

Accumulation and Transport of Cadmium by Tolerant and Susceptible Strains of *Mycobacterium scrofulaceum*

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Cadmium accumulation and transport were studied in two strains of *Mycobacterium scrofulaceum* differing in their susceptibility to Cd²⁺ toxicity. A 10-fold excess of either Zn²⁺ or Mn²⁺ partially antagonized inhibition of growth by Cd²⁺. ¹⁰⁹Cd²⁺ uptake by both the tolerant and susceptible strains was temperature dependent and inhibited by a 10-fold excess of either Zn²⁺ or Mn²⁺. There were no significant differences in either the kinetics of ¹⁰⁹Cd²⁺ uptake or the retention of accumulated ¹⁰⁹Cd²⁺ by the tolerant and susceptible strains. Both tolerant and susceptible strains removed most of the cadmium from the culture medium, but significantly more was removed by cells of the tolerant strain. Most of the accumulated Cd²⁺ in the tolerant strain was in the particulate fraction, rather than in the soluble fraction. Intracellular accumulated Cd²⁺ was primarily in the soluble fraction of the susceptible strain. Increased Cd²⁺ in culture medium resulted in decreased Mn²⁺ and Zn²⁺ in cells of the susceptible strain but did not reduce the Mn²⁺ and Zn²⁺ content of cells of the tolerant strain.

The occurrence and growth of *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum* (MAIS group) in waters (8, 12) and soils (3) of the southeastern region of the United States and the demonstration that such organisms share many characteristics with MAIS isolates recovered from hospital patients (9) support the contention that the outdoor environment is an important source of these human pathogens (29). If this contention is true, then the phenotypic and genetic properties of these isolates should permit survival in the diverse environments from which they have been recovered. For example, MAIS isolates recovered from heavy metal-polluted sites in the Delaware Bay were more likely to be resistant to a number of heavy metals than isolates recovered from relatively unpolluted sites in the Chesapeake Bay (7). That work led to the identification of plasmid-encoded mercury (22) and copper (6) resistance in *M. scrofulaceum*, which are the only known resistance markers on mycobacterial plasmids.

A number of *M. scrofulaceum* isolates have been isolated which differed widely in their susceptibility to cadmium (7). In addition to demonstrating that members of the MAIS group show evidence of adaptation to diverse environments, characterization of the influence of cadmium on mycobacteria may lead to a greater understanding of the physiology and ecology of these opportunistic pathogens. Because MAIS organisms have an optimum for growth at pH 5 to 5.5 (11) and are recovered in high numbers from soils and waters of low pH (3), and because heavy metal salts are more soluble at low pH (10), heavy metal toxicity may strongly influence mycobacterial numbers in their native habitat. Comparison of the characteristics of Cd²⁺ transport and accumulation by susceptible and tolerant strains of *M. scrofulaceum* may explain the basis for tolerance of some MAIS strains to high concentrations of cadmium. Further,

by measuring and characterizing transport of cadmium in mycobacteria, one might develop methods that will help elucidate the unusually high requirement of mycobacteria for zinc (28) and test the hypothesis that the slow growth of mycobacteria is due to the slow uptake of some key nutrient (23).

MATERIALS AND METHODS

Bacterial strains. Two isolates of *M. scrofulaceum* were used: strains W262 (22) and TMC1321, which had been isolated from a human skin lesion in 1953. Strain W262 is typical of strains with very high resistance to cadmium, while strain TMC1321 represents strains which are cadmium susceptible (7). Although strains W262 and TMC1321 are not isogenic, they are *M. scrofulaceum* representatives which share common epidemiologic markers (9) and only differ in their susceptibility to cadmium. Strain W262 has four plasmids (14.3, 150, 173, and 278 kilobases [22]); strain TMC1321 has two plasmids of 18 and 31 kilobases (P. S. Meissner, personal communication). Cultures were maintained on and subcultured from MGE agar slants (see paragraph below). Stock cultures were grown by inoculating 10 ml of MGE broth (see paragraph below) with a loopful of cellular material from the stock slants. These stock cultures were incubated at 30°C, vortexed daily, and grown to mid-log phase (i.e., 150 Klett turbidity units; 10⁸ CFU/ml; about 1 month). Slants and broth cultures were routinely stored at 5°C following growth. Fresh stock broth cultures and slants were prepared monthly and every 3 months, respectively.

