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Precipitation of Cadmium by *Clostridium thermoaceticum*

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Cadmium at an initial concentration of 1 mM was completely precipitated by cultures of *Clostridium thermoaceticum* in complex medium. The precipitation was energy dependent and required cysteine, although cysteine alone did not act as a growth substrate. Electron microscopic analysis revealed localized areas of precipitation at the surfaces of nonstarved cells as well as precipitate in the surrounding medium. The addition of cadmium had no apparent effect on growth or acetogenesis. However, nickel and cadmium were synergistically toxic at a concentration (1 mM) at which neither alone was toxic. The amount of protein extracted from cadmium-treated cultures was twofold higher than that in control extracts, and the amount of total sulfide was fourfold higher in cultures containing cadmium than in control cultures. Comparable levels of cysteine desulfhydrase activity were observed in extracts of both cadmium-treated and control cultures, but the enzyme activity was expressed maximally about 24 h earlier in the cadmium-treated cultures than in the untreated controls.

As a result of industrial release into the environment, cadmium ranks as a major anthropogenic pollutant. Concentrations of particulate cadmium in rivers and estuaries have been reported to be as high as 1,000 mg/liter (approximately 9 mmol/liter), and those in sediments have been reported to be as high as 50,000 ppm (approximately 445 mmol/kg) (9). Additionally, cadmium is more mobile than other heavy metals in many soils because the affinity of soil components for cadmium is lower. No biological function for the metal, which is transported into bacteria by energy-dependent manganese (33, 47) or magnesium (31) transport systems, is known. The toxic effects of the metal, usually due to a high affinity for sulfhydryl groups (39), include inhibition of bacterial respiration (47), proton-solute cotransport (49), cell growth (48), and phosphate transport (50). Because of the long-term toxicity of cadmium in humans and other organisms, the study of the resistance mechanisms of microorganisms to cadmium and the role they may play in removal of the metal from contaminated waters is important.

Microbial populations exposed to toxic concentrations of cadmium and heavy metals have developed four main tolerance mechanisms (41). The first involves the alteration of membrane transport systems involved in initial accumulation, thus denying or reducing entry of cadmium. This system has been demonstrated for *Bacillus subtilis* 168 (22). The second mechanism is intracellular or extracellular sequestration by specific binding to a biopolymer, usually the cell wall (3, 28). Tolerance to heavy metals can also be achieved by extracellular precipitation with microbially produced oxalate, phosphate, or sulfide (1, 2, 26, 51) or extracellular polymers as reported for cadmium-resistant strains of an *Arthrobacter* sp. (21) and *Vibrio alginolyticus* (12) in response to copper. Intracellular metallothioneins have been reported for *Pseudomonas putida* (13) and *Synechococcus* sp. (32). The *cadB* gene product of *Staphylococcus aureus* may be an inducible and/or amplifiable cadmium-binding protein (33), and an inducible zinc-binding protein was recently isolated from *Alcaligenes eutrophus* CH34 (37). The third mechanism is that of energy-dependent ion efflux. Two plasmid-determined mechanisms for cadmium efflux have been intensely studied: the *cadCA* system of *S. aureus* and the *czcCBAD* system of *A. eutrophus* (30). Plasmid-determined cadmium efflux in *P. putida* GAM-1 has also been demonstrated (16). The fourth mechanism, that of detoxification by enzymatic oxidation or reduction of the ion to a less toxic form or covalent modification, is not feasible for cadmium. Enzymatic reduction is not energetically favorable (30) and would result in a reduced state, which is not volatile; hence, cadmium would not be removed from the microbial interior. Biomethylation or other covalent modifications would result in organocadmium compounds that would be unstable, mutagenic, and/or more toxic than the divalent cation (30).

Clostridium thermoaceticum has been studied extensively with respect to its acetogenic and autotrophic capabilities (35). Of the few studies with essential or nonessential heavy metals that have been reported, the most recent is the elucidation of the nickel transport system of the organism (25). Little is known of other aspects of this acetogen, especially including mechanisms for tolerating potentially toxic concentrations of heavy metals in the environment. The purpose of this study was to evaluate the tolerance of *C. thermoaceticum* to cadmium and to study the mechanism employed by the organism.

MATERIALS AND METHODS

Cultures. *C. thermoaceticum* ATCC 39073 was a gift from Harold Drake (University of Bayreuth, Bayreuth, Germany) and was cultivated at 52°C in modified complex medium (14) containing 0.5 g each of yeast extract and tryptone per liter. For energy sources, 10 mM glucose, 50 mM methanol, and 10 mM sodium syringate were used. $NaHCO_3$ and KH_2PO_4 concentrations were decreased to 2.5 and 0.5 g/liter, respectively, and $CaCl_2$ was not used, except in maintenance cultures. When stated, KH_2PO_4 was replaced by 2 mM glycerol-2-phosphate as the source of phosphorus.

