the remainder of this report, *E. coli* containing pRock and induced with 100 µM IPTG is referred to as the control. *E. coli* containing pCysDesulf/LacI2/Rock and induced with 100 µM IPTG is referred to as *E. coli* producing cysteine desulfhydrase.

Effect of cadmium on cell proliferation

OD measurements after a culture time of 48 h demonstrated the toxic effects of cadmium on cell growth. When *E. coli* was inoculated into medium that did not contain cysteine, both the *E. coli* producing cysteine desulfhydrase and the control failed to grow (OD_{600}<0.10) at cadmium concentrations greater than 0.1 mM. When *E. coli* was inoculated into medium containing 1 mM cysteine, both the *E. coli* producing cysteine desulfhydrase and the control showed significant growth. All cultures, except the control with 0.4 mM cadmium, grew to an OD_{600} of at least 1.0.

Cadmium removal

When 1 mM cysteine was supplemented to the culture medium, the *E. coli* producing cysteine desulfhydrase consistently removed more cadmium from solution than the control (Fig. 1A). *E. coli* producing cysteine desulfhydrase removed 0.4 mM cadmium almost completely from solution (99% removal), whereas the corresponding control removed only 35%. Although the control removed considerable amounts of cadmium from solutions containing low initial cadmium concentrations, only *E. coli* producing cysteine desulfhydrase was able to reduce

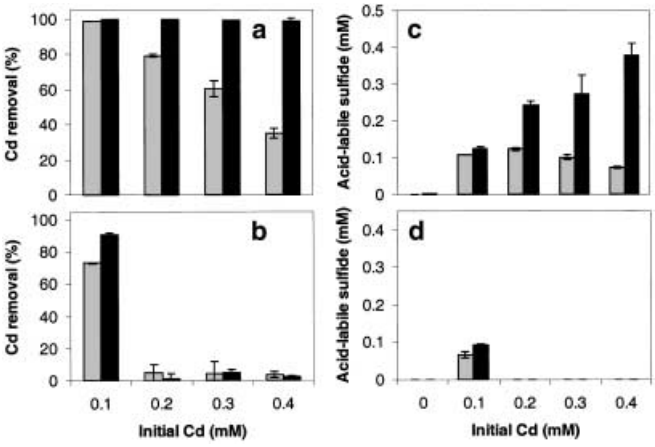

Fig. 1 Cadmium removal (**a**, **b**) and acid-labile sulfide production (**c**, **d**) by *Escherichia coli* producing cysteine desulfhydrase (*black*) and the control (*gray*) after 48 h. **a** and **c** 1 mM cysteine supplemented. **b** and **d** No cysteine supplemented. Data are the mean of duplicate experiments. *Error bars* represent the standard deviation

0.1 mM and 0.2 mM cadmium to undetectable concentrations and 0.3 mM and 0.4 mM cadmium to very low concentrations.

When cysteine was not added to the culture medium, neither *E. coli* producing cysteine desulfhydrase nor the control removed cadmium from solution when initial cadmium concentrations were greater than 0.1 mM (Fig. 1b). These non-removing cultures also failed to reach a significant cell density. With 0.1 mM cadmium and no cysteine in the growth medium, *E. coli* producing cysteine desulfhydrase and the control removed 90.9% and 73.2% of the cadmium, respectively.

Sulfide production

The acid-labile sulfide content of the cultures was analyzed after 48 h. The experimental results demonstrated a correlation between sulfide production and cadmium removal. In addition to most effectively removing cadmium, the *E. coli* producing cysteine desulfhydrase in the presence of supplemented cysteine also produced the greatest amount of sulfide (Fig. 2c). However, when cysteine was not present, cultures produced much less acid-labile sulfide (Fig. 2d).

