Cation Transport Alteration Associated with Plasmid-Determined Resistance to Cadmium in *Staphylococcus aureus*

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Plasmid-determined resistance to cadmium has only been found with plasmids from *Staphylococcus aureus*. Resistance to cadmium was associated with a lower accumulation of Cd²⁺ ions by the plasmid-bearing resistant cells. Cadmium accumulation by susceptible cells was energy dependent and had those characteristics usually associated with a transmembrane active transport system. There was a specific interrelationship between cadmium accumulation and manganese accumulation and retention. Cd²⁺ inhibited the uptake of Mn²⁺ and accelerated the loss of intracellular Mn²⁺ by the susceptible cells, but was without effect on Mn²⁺ transport in resistant *S. aureus* cells. Under similar conditions, there was no differential effect of Cd²⁺ on Mg²⁺, Zn²⁺, Co²⁺, Ni²⁺, or Rb⁺ accumulation or exchange between the susceptible and the resistant strains.

Cadmium is a highly toxic divalent cation with no known biological function (14). Cadmium resistance is the most common resistance determinant found on resistance plasmids (R plasmids) of *Staphylococcus aureus*, occurring at frequencies of 80% or greater in some clinical collections (10, 16, 17, 20–22). Resistance to antibiotics and to other heavy metals occur at somewhat lower frequencies. Plasmid-determined resistance is governed primarily by two genes on the plasmids. The cadA gene has been mapped on a number of plasmids (25, 27, 34) and causes an approximately 100-fold increase in Cd²⁺ resistance (27). Some plasmids have an additional gene, cadB, which maps quite distantly from the cadA locus (26, 34). In the presence of cadA⁺, there is no additional resistance conferred by cadB⁺ (34). However, in cadA mutants the cadB⁺ locus provided a smaller (10-fold) increase in Cd²⁺ resistance (26).

Cadmium resistance is associated with a lowered level of uptake of Cd²⁺ by the resistant, plasmid-containing cells (6, 33, 35). Protoplasts of resistant cells retain the barrier to Cd²⁺ uptake (7), suggesting that the cell membrane is involved in the barrier function. The process of uptake in susceptible cells is energy dependent, and it seems possible that the resistance barrier involves an active transport process such as is known for physiologically required divalent cations such as Mg²⁺, Mn²⁺, and Zn²⁺ (4, 15, 29, 31, 32). Alternatively, Chopra (7) has favored models attributing Cd²⁺ resistance to altered barrier proteins or lipids. We have pursued studies of Cd²⁺ uptake in *S. aureus* and concluded that Cd²⁺ is accumulated by the highly specific Mn²⁺ transport system of the cells that is altered by the Cd²⁺-resistance genes of the plasmids.

**MATERIALS AND METHODS**

**Bacterial strains and media.** *S. aureus* strains RN23 (with plasmid pI258), RN1008 (with plasmid pI258 with the cadA62 mutation leading to cadmium susceptibility), and RN1 (without a plasmid) were the three strains used in most experiments. Results were confirmed and extended in experiments with *S. aureus* strains containing different plasmids: RN885 (plasmid pII147); U71 (plasmid pU71), and RN4 (plasmid pI524). The strains and plasmids were previously described (24, 26, 27, 29, 30, 36). Plasmid pI258 has a single known locus, *cadA*, affecting cadmium resistance (26, 34). Plasmid pII147 contains both a major determinant of cadmium resistance (cadA) and a determinant of low-level resistance to cadmium (cadB), which can be observed only in the presence of a nonfunctional cadA mutation (34). To measure separately the effects of the *cadA* and *cadB* loci on radioactive cadmium uptake, strains with mutant pII147 plasmids were used: RN885 (*cadA⁺ cadB⁺*); RN1427 (*cadA114 cadB4*); RN966 (*cadA114 cadB⁺*), and AW1 (*cadA⁺ cadB4*), a revertant of RN1427 selected on nutrient agar containing 500 μM Cd²⁺.

Double-strength tryptone broth (16 g of tryptone [Difco] and 10 g of NaCl per liter of deionized water) was used as growth medium and in transport experiments. This medium has the following cation concentrations measured by atomic absorption spectroscopy or by flame emission spectroscopy (K⁺ only): 130 μM Mg²⁺; 10 μM Zn²⁺; 150 μM Ca²⁺; 0.1 μM Mn²⁺; and 100...

