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Decreased Chromate Uptake in *Pseudomonas fluorescens* Carrying a Chromate Resistance Plasmid

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CrO_4^{2-} resistance in *Pseudomonas fluorescens* LB300(pLHB1) was related to reduced uptake of CrO_4^{2-} relative to the plasmidless strain LB303. $^{51}CrO_4^{2-}$ was transported mainly via the SO_4^{2-} active transport system; thus, cells grown with 0.15 mM cysteine, a repressor of the SO_4^{2-} transport system, were much more resistant to CrO_4^{2-} than those grown with 0.15 mM djenkolic acid, which derepressed the $^{35}SO_4^{2-}$ uptake system. Kinetics of $^{51}CrO_4^{2-}$ uptake by *P. fluorescens* with and without the plasmid showed that the V_{max} for $^{51}CrO_4^{2-}$ uptake with the resistant strain was 2.2 times less than the V_{max} for the sensitive strain, whereas the K_m remained constant.

Bacterial resistance to CrO_4^{2-} has been found in several *Pseudomonas* strains (1, 3, 5, 14). Horitsu et al. (3) studied a CrO_4^{2-}-tolerant *Pseudomonas ambigua* strain obtained from activated sludge and isolated CrO_4^{2-}-sensitive derivatives to study the mechanism of CrO_4^{2-} tolerance. Bopp et al. (1) isolated a CrO_4^{2-}-resistant *Pseudomonas fluorescens* strain from CrO_4^{2-}-contaminated river sediment. CrO_4^{2-} resistance in *P. fluorescens* was plasmid specified and loss of the plasmid (pLHB1) resulted in a simultaneous loss of CrO_4^{2-} resistance. Another plasmid conferring resistance to CrO_4^{2-} was found in an antibiotic-resistant *Pseudomonas aeruginosa* isolate from the sputum of a hospital patient (14).

The biochemical and physiological mechanisms for resistance to CrO_4^{2-} are unclear. Horitsu et al. (3) showed that a CrO_4^{2-}-sensitive derivative of the CrO_4^{2-}-resistant *P. ambigua* strain accumulated six times more CrO_4^{2-} than did the resistant parental strain. Their studies involved atomic absorption measurements of chromium accumulated over periods of hours. The kinetic properties of CrO_4^{2-} transport were not studied. Enzymatic reduction of hexavalent Cr(VI) to trivalent Cr(III) has been shown in *Pseudomonas* strains (3, 4). Since Cr(III) is less toxic than Cr(VI) (8), the reduction of CrO_4^{2-} may lead to the detoxification of CrO_4^{2-}. However, it is unclear whether the reduction of CrO_4^{2-} is the mechanism of plasmid-governed resistance to CrO_4^{2-}.

The purpose of this paper is to determine the basic properties of the CrO_4^{2-} transport system(s) in *P. fluorescens* and to show whether reduced uptake of CrO_4^{2-} is responsible for CrO_4^{2-} resistance in plasmid-carrying bacteria.

P. fluorescens LB300(pLHB1) (CrO_4^{2-} resistant) and the plasmidless CrO_4^{2-}-sensitive variant, LB303, were gifts from L. H. Bopp (1) (General Electric Research and Development Center, Schenectady, N.Y.). Cells were grown in a minimal medium (modified from one described by Durham and Phibbs [2]) containing 15 mM Na_2HPO_4, 12.5 mM KH_2PO_4, 7.6 mM NH_4Cl, 0.8 mM $MgCl_2$, 20 μM $FeCl_3$, and 20 mM glucose (autoclaved separately). This medium was supplemented with 0.15 mM SO_4^{2-}, cysteine, or djenkolic acid as a sole sulfur source. Cultures were grown with aeration at 30°C. Turbidity measurements were done with a Klett-Summerson colorimeter (equipped with a no. 54 Wratten filter [Eastman Kodak Co., Rochester, N.Y.]).