Cadmium precipitation. Late-exponential- to early-stationary-phase cells were used as inocula for the precipitation experiments. The samples were removed from the following sets of flasks: (i) cultures containing glucose as the carbon

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and energy source plus or without 1 mM $CdCl_2$ (experimental cells); (ii) cultures without glucose plus or without 1 mM $CdCl_2$ (starved-cell controls); and (iii) uninoculated medium containing glucose plus or without 1 mM $CdCl_2$ (medium controls). During experiments in which cadmium precipitation by *C. thermoaceticum* was studied, cadmium chloride was added 12 h postinoculation to actively growing cultures and controls (determined by an increase in the optical density of the cultures) to an initial medium concentration of 1 mM. Samples of 4 ml were withdrawn every 12 to 24 h and centrifuged in 13-ml polycarbonate Autoclear centrifuge tubes for 10 min in a IEC Clinical centrifuge (International Equipment Company, Needham Heights, Mass.) at speed setting 6. The supernatant fluid was frozen after the addition of 80 μl of 1 M NaOH for later use in acetate and cadmium analyses. The cell pellets were washed once with wash buffer (100 mM Tris-HCl [pH 7.2], 10 mM tetrasodium EDTA, 400 mM NaCl) and frozen until protein was extracted.

Effect of cadmium on growth and acetogenesis. *C. thermoaceticum* was tested for the effect of cadmium concentration on growth of the organism (measured as acetate production). The organism was cultivated in the culture medium containing 0.1% cysteine hydrochloride and 2 mM glycerol-2-phosphate instead of potassium phosphate to reduce the formation of cadmium phosphate precipitates. $CdCl_2$ was added to the reduced medium to a final concentration of 1, 2, or 4 mM immediately before inoculation. Samples of 1 ml were withdrawn and filtered through a Metricel GN-6 filter (0.45-μm pore size, 25-mm diameter; Gelman Sciences, Inc., Ann Arbor, Mich.). NaOH was added to a final concentration of 100 μM, and the filtered medium was frozen at −20°C until it was analyzed for acetate concentration. Controls were cultures without added cadmium.

Mass balance. To determine how much of the cadmium was precipitated in an EDTA-insoluble form, samples of cultures plus or without cadmium were obtained by centrifugation as described above, washed once with 1 ml of wash buffer, and frozen. The supernatant medium and the pellets containing cells and precipitates were analyzed for cadmium content. The supernatant samples, to which 100 μM NaOH (final concentration) was added, were stored at −20°C until atomic absorption analysis.

Protein extraction. Since the precipitate interfered with optical density measurements as a means of monitoring growth, the increase in protein concentration in cultures was followed. Protein was extracted from the washed cell pellets by the method of Bhaduri and Demchick (4) with acetone and sodium dodecyl sulfate (SDS) plus 0.1 mM phenylmethylsulfonyl fluoride. Protein concentration was estimated by the bicinchoninic acid procedure of Smith et al. (42). This protein assay is sensitive to reductants and free sulfide, and it is possible that residual sulfide or the cadmium sulfide precipitate would interfere with the protein determination. To account for any cadmium sulfide-induced changes in apparent protein concentration, cadmium was precipitated from uninoculated medium with 5 mM Na_2S, pelleted by centrifugation, washed, and extracted for protein in the same manner as that used for cell pellets. The increase in A_{562} due to reaction of residual sulfide or CdS with the copper reagent accounted for less than 1% of the maximum A_{562} observed with the cadmium-treated cell extracts, indicating that the conditions used to extract the protein from the cells did not significantly affect the protein estimations.

Total sulfide production. To determine the total amount of sulfide produced during growth of *C. thermoaceticum*, zinc acetate was added to cultures plus or without cadmium to a final concentration of 124 mM after an average 168 h (7 days) of cultivation. The zinc-treated cultures were shaken for 2 h to convert free sulfide to the zinc sulfide precipitate. The controls included uninoculated medium and starved cultures treated in the same manner as the experimental cultures. Samples of 1 ml were removed from the suspensions and centrifuged in an Eppendorf model 5415 microcentrifuge (Eppendorf Gerätebau Netheler + Hinz GmbH, Hamburg, Germany) for 10 min at 14,000 rpm. The supernatant fluid was removed, and the pellet was resuspended in double-distilled water. The suspension was added to a Balch tube (Bellco Biotechnology, Vineland, N.J.) with a headspace containing 100% N_2; the tube was sealed with a black butyl rubber stopper, which was connected to a second tube containing a trap solution consisting of 10 ml of 0.23 M zinc acetate. The Balch tubes were connected to each other and to a N_2 source by polypropylene tubing and Venoject sampling needles (Terumo Medical Corp., Elkton, Md.) inserted through the serum stoppers. The tubing also extended down into the sample suspension or trap solution to allow them to be constantly sparged with 100% N_2. Injection of 4 ml of 9 M H_2SO_4 followed by incubation in boiling water for 30 min released the sulfide from the zinc sulfide precipitate. The reaction mixture was constantly sparged with N_2 to drive the evolved hydrogen sulfide into the trap solution. The total concentration of sulfide in the medium was calculated after the sulfide contents of the trap solutions for experimental and control conditions were determined.