In general, cultures removing greater than 90% of the cadmium from solution accumulated sulfide nearly equimolar to the cadmium removed. For example, *E. coli* producing cysteine desulfhydrase in the presence of cysteine removed 0.1, 0.2, 0.3, and 0.4 mM cadmium almost completely from solution. The respective sulfide concentrations measured were 0.125, 0.243, 0.273, and 0.379 mM. Because of the 1:1 stoichiometry of cadmium and sulfur in cadmium sulfide, these results strongly indicate precipitation of cadmium as cadmium sulfide. In cultures removing less than 90% of cadmium from solu-

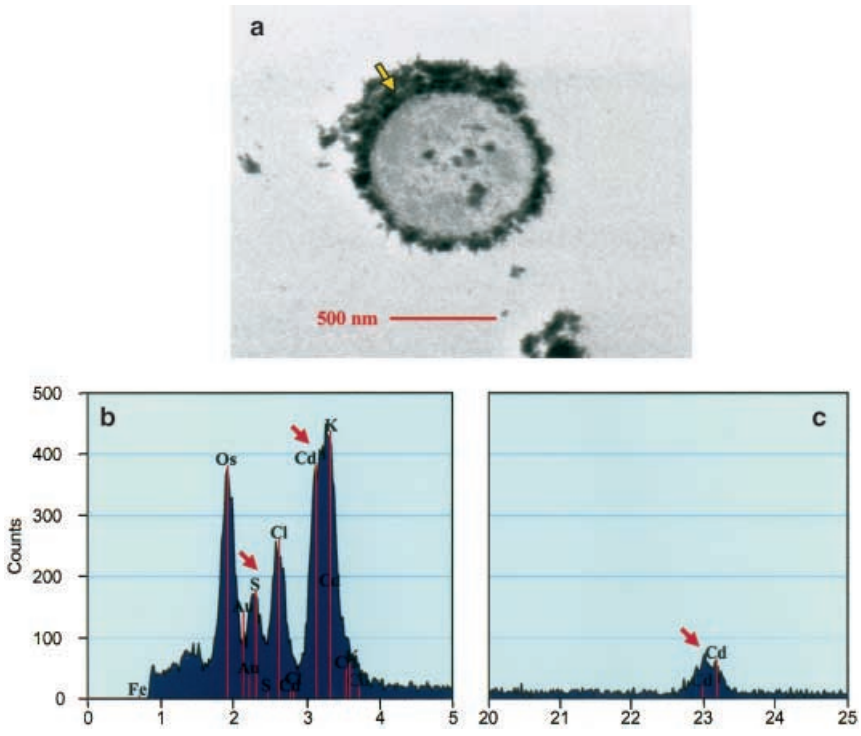

Fig. 2a–c Electron microscopy and energy dispersive X-ray spectroscopy (EDXS) of *E. coli* producing cysteine desulhfydrase. Electron microscopy (**a**) showed precipitation on the cell wall. Elemental analysis by EDXS of the precipitate (*arrow* in **a**) revealed that the precipitation was an accumulation of both sulfur and cadmium (**b**, **c**). A potassium peak (*K*) interfered with the cadmium spectrum (*Cd*) at ~3 keV, although a strong independent cadmium peak was observed at ~23 keV. The osmium peak (*Os*) was due to osmium in the sample fixative

tion, the molar ratio of sulfide produced to cadmium removed was lower than one; and in cultures that failed to remove more than 5% of the initial cadmium from solution, no sulfide was measured. When cadmium was not added to the culture medium, little or no sulfide was detected. Production of sulfide was accompanied by precipitation of a finely dispersed, yellow solid. This yellow precipitate is characteristic of cadmium sulfide formation.

Electron microscopy and EDXS

The *E. coli* culture that produced cysteine desulfhydrase and precipitated 0.2 mM cadmium from solution when 1 mM cysteine was added was analyzed by TEM and EDXS. TEM revealed dense granules on the cell wall and EDXS revealed that these granules were an accumulation of both cadmium and sulfur (Fig. 2), indicating that the cadmium precipitated on the cell wall as cadmium sulfide.

Discussion

Plasmid stability

The instability of plasmids pUC19 and pLC67 could have resulted from a combination of two factors. First, the addition of cadmium to the cultures caused a prolonged lag phase (~1–2 days). Second, during this long lag phase the ampicillin could have become inactivated, allowing plasmid-free *E. coli* to overtake the culture. This inactivation could have been induced by cadmium, which has been shown to catalyze the degradation of β-lactam antibiotics such as ampicillin (Navarro et al. 1998). Furthermore, the metabolic burden of expressing a protein such as cysteine desulfhydrase may select for plasmid loss.