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μM K+, Cd2+ and other divalent cations were not determined. Reagent-grade salts were used, and no further or special efforts at regulating cation contents were made. The uncouplers carbonyl cyanide m-chlorophenylhydrazone (Sigma Chemical Co.) and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (gift of P. G. Heytler) were used. In some experiments, nutrient broth was used according to the procedures of Chopra (6, 7). To reduce cation levels further, experiments were also run in 15 mM glycylglycine buffer (pH 6.8) plus 0.17 M NaCl. Cells were grown to 50 to 100 Klett units of turbidity (no. 54 filter in a Klett colorimeter; 100 Klett units of turbidity is equivalent to 0.22 mg [dry weight] of cells per ml), harvested by centrifugation, and suspended in the original volume of fresh broth or buffer.

Transport and retention assays. After the addition of radioactivity and other components to the cultures incubating with aeration at 37°C, 1.0-ml samples were removed and filtered through HA membrane filters (Millipore Corp.) After two washings with 5 ml each of nonradioactive incubation medium, the radioactivity on the filters was determined either on planchets with a gas-flow Geiger counter (58Rb, 60Co, and 115mCd), in a liquid scintillation counter in water (by Cerenkov radiation; 28Mg), or in scintillation counting fluid (54Mn, 65Zn, 60Ni, and 109Cd). Growth was not generally monitored during the 20- to 60-min incubations with radioactive cations, and uptake was calculated as micromoles per initial cell mass.

RESULTS

Figure 1 demonstrates the increased Cd2+ resistance afforded by the plasmids in liquid growth experiments. Comparable data (not shown) were obtained by placing filter paper disks containing 0.1 to 2.0 μmol of Cd2+ onto petri dishes spread with 0.1 ml of log-phase cultures of S. aureus and measuring the inhibition zones surrounding the disks (38). The plasmid with the single cadA gene (pII258) conferred the same level of resistance as the plasmid with both cadA+ and cadB+ (pII147). Similarly, the cadA+ cadB mutant plasmid conferred the same level of resistance to Cd2+ (Fig. 1). The cadA cadB+ plasmid conferred an intermediate level of resistance to Cd2+, clearly higher than that with the plasmidless strain or the cadA cadB double mutant. Smith and Novick (34) found that the strain with the PII147 cadA cadB double mutant plasmid was slightly more susceptible to cadmium than the plasmidless strain when tested by liquid growth tests with inocula about 1,000 times lower than we have used.

Decreased Cd2+ uptake at low temperature (4°C) and in the presence of inhibitors of energy-dependent processes is shown in Fig. 2 for the plasmidless strain RN1. Cd2+ uptake appeared to be a saturable function of added Cd2+ (Fig. 3). When plotted with reciprocal coordinates, the affinity constant (Km) was 4.2 μM Cd2+ for the susceptible cells and 7.5 μM Cd2+ for the resistant cells (not a significant difference). The V_max values for Cd2+ uptake also differed and were greater for the susceptible cells (28 nmol/min per g [dry weight] of cells) than for the resistant.

![Fig. 1. Cadmium resistance of strains carrying plasmids with different cad alleles. Overnight cultures were diluted 1:100 into tryptone broth containing CdCl₂ and the culture turbidity was measured after 6 h of growth at 37°C.](http://aac.asm.org/DownloadedFrom)
midless *S. aureus* and the *S. aureus* strain with the cadA mutant variant of plasmid pI258 (Fig. 3). With plasmid pII147, one could distinguish between the levels of Cd\(^{2+}\) uptake affected by the different genes (Fig. 4). The double mutant cadA cadB strain accumulated cadmium at a rate similar to the strain without plasmid. The cadA\(^{+}\) cadB mutant strain accumulated as little Cd\(^{2+}\) as did the strain with the wild-type cadA\(^{+}\) cadB\(^{+}\) plasmid. (In this experiment, *S. aureus* with either of two additional wild-type plasmids, pI258 or pI524, was indistinguishable from the strain with plasmid pII147; data not shown.) The cadA cadB\(^{+}\) strain showed an intermediate level of Cd\(^{2+}\) uptake (Fig. 4) consistent with its intermediate level of Cd\(^{2+}\) resistance (Fig. 1; 34).