Cysteine desulfhydrase activity. Cysteine desulfhydrase-catalyzed reactions were incubated at 52°C in serum-stoppered test tubes (11 by 100 mm) under 100% N_2. The reaction mixture contained 100 mM Tris-HCl (pH 8.6) 2 mM cysteine hydrochloride monohydrate, and 0.005 mM pyridoxal 5-phosphate. The reaction was initiated by injection of 10 μl of cell extract and was terminated after 15 min by addition of 0.5 ml of 1.5 M H_2SO_4. The assays were developed colorimetrically at 540 nm by following the development of the 2,4-dinitrophenylhydrazone derivative of pyruvate by the method of Friedemann and Haugen (10) as modified by Kredich et al. (20) in the presence of 10 mM $HgCl_2$.

The effect of cadmium on the expression of cysteine desulfhydrase activity in *C. thermoaceticum* during batch growth was monitored. Cadmium was added to the medium of treated cultures after the addition of cysteine but before inoculation. Cell extracts were prepared anaerobically in a Coy anaerobic chamber (Coy, Ann Arbor, Mich.). Aliquots of 10 ml were removed at approximately 24-h intervals. The cells were pelleted by centrifugation in a Sorvall RC2B centrifuge with an SS34 rotor for 15 min at 29,000 × *g* and room temperature in sealed glass centrifuge tubes. The pellets were lysed with buffered lysozyme as previously described (24). The debris was removed by centrifugation in the Eppendorf microcentrifuge. The supernatant fluid, designated the cell extract, was stored on ice under 100% N_2 until it was assayed for cysteine desulfhydrase activity. Protein concentration was estimated by the method of Bradford (5) with bovine serum albumin as the standard.

Analytical methods. Acetate concentration was estimated by utilizing an adaptation of the hydroxamate method of Lippmann and Tuttle (23) and Rose et al. (38). Sodium acetate was used as the standard. Total sulfide was assayed as methylene blue by an adaptation of the method of Kirsten (18). The assay was performed with 1 ml of the trap solution (described above), which was diluted to 3 ml with additional

TABLE 1. Mass balance of cadmium precipitate

Cells	[Cysteine] (mM)	Δ[cadmium][a] ± SE[b] (mM)		
		Medium	Pellet	EDTA-soluble fraction[c]
Nonstarved	2.85	0.84 ± 0.09	0.72 ± 0.05	0.12 ± 0.13
Starved	2.85	0.41 ± 0.09	0.10 ± 0.03	0.31 ± 0.12
Nonstarved	5.70	0.80 ± 0.08	0.75 ± 0.05	0.06 ± 0.13
Starved	5.70	0.59 ± 0.00	0.17 ± 0.08	0.42 ± 0.08
Nonstarved	8.55	0.77 ± 0.06	0.76 ± 0.07	0.01 ± 0.13
Starved	8.55	0.74 ± 0.03	0.25 ± 0.13	0.48 ± 0.17

[a] Net removal from the medium or appearance in the pellet after washing.
[b] Standard errors of six cultures.
[c] Cadmium lost from the pellet during washing with 10 mM EDTA.

zinc acetate trap solution. To this was added 0.5 ml of 19.92 mM *N,N*-diethyl-*p*-phenylenediamine sulfate in 1.8 M sulfuric acid and then 0.25 ml of 74.7 mM $FeNH_4(SO_4)_2 \cdot 12H_2O$. The solution was mixed and allowed to develop in the dark for 30 min, and then 1 ml of 0.4% SDS–0.31% tetrasodium EDTA was added. The A_{670} was read, and the concentration of sulfide in the zinc acetate trap solution was calculated with reference to sodium sulfide standards.

Atomic absorption spectroscopy. The supernatant growth medium saved for cadmium determination was treated by the addition of 100 μl of concentrated nitric acid and overnight incubation to hydrolyze cellular constituents. The resultant liquid was diluted in 10 ml of double-distilled water. The cadmium concentration in the fluid was determined by atomic absorption spectroscopy on a model 451 aa/ae spectrophotometer (Instrumentation Laboratory, Inc., Lexington, Mass.) with a cadmium lamp. Dilutions of cadmium chloride in growth medium treated like the experimental samples were used as standards.

Electron microscopy. Electron microscopy was performed by the Electron Microscope Laboratory at New Mexico State University. Pellets of 90-h cultures were fixed in 2.5% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 5.5) for 2 h at room temperature and postfixed in 1% osmium tetroxide overnight. All samples were dehydrated in a graded ethanol series and embedded in Spurr's low-viscosity resin. Ultrathin sections were cut with an LKB Ultratome III microtome. Stained and unstained samples were examined in an Hitachi 7000 transmission electron microscope. The cultures analyzed were a control culture (without cadmium), a starved culture plus 1 mM cadmium, and a nonstarved culture plus 1 mM cadmium.