Media and heavy metal solutions. The broth medium used was Middlebrook 7H9 medium (BBL Microbiology Systems, Cockeysville, Md.) containing 0.4% (vol/vol) glycerol (MG broth). The agar medium used was Middlebrook 7H10 (BBL Microbiology Systems, Cockeysville, Md.) containing 0.4% (vol/vol) glycerol (MG agar). The pHs of these media were adjusted as desired by the addition of 1 M NaOH or HCl prior to sterilization. Glass-distilled water was used in the preparation of all media and stock solutions. Oleic acid-

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albumin-glucose-catalase enrichment was added to the media to a final concentration of 10% (vol/vol) when enhanced growth of isolates was desired, but only to media lacking exogenous heavy metal salts. The oleic acid-albumin-glucose-catalase enrichment was prepared in the following manner. First, 8.5 g of NaCl, 30 mg of bovine catalase (Sigma Chemical Co., St. Louis, Mo.), and 50 g of bovine serum albumin, fraction 5 (Sigma Chemical Co., St. Louis, Mo.), were dissolved in 1 liter of water at ambient temperature. The solution was then placed in a cold (8°C) room; 0.6 ml of oleic acid (Sigma Chemical Co., St. Louis, Mo.) was added, and the solution was mixed at medium speed on a magnetic stirring device until the oleic acid went into solution (overnight). The solution was then filtered successively through 5-, 1.2-, 0.45-, and 0.2- μm -pore-size Metrical membrane filters (Gelman Sciences, Inc., Ann Arbor, Mich.) and finally filter sterilized by passage through a sterile 0.2- μm -pore-size Metrical membrane filter. The sterile enrichment was stored at 8°C. Sterile glucose was added to a final concentration of 2% (wt/vol) prior to the addition of the enrichment to any medium. MG broth medium and MG agar medium containing 10% (vol/vol) oleic acid-albumin-glucose-catalase enrichment were designated MGE broth and MGE agar, respectively. Stock solutions (100 mM) of CaCl_2 , CdCl_2 , CoCl_2 , CuCl_2 , CrCl_2 , FeCl_2 , MnCl_2 , HgCl_2 , NiCl_2 , and ZnCl_2 were prepared in 10 mM HCl and filter sterilized. Stock solutions of the metal salts were diluted in sterile 1 mM HCl as necessary. Reagent grade salts of heavy metals were used throughout.

Heavy metal susceptibility testing. Susceptibility, expressed as the growth yield of cultures in the presence of heavy metal salts divided by that in their absence, was measured in MG broth medium. In the metal-free cultures, an equal volume of 10 mM HCl (or 1 mM if diluted) was added in place of the metal(s). Changes in the pHs of media due to the addition of metals, as has been documented to occur with Cu^{2+} (24), were corrected prior to inoculation. Cultures were incubated at 30°C vertically without shaking and vortexed daily. Turbidity measurements were done with a Klett-Summerson photoelectric colorimeter (no. 66 filter).

Uptake and retention of $^{109}\text{Cd}^{2+}$. Cells were grown to log phase in 100 ml of MG broth at 30°C with continuous shaking (60 cycles per min in a model 2156 Reciprocating Water Bath Shaker; American Optical Corp., Buffalo, N.Y.). Cells were harvested by centrifugation at $10,000 \times g$ for 10 min at 25°C, suspended in an equal volume of half-strength MG broth at pH 5.5, and incubated with shaking at 30°C for 4 h prior to the initiation of $^{109}\text{Cd}^{2+}$ transport studies. Uptake was initiated by the addition of 10 μM Cd^{2+} (0.5 μCi of $^{109}\text{Cd}^{2+}$; Dupont, NEN Research Products, Boston, Mass.) to 10 ml of cells. The final cell density during the transport assays was approximately 2.0 mg (dry weight) per ml. Samples (0.2 ml) were collected on 0.45- μm -pore-size cellulose triacetate membrane filters (Gelman Sciences, Inc., Ann Arbor, Mich.) and washed with 10 ml of 1 mM HCl. Nonspecific binding of $^{109}\text{Cd}^{2+}$ to filters and cells was minimal when they were washed with 1 mM HCl compared with when they were washed with MG broth containing 0.5 mM Cd^{2+} . ^{109}Cd on filters was determined by gamma ray spectrometry (Biogamma II gamma counter; Beckman Instruments, Inc., Fullerton, Calif.). To study the effect of inhibitors of energy-dependent processes on transport, the cell suspensions were preincubated for 10 min in either 10 mM sodium arsenate, 20 mM sodium azide, 100 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), or 1 mM dinitrophenol (DNP) prior to the addition of cadmium. Where indicated, $^{109}\text{Cd}^{2+}$ was