**Specificity of Cd\(^{2+}\) uptake.** Specificity of Cd\(^{2+}\) uptake was tested by measuring the effects of other divalent cations on Cd\(^{2+}\) uptake and retention and the effects of Cd\(^{2+}\) on the uptake and retention of other cations. Neither 1 mM Ca\(^{2+}\) nor 1 mM Mg\(^{2+}\) affected the uptake or retention of \(^{100}\)Cd\(^{2+}\) (Fig. 5A). Addition of 100 \(\mu\)M Ni\(^{2+}\) or Co\(^{2+}\) was also without effect on Cd\(^{2+}\) uptake (data not shown), but 100 \(\mu\)M Fe\(^{3+}\), Mn\(^{2+}\), or Zn\(^{2+}\) inhibited the uptake of 1.0 \(\mu\)M \(^{100}\)Cd\(^{2+}\) significantly, with Mn\(^{2+}\) appearing to have the greatest effect. That the slight inhibitions by Fe\(^{3+}\) and Zn\(^{2+}\) occurred only after a delay of 30 min makes these effects questionable. Once Cd\(^{2+}\) was accumulated by the cells, the addition of excess Mg\(^{2+}\), Mn\(^{2+}\), Zn\(^{2+}\), or Fe\(^{3+}\) did not displace

[Diagram of Cd\(^{2+}\) uptake by resistant and susceptible *S. aureus*. Strains RN1008 (susceptible) and RN23 (resistant) were grown, centrifuged, and suspended in broth at 0.22 mg (dry weight) per ml. \(^{100}\)Cd\(^{2+}\) was added, and the initial rate of uptake was measured.]
the accumulated Cd$^{2+}$. The addition of excess nonradioactive Cd$^+$ caused the loss of 50% of the radioactivity from the cells (Fig. 5B).

The results of testing Cd$^{2+}$ inhibition of uptake and stimulation of exit of other divalent cations were more clear-cut. Cd$^{2+}$ specifically inhibited the uptake of radioactive $^{54}$Mn$^{2+}$ by the susceptible cells (Fig. 6A) but was without effect on Mn$^{2+}$ uptake by resistant cells. Similarly, Cd$^{2+}$ simulated the loss of $^{54}$Mn$^{2+}$ from susceptible but not from resistant cells (Fig. 6B). This effect of Cd$^{2+}$ was highly specific for Mn$^{2+}$ uptake and retention. Cd$^{2+}$ did not differentially inhibit $^{26}$Mg$^{2+}$ uptake with susceptible and with resistant cells (Fig. 7A), and Cd$^{2+}$ was without effect on $^{26}$Mg$^{2+}$ retention (Fig. 7B). With $^{60}$Zn$^{2+}$-labeled cells, Cd$^{2+}$ caused a slight inhibition of uptake equal in both susceptible and resistant cells (Fig. 7C) and slight but equal loss of accumulated $^{60}$Zn$^{2+}$ (Fig. 7D). Because of the known difficulties determining Zn$^{2+}$ active transport in microbes (12) and the substantial evidence for interactions between Cd$^{2+}$ and Zn$^{2+}$ in microbial and mammalian cells (12, 13), the negative results with regard to Zn$^{2+}$ and Cd$^{2+}$ should be considered tentative. Cd$^{2+}$ at 50 μM was also essentially without effect on the uptake and retention of $^{86}$Rb$^+$, $^{60}$Co$^{2+}$, or $^{63}$Ni$^{2+}$ (data not shown). These results suggest a specific interaction of Cd$^{2+}$ with the Mn$^{2+}$ transport system, and we proceeded to look at the kinetics of this inhibition. Cd$^{2+}$ uptake appeared to be a saturable function of external Cd$^{2+}$ (Fig. 3), but the limited
Fig. 5. Effect of divalent cations on cadmium uptake and retention. (A) Strain RN1 (susceptible; without plasmid) was grown and harvested as in Fig. 2. $^{109}\text{Cd}^{2+}$ at 1.0 $\mu\text{M}$ was added to cells with or without additional cations. Samples were filtered and washed as described in the text. (B) Cells were grown with 5 $\mu\text{M}^{109}\text{Cd}^{2+}$ in broth for 30 min, centrifuged, and suspended in fresh broth. The single point (x) represents the $\text{Cd}^{2+}$ retained by unwashed membrane-filtered cells. The other points show the retained $\text{Cd}^{2+}$ with cells exposed to the indicated cations and washed twice with 5 ml of medium.