RESULTS

Precipitation of cadmium. Within 24 to 48 h after cadmium was added to actively growing cultures of *C. thermoaceticum* containing 0.05% cysteine, a fine white precipitate, presumably $CdCO_3$ formed from CO_2 released during growth, appeared in the growth medium. Approximately 12 to 24 h after the precipitate formed, it was gradually converted to a bright yellow form that was physically identical to cadmium sulfide (27). Addition of concentrated nitric acid to the precipitate released hydrogen sulfide gas, indicating that cadmium sulfide was being precipitated by the organism. Mass balance experiments (Table 1) show that of the cadmium removed from the medium of starved cultures, 50 to 75% was lost after the pellet was washed with EDTA,

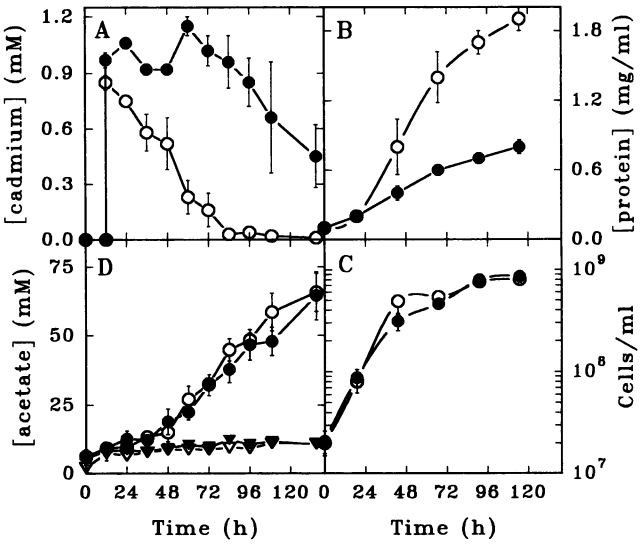

FIG. 1. Effect of cadmium on *C. thermoaceticum*. (A) Cadmium precipitation by *C. thermoaceticum*. Cadmium chloride was added at 12 h. Cultures were grown in medium containing 1 mM $CdCl_2$ and 20 mM glucose (○) or no glucose (starved) (●). (B) SDS-extractable protein. Cultures were grown in medium containing 20 mM glucose plus (○) or without (●) 1 mM $CdCl_2$. (C) Cell numbers. Cultures were grown in medium containing 20 mM glucose plus (○) or without (●) 1 mM $CdCl_2$. (D) Acetogenesis. Cadmium chloride was added at 12 h. Cultures were grown in medium containing 20 mM glucose plus (○) or without (●) 1 mM $CdCl_2$ or in medium containing no glucose plus (▽) or without (▼) 1 mM $CdCl_2$.

whereas 1 to 14% of the cadmium removed from nonstarved cultures was EDTA soluble. Cadmium sulfide was not EDTA soluble under these conditions, whereas the phosphate and carbonate salts of the metal were (data not shown). Binding to glass accounted for less than 1% of the cadmium removed under these conditions (data not shown).

Atomic absorption analysis of medium from which cells and precipitate were removed showed that precipitation of cadmium commenced approximately 12 h after the addition of $CdCl_2$. Complete removal of the metal from the growth medium was accomplished by *C. thermoaceticum* about 72 h after the addition of the metal (Fig. 1A). In contrast, the concentration of cadmium remained high throughout most of the experiment in the starved-cell controls. The abiotic precipitation of cadmium in the starved controls (Fig. 1A) and medium controls (data not shown) at 95 h and later is possibly due to reaction of the metal with medium components such as bicarbonate and phosphate. The concentration of cadmium in the controls never dropped below 0.2 mM, however. With 0.075 and 0.1% cysteine, precipitation occurred more rapidly (data not shown) and more completely (Table 1), but abiotic precipitation also increased and approximately twice as much EDTA-insoluble cadmium appeared in the pellet. Nonetheless, more cadmium was precipitated by actively metabolizing cells in the insoluble form.