added simultaneously with a 10-fold excess of Ca^{2+} , Co^{2+} , Cu^{2+} , Fe^{2+} , Mn^{2+} , or Zn^{2+} .

To assess ^{109}Cd retention, cells were incubated in MG broth containing 10 μM $^{109}\text{Cd}^{2+}$ for 60 min before being harvested by centrifugation at $10,000 \times g$ for 10 min at 4°C. After suspension in ice-cold, half-strength MG broth, samples were transferred to 50-ml flasks in a shaking water bath at either 0 or 30°C and test substances were added. After 60 min, cells were collected by filtration and washed and $^{109}\text{Cd}^{2+}$ was measured as described above.

Long-term Cd^{2+} , Zn^{2+} , and Mn^{2+} accumulation and cellular distribution of Cd^{2+} . Organisms were cultured in MG broth containing different concentrations of Cd^{2+} for 30 days at 30°C. Then, cells were collected by centrifugation and spent media were retained for metal analysis. Cells were suspended in 10 ml of distilled water and divided into two aliquots, and the cells were again harvested by centrifugation. For each pair of cell pellets, one pellet was suspended in 5 ml of distilled water, frozen, and lyophilized. The second pellet was suspended in 10 ml of phosphate buffer (pH 6.0), and the cells were disrupted by two passages through a French pressure cell (American Instruments Co., Silver Spring, Md.) at 1.37 kPa. Unbroken cells and cellular debris were pelleted by centrifugation at $15,000 \times g$ for 15 min at 4°C. The particulate and soluble fractions of the crude extract were isolated by following a modification of the procedure of Darter and Millman (5). The supernatant suspension was centrifuged at $100,000 \times g$ for 60 min at 4°C. The soluble fraction (supernatant) was removed, and the particulate fraction was suspended in 10 ml of phosphate buffer (pH 6.0). To determine the quantity of cadmium, zinc, and manganese in the soluble and particulate fractions, a modification of the procedure of Hill et al. (14) was used. Each fraction was sonicated (Ultrasonic Microson Cell Disrupter; Heat Systems-Ultrasonics, Inc., Farmingdale, N.Y.) for 30 s at an output power of 40% maximum, transferred to metal-free glass tubes, and dried for 20 h at 95°C. Lyophilized samples of intact cells were similarly dried at 105°C for 20 h. Organic materials were digested by wet ashing samples with concentrated ultrapure HNO_3 (Ultrex grade; J. T. Baker Chemical Co., Phillipsburg, N.J.) and H_2O_2 . Ash was dissolved in 4 ml of 1 N ultrapure HCl (Ultrex grade; J. T. Baker Chemical Co., Phillipsburg, N.J.), diluted when necessary, and analyzed by atomic absorption spectrophotometry (model 5000 spectrophotometer; The Perkin-Elmer Corp., Norwalk, Conn.) with an air-acetylene flame. The recovery of cadmium, manganese, and zinc exceeded 97% as assessed by identical treatment and analysis of samples of certified Standard Reference Material Oyster Tissue 1566 from the U.S. National Bureau of Standards.

RESULTS

Effect of Cd^{2+} on growth yield. Growth yields of *M. scrofulaceum* TMC1321 and W262 were similar in MG medium in the absence of Cd^{2+} (Fig. 1). The addition of 5 to 30 μM Cd^{2+} markedly inhibited the growth of the susceptible strain (TMC1321), while the growth of the tolerant strain (W262) was relatively unaffected (Fig. 1). In contrast, the growth yields of both strains were similarly affected by the addition of Ca^{2+} , Co^{2+} , Cu^{2+} , Cr^{2+} , Fe^{2+} , Mn^{2+} , Hg^{2+} , Ni^{2+} , or Zn^{2+} (data not shown).