Fig. 6. Effects of cadmium on manganese uptake and retention. (A) Strains RN1008 and RN23 were grown, centrifuged, and suspended in broth at 0.11 mg of cells per ml $^{54}\text{Mn}^{2+}$ at 5 $\mu\text{M}$ was added with or without 50 $\mu\text{M} \text{Cd}^{2+}$. (B) Cells were grown in broth containing 0.1 $\mu\text{M}^{54}\text{Mn}^{2+}$ for the final 20 min before harvesting by centrifugation. After resuspension in fresh broth at 0.12 mg of cells per ml, 50 $\mu\text{M} \text{Cd}^{2+}$ was added to half of each culture as indicated.
Fig. 7. Effects of cadmium on magnesium and zinc uptake and retention. Late-log-phase cells of strains RN23 (resistant) and RN1008 (susceptible) were centrifuged and suspended at 0.11 (dry weight) of cells per ml in glycylglycine buffer (magnesium experiments) or broth (zinc experiments). \(^{28}\)Mg\(^{2+}\) (12.5 \(\mu\)M) or carrier-free \(^{65}\)Zn\(^{2+}\) (total concentration in broth, 10 \(\mu\)M Zn\(^{2+}\)) was added 5 min after the addition of 50 \(\mu\)M Cd\(^{2+}\) to part of each culture. For the efflux experiments, cells were incubated with trace \(^{28}\)Mg\(^{2+}\) for 3 h during growth or with trace \(^{65}\)Zn\(^{2+}\) for the final 20 min before the cells were harvested and resuspended at 0.11 mg/ml in glycylglycine buffer (\(^{28}\)Mg\(^{2+}\)) or broth (\(^{65}\)Zn\(^{2+}\)). Cd\(^{2+}\) at 50 \(\mu\)M was added 5 min after the initial filtration points were taken.
inhibition of Cd²⁺ uptake by 100 μM Mn²⁺ (Fig. 5A), coupled with the experiments showing a $K_m$ for Mn²⁺ transport between 6 and 8 μM Mn²⁺ (Fig. 8 and additional data), is not consistent with a simple model of two alternative substrates (and competitive inhibition) for a common transport mechanism. Cd²⁺ inhibited Mn²⁺ uptake more strikingly (Fig. 6A) than the converse. When the data from such experiments were forced to fit a kinetic model with either Lineeweaver-Burk plots (Fig. 8) or Dixon plots (data not shown), it appeared possible that Cd²⁺ is a competitive inhibitor of Mn²⁺ uptake with a $K_i$ of about 10 μM Cd²⁺ for the susceptible cells and a $K_i$ much greater than 50 μM Cd²⁺ for the resistant cells. Repeated experiments with varying experimental conditions failed to produce more definitive data.

**DISCUSSION**

Plasmid-determined resistance to cadmium has been found only with *S. aureus* (6, 7, 35). We never observed increased resistance to cadmium associated with any of the *Escherichia coli* and *Pseudomonas aeruginosa* plasmids that were tested. Part of the reason for this may be the inherently greater resistance to cadmium shown by *E. coli* and *P. aeruginosa* even in the absence of plasmids (9, 22).

Although it has been recognized for several years that the mechanism of resistance to cadmium occurs at the membrane level (7), the molecular basis for this resistance is not established. Chopra (7) hypothesized that the decreased level of uptake of cadmium by the resistant cells might be due to changes in the lipid structure of the membrane in the resistant cells. However, he could find no difference in the membrane lipids (7). Chopra also reported that he could find no difference in the membrane proteins between the susceptible and resistant strains with one-dimensional gel electrophoresis. In our hands, both one-dimensional and two-dimensional (1, 28) gel electrophoresis revealed the presence of new proteins in the membranes of the plasmid-bearing strains. Unfortunately, careful analysis of membranes from various point mutants in cadA and cadB, and from mutants deleted in the cadA region, failed to establish a consistent relationship between any of these proteins and either of the cadmium resistance alleles. If there are such cad-related proteins, they may be very minor membrane constituents beyond our limits of resolution, or perhaps the proteins are lost during the membrane isolation. Only subsequent experiments will clarify this situation. Attempts to measure Mn²⁺ (2, 3), Cd²⁺, or amino acid (30) transport in membrane vesicles from *S. aureus* were consistently unsuccessful, as well.