Effects of cadmium on growth and acetogenesis. Cells treated with cadmium produced nearly twice as much SDS-extractable protein as untreated cells did by the end of the experiments (Fig. 1B). An increase in total extractable protein indicated that cadmium may have had a positive effect on the growth of the organism. To determine whether cadmium was stimulating growth, samples from cultures of *C. thermoaceticum* plus or without cadmium were counted

TABLE 2. Cadmium tolerance of *C. thermoaceticum*

[Cadmium] (mM)	$g^a \pm SE^b$ (h)	Formation of precipitate
0	32.50 ± 0.72	NA[c]
1	29.76 ± 2.41	Yes
2	31.61 ± 0.54	Yes
4	44.46 ± 4.34	Yes

[a] Generation time calculated from acetate production at log phase.
[b] Standard errors of three cultures with triplicate samples from each.
[c] NA, not applicable.

in a Petroff-Hauser counting chamber. There was no apparent increase in the average number of cells per milliliter when cadmium was present during growth (Fig. 1C), although the protein concentration was still nearly twofold higher in cadmium-supplemented cells (Fig. 1B). There was also no significant difference between the cadmium-treated and control culture doubling times, which approximated 13 h (Fig. 1C). When 1 mM $NiCl_2$ was added concurrently with cadmium, growth was completely inhibited. The nickel at this concentration was slightly stimulatory in the absence of cadmium but became somewhat inhibitory with 10 mM (data not shown). Neither metal was precipitated during the coincubation; nickel at 10 mM was not precipitated in any significant amount, and nickel at 1 mM was not precipitated at all without cadmium.

The toxic action of cadmium is due, in part, to the affinity of the metal for sulfhydryl groups in enzymes and proteins (36). Acetogenesis in *C. thermoaceticum* is dependent on sulfhydryl-containing enzymes and proteins (e.g., CO dehydrogenase) (34). Thus, it was necessary to determine whether cadmium was disrupting acetate production by the organism. The generation of acetate by cadmium-supplemented cultures was not significantly different than that by the control cultures (Fig. 1D). The starved controls (Fig. 1D) and uninoculated controls (data not shown) did not produce significant amounts of acetate above what would be expected from glucose carryover. Increasing the cadmium concentration to 2 mM did not affect the rate of acetogenesis by the organism in the complex medium (data not shown). However, attempts to ascertain the effect of cadmium at concentrations greater than 2 mM were unsuccessful because the metal precipitated in media that supported growth of the organism, including a defined medium that was deficient in yeast extract and tryptone.

One possible reason for the precipitation at higher concentrations of cadmium was the formation of cadmium phosphate. In an attempt to overcome this problem, 2 mM glycerol 2-phosphate replaced potassium phosphate in the growth media (defined and complex). The results of the tolerance studies with the complex form of the glycerol phosphate medium are shown in Table 2. *C. thermoaceticum* was able to grow and produce acetate in the presence of up to 4 mM cadmium. The lag phase for the cultures with 4 mM cadmium increased from 24 h to 48 h, and the generation time increased from 30 h to nearly 45 h. At this concentration of cadmium, however, the metal precipitated with medium components and may have caused other micronutrients in the medium to coprecipitate, resulting in the increased lag period and generation time. This phenomenon has been reported to occur with cadmium in Luria-Bertani broth (36), which also contains yeast extract and tryptone. The amount of the precipitate decreased after 24 to 48 h of incubation but never completely disappeared. Experiments with a defined medium in which yeast extract and tryptone were deleted yielded similar results (data not shown). Precipitation at cadmium concentrations higher than 2 mM also occurred with the defined glycerophosphate medium. However, all cultures with cadmium produced the bright yellow cadmium sulfide precipitate, although the 4 mM cadmium cultures produced much less.

Energy dependence of cadmium precipitation. Cadmium sulfide was not formed in the medium or starved controls even when incubation was for 7 days. However, when glucose was added to starved control cultures, cadmium sulfide precipitation commenced within 24 h. Cultures containing methanol and syringic acid, both substrates utilized by *C. thermoaceticum* for energy, also supported the formation of cadmium sulfide (data not shown). Additionally, cysteine added as the sole source of carbon and energy at 14.25 mM (0.25%, wt/vol) would not support growth or cadmium precipitation until glucose was added to the medium (data not shown).

Analysis of starved and nonstarved cells treated with 1 mM $CdCl_2$ under the electron microscope (Fig. 2) revealed localized sites of precipitation at the surfaces of nonstarved cells only (Fig. 2A, arrow a). Additionally, a large quantity of the extracellular precipitate was present in the medium as large electron-dense granules (Fig. 2A, arrow b). The precipitate in starved cells appears as an amorphous mass with a few very small electron-dense granules (Fig. 2B, arrow c). A small amount of general binding to the cell wall of *C. thermoaceticum* occurs in both cultures, although it appears to be minor compared with the localized precipitation of the nonstarved cells. Cadmium did not appear to be accumulated in the interior of the cells. The control cells (nonstarved, no cadmium) showed no extracellular binding of electron-dense material with very little precipitated material (data not shown).

Sulfide production and cysteine desulfhydrase activity. To assess the possibility that *C. thermoaceticum* was producing sulfide in response to the addition of cadmium, total sulfide concentration was determined after the organism had grown on the complex medium for 7 days (Table 3). The organism produced an average of 0.38 mM sulfide during normal growth. However, over fourfold more (1.68 mM) sulfide was generated when cadmium was added to the growth medium. When the amount that would have theoretically formed complexes with cadmium (1 mM) was subtracted, nearly 1.8-fold more sulfide was generated by cadmium-supplemented cultures than by controls. Sulfide was not produced in significant amounts under abiotic conditions or when the cells were starved (Table 1).