In the presence of 20 μM Cd^{2+} , the growth yield of strain TMC1321 was 6% of that of the metal-free control. Mn^{2+} or Zn^{2+} at 200 μM increased its growth yield to 30 and 53% of that of the control, respectively. None of the other divalent

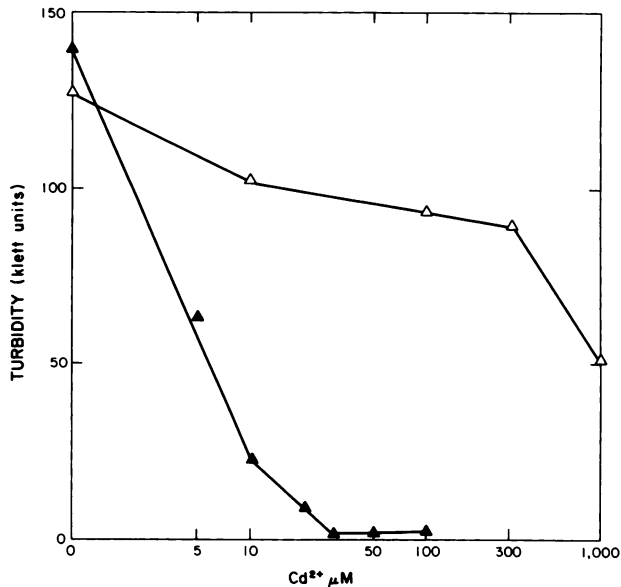


FIG. 1. Effect of Cd^{2+} on the growth of *M. scrofulaceum* TMC1321 (▲) and W262 (Δ). Growth yield is expressed as turbidity in Klett units after 30 days of incubation at 30°C in MG broth containing different concentrations of Cd^{2+} .

cations tested (see above) altered Cd^{2+} -mediated inhibition of the growth of strain TMC1321 (data not shown).

$^{109}\text{Cd}^{2+}$ uptake and retention. Cells of strain TMC1321 accumulated $^{109}\text{Cd}^{2+}$ from half- and full-strength MG medium but not from a minimal salts buffer containing glycerol

TABLE 1. Effect of temperature, energy inhibitors, and metals on $^{109}\text{Cd}^{2+}$ accumulation by *M. scrofulaceum* TMC1321 and W262

Parameter	$^{109}\text{Cd}^{2+}$ uptake ^a	
	TMC1321	W262
Temp		
30°C	113	100
0°C	18	25
Energy inhibitors (30°C)		
CCCP	82	87
DNP	63	70
Divalent metals (100 μM at 30°C)		
Cd^{2+}	8	11
Mn^{2+}	44	54
Zn^{2+}	36	67

^a Expressed as picomoles of $^{109}\text{Cd}^{2+}$ accumulated in 10 min at 30°C per milligram (dry weight) of cells with the addition of 10 μM $^{109}\text{Cd}^{2+}$.

(data not shown). $^{109}\text{Cd}^{2+}$ uptake by both strains increased rapidly to a maximal value within 30 to 60 min in medium containing 1, 10, or 100 μM Cd^{2+} at 30°C (Fig. 2). $^{109}\text{Cd}^{2+}$ uptake by the susceptible strain (Fig. 2A) was higher than that by the tolerant strain (Fig. 2B), though the differences were not statistically significant. The amount of $^{109}\text{Cd}^{2+}$ accumulated by the susceptible strain was sixfold greater at 30°C than at 0°C (Table 1). Possibly, the $^{109}\text{Cd}^{2+}$ accumulated by cells at 0°C represented binding to the surface, while that at 30°C was due to both surface binding and transfer across the cytoplasmic membrane. $^{109}\text{Cd}^{2+}$ uptake was slightly inhibited by DNP and CCCP and unaffected by either azide or arsenate. The presence of a 10-fold excess of Cd^{2+} , Mn^{2+} , and Zn^{2+} significantly decreased $^{109}\text{Cd}^{2+}$ uptake (Table 1). This antagonism was specific, since equiva-