Mid-log-phase cells for transport assays were harvested by centrifugation at 6,000 × *g* for 10 min at 4°C and washed once with SO_4^{2-}-free minimal medium. The transport assays were with 1.0 mg (dry weight) of cells in 1 ml of minimal medium that contained various concentrations of $Na_2{}^{51}CrO_4$ or $Na_2{}^{35}SO_4$. Samples (0.1 ml) were filtered (filter pore size, 0.45 μm; Nucleopore Corp., Pleasonton, Calif.; mixed cellulose esters) and rinsed twice with 5-ml volumes of nonradioactive uptake solution. Kinetic parameters were determined from the linear uptake phase, generally from 15 s to 5 min at 30°C. Measurements of $^{51}CrO_4^{2-}$ efflux from preloaded cells were carried out at 30°C by the procedures of Silver and Keach (12). [^{35}S]sulfate was obtained from New

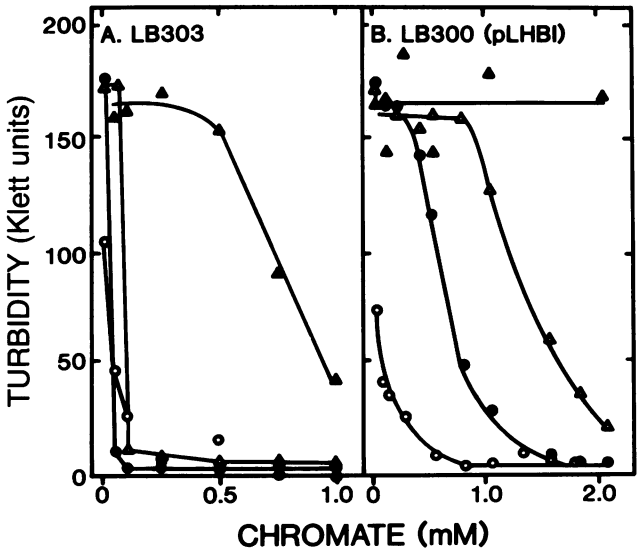

FIG. 1. Resistance to chromate in *P. fluorescens* strains. (A) Strain LB303 (sensitive); (B) strain LB300(pLHB1) (resistant). Growth was determined by turbidity after 22 h at 30°C in minimal medium with 0.15 mM djenkolic acid (○); 0.15 mM (●) or 0.75 mM (△) SO_4^{2-}; or 0.15 mM cysteine (▲).

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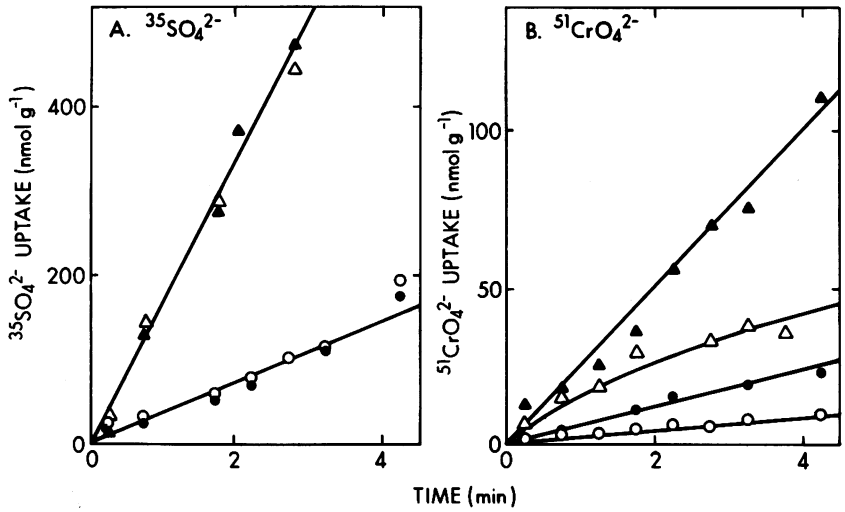

FIG. 2. Uptake of (A) $^{35}SO_4^{2-}$ and (B) $^{51}CrO_4^{2-}$ by chromate-resistant and -sensitive *P. fluorescens* at 30°C. Sensitive cells were assayed at 1 μM SO_4^{2-} or CrO_4^{2-} (●) or 5 μM SO_4^{2-} or CrO_4^{2-} (▲); resistant cells were assayed at 1 μM SO_4^{2-} or CrO_4^{2-} (○) or 5 μM SO_4^{2-} or CrO_4^{2-} (△).

England Nuclear Corp., Boston, Mass., and [^{51}Cr]chromate was obtained from ICN Pharmaceuticals Inc., Irvine, Calif. All samples were counted in a liquid scintillation spectrometer.