Cysteine hydrochloride (0.05%, 2.85 mM) was added to the growth medium as a reductant and was the most likely source for sulfide. Two enzymes are responsible for sulfide production from cysteine and cystine in soils: cysteine desulfhydrase and cystathionine γ-lyase (both listed as EC 4.4.1.1). Cysteine desulfhydrase activity has been found in eubacteria (11), whereas cystathionine γ-lyase is usually found in actinomycetes such as *Streptomyces phaeochromogenes* (29) and fungi (7). Cysteine desulfhydration activity was discovered in cell extracts from midexponential-phase cultures of *C. thermoaceticum* (data not shown) at a specific activity of approximately 1.25 μmol of pyruvate formed per min per mg of protein. Because of the lack of inhibition by 25 mM propargylglycine, a specific inhibitor of cystathionine γ-lyase (45), and the lack of activity with 1 mM cystine in place of cysteine, we believe that the enzyme catalyzing the reaction is cysteine desulfhydrase. Cysteine desulfhydrase

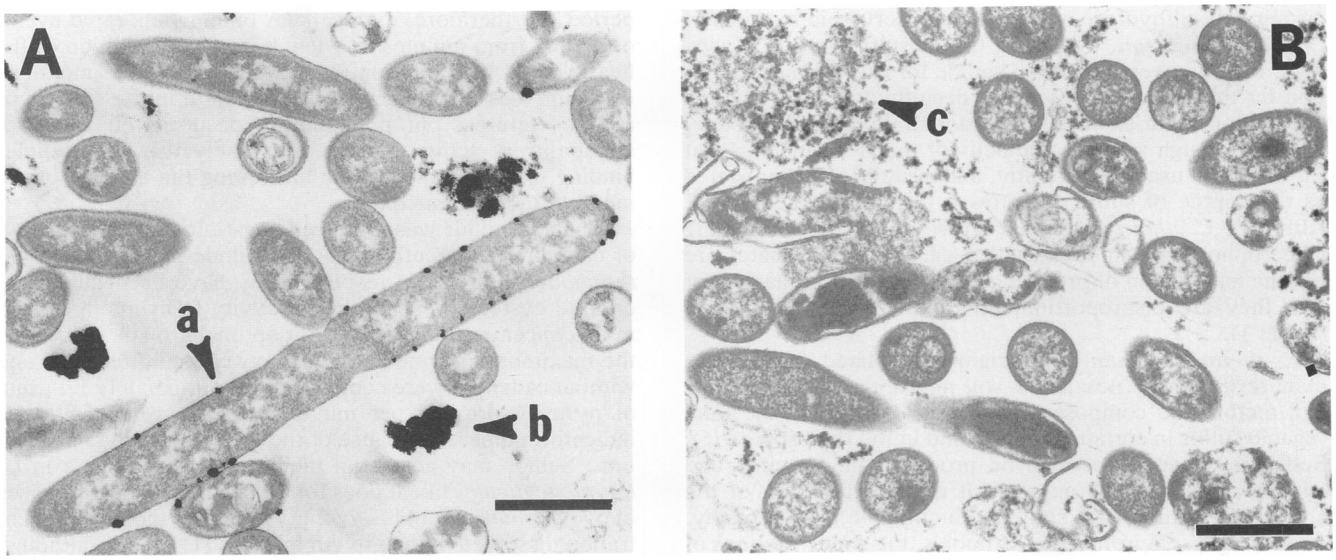

FIG. 2. Electron micrographs of *C. thermoaceticum* incubated with 1 mM $CdCl_2$ for 3 days. (A) Nonstarved cells, showing localized cadmium precipitate at the cell surface (a) and extracellular cadmium precipitate (b). (B) Starved cells, showing an amorphous precipitate with small electron-dense granules (c). Bars, 1 μm.

activity has also been reported in an acid-tolerant strain of *C. thermoaceticum* grown in a minimal medium (19).

The specific activity of cysteine desulfhydrase from extracts of cadmium-treated cultures was similar to that of the control cultures. However, the activity appeared in cadmium-containing cultures an average of 12 h earlier than in control cultures and peaked an average of 22 h earlier in the extracts from cadmium-treated *C. thermoaceticum* (Fig. 3). Using a two-way analysis of variance in which each experiment was treated as a block effect (43) and plus or without cadmium was used as the treatment, we found that there was a significant difference in time (hours) to peak enzyme activity between the cultures with and without cadmium ($F_{1,12} = 170.74$; $P < 0.0001$). These data were found to be normally distributed by the Shapiro-Wilks test.