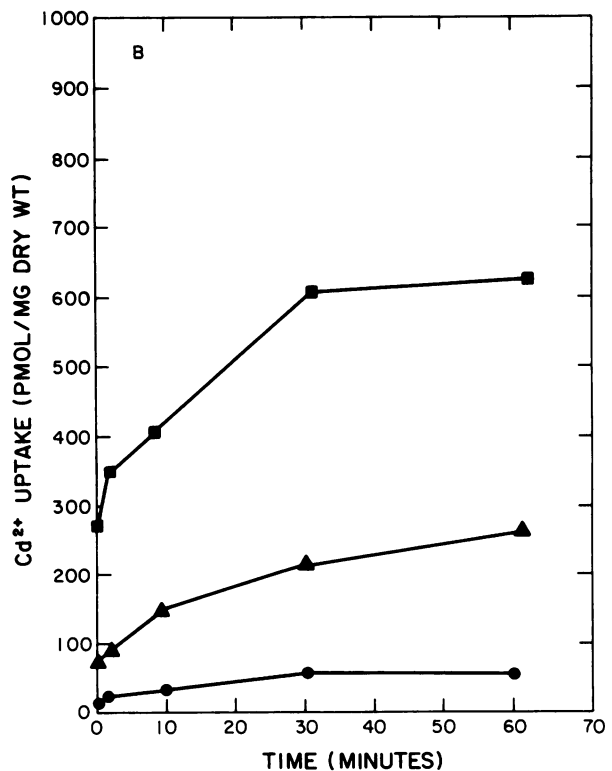
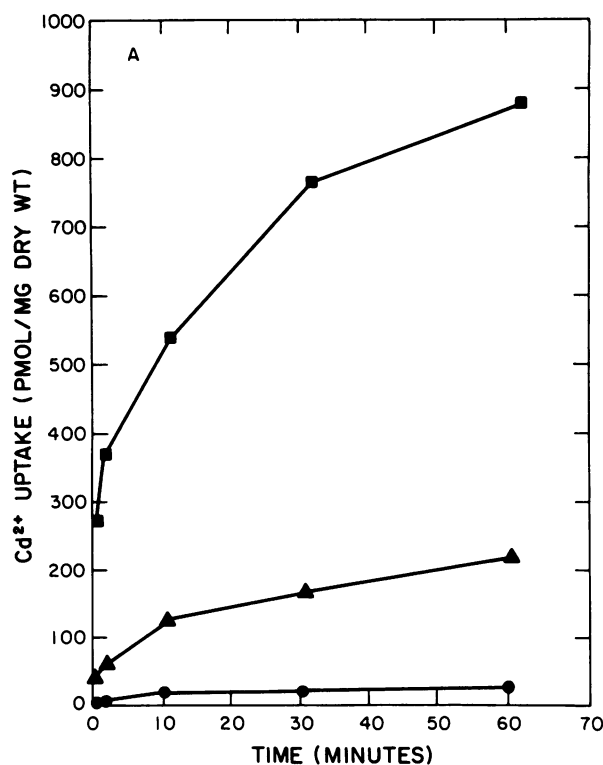


FIG. 2. Cadmium uptake by susceptible strain TMC1321 (A) and tolerant strain W262 (B) of *M. scrofulaceum*. Cells were grown and $^{109}\text{Cd}^{2+}$ uptake was measured as described in Materials and Methods. ●, 1 μM $^{109}\text{Cd}^{2+}$; ▲, 10 μM $^{109}\text{Cd}^{2+}$; ■, 100 μM $^{109}\text{Cd}^{2+}$.

TABLE 2. Accumulation of Cd²⁺ by *M. scrofulaceum* TMC1321 and W262

Cd ²⁺ added (μM)	Accumulation of Cd ²⁺ in strain:					
	TMC1321			W262		
	Total ^a	Soluble (%)	Particulate (%)	Total	Soluble (%)	Particulate (%)
0	1	50	50	1	33	67
1	88	39	61	88	30	70
10	702	40	60	640	14	86
50	NT ^b			3,777	6	94

^a Total Cd²⁺ accumulated in MG medium is expressed as micrograms of Cd per gram (dry weight).