The specific effect of cadmium on the manganese transport in the susceptible cells and the lack of effect of cadmium on manganese transport in the resistant cells point to an energy-dependent manganese transport system as the primary avenue of entry of cadmium into *S. aureus* cells. In cells containing the plasmid-determined resistance gene for cadmium, the manganese transport system no longer accepts cadmium as an alternate substrate. None of the other cation uptake systems tested showed a difference between the susceptible and resistant strains. This specificity is in keeping with the known specific nature of the cation transport systems that have been studied primarily in other bacteria, but which appear to be quite similar in *S. aureus* (11, 15, 31; Weiss, unpublished data). All bacteria studied thus far have highly specific Mn²⁺ transport systems that discriminate between Mn²⁺ and the abundant divalent cations Mg²⁺ and Ca²⁺ (37). Mg²⁺ and Ca²⁺ would not be expected to affect transport of Cd²⁺ through these Mn²⁺ systems. Zn²⁺ may be transported by a highly specific transport system in bacteria (4, 12) or may be transported as an analog via the basic Mg²⁺ transport system (15). *E. coli* has two distinguishable Mg²⁺ transport systems, one of which also transports a range of “trace” cations including Co²⁺, Mn²⁺, and Ni²⁺ (15, 23, 29). Cd²⁺ apparently is not transported by these systems since high Mg²⁺ does not inhibit Cd²⁺ uptake. A more difficult question involves the relationship between Cd²⁺ and Zn²⁺ uptake. Experiments with animal cells and tissues have implicated Cd²⁺ in normal Zn²⁺ transport and metabolism (5, 13, 14). Novick and Roth (27) showed that the resistance plasmids studied here also confer three fold resistance to Zn²⁺, and that mutants isolated as Cd²⁺ susceptible also became Zn²⁺ susceptible. Furthermore, the basic chemistries of Cd²⁺ and Zn²⁺ are rather similar to one another, whereas those of Cd²⁺ and Mn²⁺ are rather different. Yet Zn²⁺ had little effect on Cd²⁺ uptake and retention (Fig. 5), and Cd²⁺ had little effect on Zn²⁺ uptake and retention (Fig. 7C and D). It was only with Mn²⁺ that we saw any striking effect with difference between the Cd²⁺-resistant and the Cd²⁺-suscepti-

**FIG. 8.** Kinetics of Mn²⁺ accumulation in susceptible and resistant cells. The initial rates of Mn²⁺ uptake in the presence and absence of 50 μM Cd²⁺ were determined in experiments as in Fig. 6A with strains RN1008 (susceptible) and RN29 (resistant).
ble cells (Fig. 6). Nevertheless, it is possible that interactions of Cd$^2+$ with the Zn$^2+$ transport system (4, 12) will be observed when optimum conditions for studying this system are found.

An additional hypothesis could be constructed based on a differential efflux of Cd$^2+$. The resistant cells might thus lower the intracellular Cd$^2+$ levels and prevent a secondary displacement of cellular Mn$^{2+}$ by Cd$^{2+}$ accumulated by a non-Mn$^{2+}$ system.

Plasmid-determined resistances having mechanisms based on altered energy-dependent transport are not without precedent. Tetracycline uptake by susceptible cells occurs by an energy-dependent concentrative process that is altered in strains bearing tetracycline-resistance plasmids (8, 18). Although a membrane protein associated with tetracycline resistance has been identified (8, 37), the role of this protein in decreasing tetracycline uptake by cells is not known.

Whereas plasmid-gene-determined Cd$^2+$ resistance has been reported only with S. aureus (10, 16, 17, 20, 35), and no specific cases of chromosomal-gene-determined Cd$^2+$ resistance are known, different bacterial species differ in inherent resistance to Cd$^2+$ (e.g. 9). Furthermore, there is a report by Mitra et al. (19) of "accommodation" to high cadmium levels in E. coli. This resistance was not genotypically based, in that growth without Cd$^{2+}$ led the cells to become Cd$^{2+}$ susceptible again (19). The mechanism of accommodation in E. coli appeared to involve a permeability block (19). It is not clear whether this process is similar to plasmid-determined resistance in any other way.

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LITERATURE CITED