While the plasmids could possibly have been maintained by using a different antibiotic selection, this strategy was not pursued. Instead, plasmids were constructed with the *hok/sok* stability cassette from the *parB* locus of plasmid R1. This system utilizes a suicide gene with an extraordinarily stable mRNA and an unstable antisense RNA to enforce plasmid maintenance (Gerdes 1988).

Toxicity of cadmium

Adding cysteine to the culture medium reduced the toxicity of cadmium to *E. coli*. In medium without cysteine, *E. coli* producing cysteine desulfhydrase and the control both ceased to grow at cadmium concentrations of 0.2 mM and greater. One should note that the inhibition of growth at these high concentrations is one of the primary reasons that very little cadmium was removed from solution. In medium with 1 mM cysteine, both *E. coli* strains grew to a high density, even in the presence of 0.4 mM Cd, indicating that cysteine may be partially responsible for reducing Cd toxicity. This reduction in toxicity may be due to cadmium chelation by the cysteine thiol group, thus reducing the bioavailability of cadmium.

With 0.4 mM cadmium and 1 mM cysteine added to the culture medium, *E. coli* producing cysteine desulfhydrase appeared to have a higher resistance than the control, based on cell density. However, precipitation of cadmium sulfide and subsequent aggregation of cells made OD measurements less reliable. Higher cadmium concentrations could not be reliably tested, because cadmium concentrations greater than 0.4 mM resulted in a small degree of abiotic precipitation in the culture medium.

Cadmium removal and precipitation as cadmium sulfide

The measurement of acid-labile sulfide represents the sulfide present as cadmium sulfide for three reasons. First, because free sulfide ions and hydrogen sulfide readily react with oxygen to form sulfite and sulfate, the production of free sulfide ions and hydrogen sulfide cannot be detected with the acid-labile sulfide assay. Second, because metal sulfides are stable in the presence of oxygen (over the period of the experiment), the sulfide concentration measured in the assay primarily represents the concentration of metal sulfides in the culture. Third, because the concentration of cadmium added to the culture medium was much greater than other medium components that could precipitate with sulfide, the sulfide measured was primarily present as cadmium sulfide in the culture. However, it should be noted that because precipitation causes aggregation of cells and therefore introduces some heterogeneity to the cultures, some experimental error could be due to imperfect sampling of the cultures.

Clearly, *E. coli* producing cysteine desulfhydrase in the presence of cysteine consistently removed more cadmium than the control. The near equimolar ratio of sulfide to removed cadmium and EDXS analysis indicated that cadmium removal was due almost completely to precipitation as cadmium sulfide. In some cases, the control *E. coli* was able to produce sulfides and partially remove cadmium from solution. Sulfide production from cysteine by the control could be due to native tryptophan aminotransferase, an enzyme with some cysteine desulfhydrase activity (McFall and Newman 1996). Sub-equimolar ratios of sulfide to cadmium removed in some control cultures indicated that cadmium was removed in ways other than sulfide precipitation. This removal might be attributed to biosorption or precipitation as carbonate or other complexes. When varying concentrations of glycerol phosphate were added to the culture medium, cadmium removal did not change (data not shown), suggesting that precipitation as cadmium phosphate did not contribute to cadmium removal.

This study has three notable conclusions. First, cysteine desulfhydrase is functional in vivo in the presence of high concentrations of cadmium outside the cell. Second, production of cysteine desulfhydrase in the presence of cysteine results in near complete precipitation of aqueous cadmium (up to concentrations of 0.4 mM) as cadmium sulfide. Third, while cysteine desulfhydrase has been previously associated with cadmium precipitation in a wild-type microorganism (e.g., *Clostridium thermoaceticum*), we demonstrated that an engineered microorganism could produce cysteine desulfhydrase and sulfide under aerobic conditions and use the sulfide to precipitate cadmium sulfide.

Applications utilizing cysteine desulfhydrase would require a source of cysteine. However, if a microorganism that over-produces cysteine can be engineered or isolated, cysteine produced by the organism could be converted to sulfide with cysteine desulfhydrase (Wang et al. 2000). The sulfide produced, without addition of cysteine, could be used to precipitate cadmium or other heavy metals from solution.

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