^b NT, Not tested because of lack of growth.

lent concentrations of Co²⁺, Cu²⁺, and Fe²⁺ did not affect ¹⁰⁹Cd²⁺ uptake (data not shown). ¹⁰⁹Cd²⁺ uptake by strain W262 was also temperature dependent, weakly inhibited by DNP and CCCP, and inhibited by Cd²⁺, Mn²⁺, and Zn²⁺ (Table 1).

Cd²⁺ retention was investigated by using cells previously incubated in medium containing 10 μM ¹⁰⁹Cd²⁺. Neither the tolerant (W262) nor the susceptible (TMC1321) strain lost ¹⁰⁹Cd²⁺ when incubated in MG medium at either 0 or 30°C for 60 min. Only 26 and 17% of the accumulated ¹⁰⁹Cd²⁺ was lost from cells of the susceptible and tolerant strains, respectively, when 100 μM Cd²⁺ was added to the medium at 30°C. Zn²⁺ or Mn²⁺ (100 μM) failed to induce the loss of ¹⁰⁹Cd from cells of either strain (data not shown).

Removal of Cd²⁺ from culture medium. Because there was little difference in the characteristics of short-term uptake and accumulation of ¹⁰⁹Cd²⁺, the long-term removal from medium and accumulation of Cd²⁺ by cells of the two *M. scrofulaceum* strains were examined. Almost all (92%) of the Cd²⁺ in the original medium was removed when either strain was grown in medium containing 1 μM Cd²⁺. When cells were grown in 10 μM Cd²⁺, significantly more Cd²⁺ was removed from the culture medium by the tolerant strain, W262 (95%), than by the susceptible strain, TMC1321 (66%). Part, if not all, of that difference could have been due to the reduced growth of the susceptible strain (Fig. 1). Growth of the tolerant strain in 50 μM Cd²⁺ resulted in substantial removal (i.e., 86%) of the Cd²⁺ from the culture medium.

Accumulation of Cd²⁺ by the susceptible and tolerant strains. Although both strains accumulated approximately the same amount of total Cd²⁺ when grown in medium containing either 1 or 10 μM Cd²⁺ (Table 2), the distributions of Cd²⁺ between the soluble and particulate fractions of these cells were significantly different. More cadmium was found in the soluble fraction of the susceptible strain, TMC1321, than in the tolerant strain, W262 (Table 2). As the concentration of Cd²⁺ in the culture medium was increased, the percentage of accumulated Cd²⁺ present in the particulate fraction of cells of the tolerant strain increased (Table 2). That was not observed in cells of the susceptible strain.

Effect of Cd²⁺ on cellular Zn²⁺ and Mn²⁺ concentrations. At the level of Cd²⁺ which prevented normal growth of the susceptible strain (10 μM), cellular contents of Mn²⁺ and Zn²⁺ were significantly lower (Table 3). In contrast, the levels of Mn²⁺ and Zn²⁺ in the tolerant strain were maintained as the concentration of Cd²⁺ in the medium was increased (Table 3).

DISCUSSION

It is unlikely that the cadmium tolerance of *M. scrofulaceum* W262 was due to a barrier to Cd²⁺ influx or the

existence of an efflux mechanism to reduce the concentration of intracellular Cd²⁺. Though the ¹⁰⁹Cd²⁺ uptake rate by the susceptible strain was slightly higher than that of the resistant strain (Fig. 2), that difference was not reflected in the amount of cadmium accumulated over a long period of time (Table 2). Although no efflux of ¹⁰⁹Cd²⁺ was observed in either strain, it is possible that the 60-min incubation period was too short to produce observable efflux. The observation that increased resistance to cadmium was not associated with decreased accumulation of the toxic heavy metal (Table 2) is unusual, because studies of cadmium-resistant isolates of *Pseudomonas putida* (15), *Bacillus subtilis* (4, 18), a *Bacillus* sp. (21), and *Staphylococcus aureus* (17, 25–27) have all shown reduced Cd²⁺ accumulation by cadmium-resistant isolates. The cadmium tolerance of *M. scrofulaceum* W262 is not unique, because a number of other clinical and environmental MAIS isolates grow in the presence of 0.3 to 1.0 mM Cd²⁺ (7). Although strains TMC1321 and W262 are not isogenic, they are representatives of the same species, share common epidemiologic markers (9), and are only known to differ in their susceptibility to cadmium. The cadmium tolerance of strain W262 is unrelated to its plasmid-encoded, sulfate-dependent precipitation of copper (6), because the copper-sensitive, plasmid-free derivative of strain W262 is equally cadmium tolerant (data not shown).