DISCUSSION

Most studies on cadmium tolerance have been performed with aerobic or facultatively anaerobic organisms. Cadmium in sulfidogenic environments would tend to be precipitated as a sulfide and therefore is not a problem. However, little has been done to investigate the fate of cadmium in nonsulfidogenic anaerobic environments. *C. thermoaceticum* tolerated up to at least 2 mM cadmium by producing sulfide, which forms an insoluble precipitate with the metal. This concentration is similar to that reported for *P. putida* GAM1 (16) and *Escherichia coli* containing the pGU100 plasmid (17). In the presence of 1 mM nickel, a synergistic toxic effect occurs, as reported for other microorganisms (15).

The mechanism of cadmium precipitation utilized by *C. thermoaceticum* appears to be very similar to those of *Cyanidium caldarium* (51) and *Klebsiella aerogenes* (1), which utilize the reduction of sulfate to sulfide, or to that of *Citrobacter* sp., which enzymatically cleaves the phosphate from glycerophosphate (26) to mediate resistance. The sulfate concentration of the medium is only 18.7 μM, and it is unlikely that sulfide generated by sulfate reduction is responsible for the formation of cadmium sulfide by *C. thermoaceticum*. Additionally, it was reported that an acetate-tolerant strain of the organism could not utilize sulfate by reductive dissimilation (19). Cysteine, added to the growth medium as a reducing agent, was the largest source of sulfur, and

TABLE 3. Total sulfide produced by *C. thermoaceticum*

Culture and growth conditions[a] (*n*)	[Sulfide][b] ± SE (mM)
Experimental cultures	
No Cd (12)	0.38 ± 0.06
Cd (12)	1.68 ± 0.09
Starved controls	
No Cd (12)	0.01 ± 0.02
Cd (15)	0.03 ± 0.02
Medium controls	
No Cd (9)	0.03 ± 0.01
Cd (9)	0.10 ± 0.01

[a] See Materials and Methods.
[b] Sulfide concentration after 7 days.

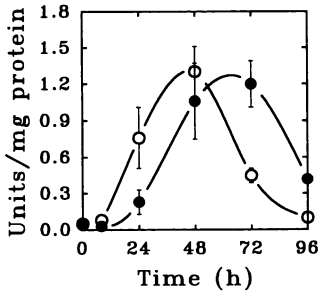

FIG. 3. Effect of cadmium on cysteine desulfhydrase expression in *C. thermoaceticum*. Cadmium chloride was added before inoculation. Cultures were grown in medium containing 20 mM glucose plus (○) or without (●) 1 mM $CdCl_2$. Units are micromoles of pyruvate formed per minute.

cysteine desulfhydrase activity was observed in extracts of *C. thermoaceticum*, indicating that desulfhydration of cysteine was the likely source of sulfide for cadmium precipitation by this organism. Cysteine desulfhydrase activity has been reported for an acetate-tolerant strain of *C. thermoaceticum*, although at a specific activity much lower than that for the strain used in this study, and cysteine was implicated as the source of sulfur for growth in minimal media (19). Although *C. thermoaceticum* grew on media containing glycerophosphate, it appears that cadmium phosphates are not the major form of precipitate formed by the organism or that they are disproportionated to the sulfide precipitate (Table 1).

C. thermoaceticum was originally isolated from horse manure (8) and is possibly a soil microorganism. Cysteine and methionine compose a significant portion of the total organic sulfur in certain soils (40) and in the rhizosphere (6). Sulfide has been shown to be produced in cysteine- and cystine-amended soils as a result of mineralization of the amino acids rather than sulfate reduction (46). Additionally, depending on environmental variables, the concentrations of amino acids from residue decomposition can vary (44). However, little is known about the importance of this mechanism of sulfide release in the soil environment, especially in anoxic soils. It is possible that *C. thermoaceticum* produces sulfide from cysteine in soil to reduce the redox potential for optimal growth. This would result in the precipitation of heavy metals by normal metabolic activity of the organism. This hypothesis is supported by previous work with cultures of the acetate-tolerant strain of the organism on a minimal medium (19). The redox potentials of cultures with cysteine ranged between −370 and −400 mM, whereas those of thioglycolate-supplemented cultures ranged between −350 and −370 mM (19).

A source of energy was required for CdS formation. In the absence of any energy source besides cysteine, *C. thermoaceticum* was unable to precipitate cadmium. Although approximately 60% of the metal eventually precipitated under abiotic or starvation (Fig. 1A) conditions, this was after nearly 100 h of incubation at 53°C. Precipitation of cadmium with medium components has been reported for Luria-Bertani broth, a medium with a high concentration of yeast extract and tryptone (36). The medium utilized in these studies contains 0.5 g of each of these components per liter as well as phosphate. It is quite likely that the white precipitate observed in both medium (abiotic) and starved controls is a precipitate of cadmium with components of the growth medium. Our mass balance results suggest that the precipitate formed under starvation and abiotic conditions is composed of cadmium complexes from medium components such as phosphate or bicarbonate, whereas the precipitate formed by actively metabolizing cells, which are producing sulfide, is eventually completely insoluble in EDTA. Thus, the formation of sulfide and the EDTA-insoluble precipitate by the cell requires a utilizable energy substrate that allows the organism to convert more readily mobilized forms of cadmium to a much less soluble precipitate.

Although this study does not rule out the possibility that the cells accumulate cadmium extracellularly by binding to the cell wall, the electron microscopic evidence points to an energy-dependent deposition of cadmium at distinct locations in the cell wall. These locations may represent the location cysteine desulfhydrase. Additionally, the removal of cadmium by extracellular binding under starvation conditions (Fig. 1A) requires far more cell mass than is present, and the cells do not increase in number during the starvation period. Furthermore, extracellular binding is a rapid event, occurring from minutes to a few hours after addition of the heavy metal to a solution (3, 28). No significant amount of cadmium was removed after the first 24 h in the starved or abiotic cultures, but precipitation occurred quickly with metabolically active cells. It is unlikely that extracellular binding plays a major role in alleviating the toxicity of the metal to *C. thermoaceticum*.

Whereas sulfide was produced constitutively, the addition of cadmium apparently enhanced sulfide production by *C. thermoaceticum*. This response may have been due to increased cysteine desulfhydrase activity to maintain a minimum concentration of sulfide for optimal growth. However, the maximum enzyme activities between cultures with and without cadmium were comparable (approximately 1.2 μmol of pyruvate formed per min per mg of protein; Fig. 2), indicating some other cause of the increased sulfide production. Sulfide may act as an inhibitor of the enzyme in *C. thermoaceticum*, like it does for the cysteine desulfhydrases of *S. typhimurium* and *E. coli* (11), even at the low concentration dissolved in the growth medium (19). Thus, although the specific activities of the enzyme were similar in cultures with and without cadmium, removal of extracellular sulfide by cadmium sulfide formation may have prevented enzyme inhibition. The effect would have been an overall increase in the amount of total sulfide produced. This does not fully explain the overproduction of sulfide, as represented by the amount remaining when sulfide theoretically bound up as CdS is subtracted from the total generated by cadmium-treated cultures.

Like the cysteine desulfhydrases in *E. coli* and *S. typhimurium* (11, 20), the *C. thermoaceticum* enzyme appears to be induced by the addition of cysteine to the growth medium. When dithioerythritol instead of cysteine was added to *C. thermoaceticum* cultures, no cysteine desulfhydrase activity was observed (data not shown). The earlier expression of enzyme activity in the cadmium-treated cultures (Fig. 3) indicates some role for cadmium in the regulation of the enzyme. The cell may express the enzyme earlier in response to an initial increase in intracellular cadmium concentration. Earlier expression would allow the cell to produce sulfide more quickly to precipitate the metal and reduce its toxic action on growth.

For cadmium to exert an effect on the expression of cysteine desulfhydrase, it must interact with internal components of the cell. It is unknown by what mechanism cadmium enters *C. thermoaceticum* or whether it enters the cell at all after initial transport or uptake. Because of its high affinity for sulfhydryls, cadmium could be transported into the cell already complexed to cysteine. Additionally, free Cd^{2+} may be transported via energy-dependent manganese or magnesium transport, as reported for other bacteria (31, 33, 47). We are currently attempting to determine whether cadmium is taken up by *C. thermoaceticum* and, if so, by what mechanism.

It is likely that cadmium was detoxified quickly, since neither acetogenesis nor growth was affected. The apparent increase in the protein concentration in cell extracts from cadmium-supplemented cultures may be a result of expression of an intracellular cadmium-binding protein similar to metallothionein (13), the *cadB* product from *S. aureus* (33), the zinc-binding protein of *A. eutrophus* (37), or an extracellular cadmium-binding protein similar to those produced by *Arthrobacter* sp. (21) and *V. alginolyticus* (12) for copper. An alternative explanation is that the cadmium formed complexes with the protein in tryptone, resulting in an

artifactually higher protein concentration. However, such a complex is easily dissolved by an EDTA solution (36); we believe the increased protein in cadmium-treated cells to be a response to cadmium, since the cells are washed with 10 mM EDTA before lysis.

It is unknown whether the precipitation mechanism is coupled with an altered transport for cadmium, production of extracellular or intracellular sequestration biopolymers, or cadmium efflux. The greatly increased extractable protein levels of the cadmium-treated cultures supports possible sequestration of the metal. Preliminary experiments with SDS treatment of washed pellets after lysozyme extraction of cells indicates that *C. thermoaceticum* may produce an extracellular protein in response to cadmium (23). The electron microscopic evidence also appears to support an extracellular binding protein rather than an intracellular protein, since the only electron-dense particles observed besides those in the medium were deposited around the exteriors of the nonstarved cells (Fig. 2A) and not the interiors. We are presently working to determine the nature of the higher protein concentration in cadmium-treated cultures of *C. thermoaceticum* and the role of cadmium in the expression of cysteine desulfhydrase.

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