Both the tolerant and susceptible strains had a temperature-dependent system for Cd²⁺ uptake (Table 1) whose rates and extent of accumulation were similar (Fig. 2). Because of the weak influence of the inhibitors DNP and CCCP on uptake, it is possible that only a fraction of the extracellular ¹⁰⁹Cd²⁺ was taken up into the cytoplasm. Uptake of ¹⁰⁹Cd²⁺ was reduced by 10-fold excesses of either Mn²⁺ or Zn²⁺ (Table 1). In *B. subtilis* (4, 18), *S. aureus* (25–27), and *Lactobacillus plantarum* (2), Cd²⁺ is accumulated by the Mn²⁺ transport system. By contrast, in *Escherichia coli* Zn²⁺, not Mn²⁺, is a competitive inhibitor of Cd²⁺ uptake (19). It would appear from our data that a

TABLE 3. Effect of Cd²⁺ on cellular content of Mn²⁺ and Zn²⁺

Cd ²⁺ added (μM)	Cation content ^a			
	TMC1321		W262	
	Mn ²⁺	Zn ²⁺	Mn ²⁺	Zn ²⁺
0	1.9	55	0.80	58
1	2.0	51	0.76	62
10	0.76	45	0.88	60
50	NT ^b	NT	0.88	98

^a Expressed as micrograms of Mn²⁺ or Zn²⁺ per gram (dry weight) of MG-grown cells. Concentrations of Mn²⁺ and Zn²⁺ in uninoculated MG were 0.04 and 0.3 μg/ml, respectively.

^b NT, Not tested because of lack of growth.

portion of the nonessential Cd^{2+} enters cells by either a common or separate Mn^{2+} and Zn^{2+} transporter(s) in *M. scrofulaceum*.

The relative resistance of *M. scrofulaceum* W262 to Cd^{2+} may be due to its ability to partition Cd^{2+} once it is accumulated in the particulate fraction of the cells. Although equivalent concentrations of total Cd^{2+} were accumulated by the susceptible and tolerant strains, more of the metal was found in the soluble fraction of the susceptible strain (Table 2). In addition, cells of the susceptible strain grown in toxic concentrations of Cd^{2+} (i.e., 10 μ M) contained less intracellular Mn^{2+} and Zn^{2+} than did cells of the tolerant strain (Table 3). It is not clear whether the loss of these essential metals is specific and leads to impaired cell function or, perhaps, reflects a Cd^{2+} -mediated inhibition of cellular processes. The ability of a Cd^{2+} -tolerant isolate to place more Cd^{2+} into the particulate fraction than is observed in a susceptible isolate has been previously reported for *S. aureus* (17) and *Saccharomyces cerevisiae* (16).

Because of the reported correlation between cadmium resistance of *Pseudomonas putida* and the production of cysteine-rich soluble proteins (i.e., metallothioneins [13]), we investigated whether such proteins were produced by the two mycobacterial strains. Although a cadmium-inducible, soluble protein was discovered in only the tolerant strain, its inability to bind Cd^{2+} and exclusive production during the stationary phase of growth argued against its role in cadmium tolerance (data not shown).

It is possible that the relative resistance of strain W262 compared with that of strain TMC1321 is due to the ability of the former to sequester Cd^{2+} in an insoluble form in the particulate fraction of the cell (Table 2) and to maintain intracellular Zn^{2+} and Mn^{2+} levels in the presence of increased Cd^{2+} concentrations (Table 3). Starvation of *Mycobacterium smegmatis* for Zn^{2+} resulted in a fivefold increase in the amount of insoluble polyphosphate (28). *Klebsiella aerogenes* (1) and a *Citrobacter* sp. (20) detoxify accumulated Cd^{2+} , in part, by formation of an insoluble phosphate. Additional measurements are required to determine whether the Cd^{2+} is sequestered in the particulate fraction of *M. scrofulaceum* as an insoluble cadmium-phosphate complex.

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