The effect of CrO_4^{2-} on the growth of sensitive and resistant *P. fluorescens* strains was tested (Fig. 1). The plasmidless strain *P. fluorescens* LB303 did not grow in the presence of 0.1 mM CrO_4^{2-} in minimal medium with 0.75 mM SO_4^{2-}, while strain LB300(pLHB1) could grow in medium with above 0.8 mM CrO_4^{2-}. The addition of high SO_4^{2-} significantly increased the growth of both *P. fluorescens* strains in the presence of CrO_4^{2-} and the ratio of resistance between plasmid-containing and plasmid-free cells (data not shown).

The level of CrO_4^{2-} resistance in *P. fluorescens* depended on the sulfur source in minimal medium (Fig. 1). Djenkolic acid-grown cells were most sensitive to CrO_4^{2-} toxicity in the presence of the plasmid pLHB1. Cells growing with cysteine were much more resistant to CrO_4^{2-} than cells growing on djenkolic acid or SO_4^{2-}. Similar results were obtained for the degree of CrO_4^{2-} resistance with various sulfur sources in *P. aeruginosa* with and without chromate resistance plasmids (data not shown).

There was a clear difference in $^{51}CrO_4^{2-}$ uptake between *P. fluorescens* LB300(pLHB1) and LB303 (Fig. 2). While both the sensitive and the CrO_4^{2-}-resistant strains took up about the same amount of $^{35}SO_4^{2-}$ (Fig. 2A), the sensitive cells took up about 2.2 times more $^{51}CrO_4^{2-}$ than did the resistant cells (Fig. 2B).

Kinetics of $^{35}SO_4^{2-}$ and $^{51}CrO_4^{2-}$ accumulation by *P. fluorescens* in minimal medium followed the Michaelis-Menten kinetics (Fig. 3). From a series of such experiments, it appeared that CrO_4^{2-} and SO_4^{2-} were competitive inhibitors of each other's transport. Kinetic parameters for $^{35}SO_4^{2-}$ uptake were similar for sensitive and resistant strains (Table 1). The V_{max} for $^{51}CrO_4^{2-}$ uptake with the resistant *P. fluorescens* LB300(pLHB1) was 2.2 times less than the V_{max} for the sensitive strain LB303. The K_m for $^{51}CrO_4^{2-}$ transport by the *P. fluorescens* strains was similar to the K_i for CrO_4^{2-} as an inhibitor of $^{35}SO_4^{2-}$ uptake.

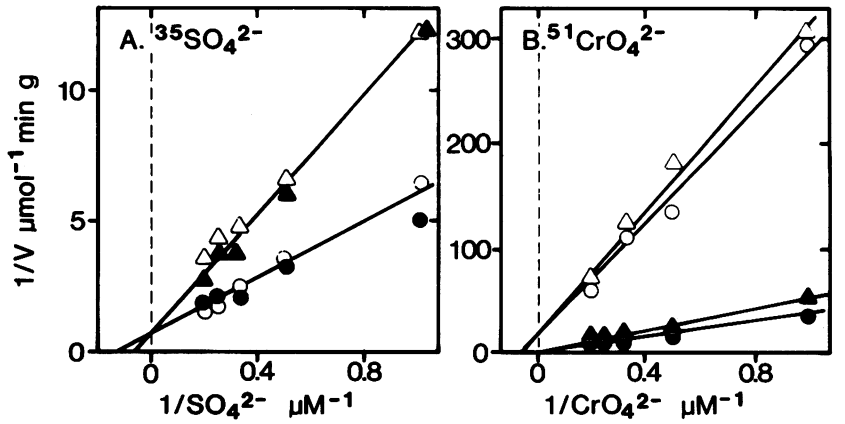

FIG. 3. Kinetics of $^{35}SO_4^{2-}$ and $^{51}CrO_4^{2-}$ transport in *P. fluorescens*. (A) $^{35}SO_4^{2-}$ or (B) $^{51}CrO_4^{2-}$ uptake with sensitive (●,▲) or resistant (○,△) cells at 30°C without added chromate or sulfate (○,●) or with 20 μM CrO_4^{2-} (△,▲ [panel A]) or 100 μM SO_4^{2-} (△,▲ [panel B]). Cells were grown into log phase with 0.15 mM SO_4^{2-}.

TABLE 1. Kinetic parameters for sulfate and chromate transport by *Pseudomonas fluorescens*

Type of transport and parameter	Mean value ± SD (no. of expts) at 30°C for strain[a]:	
	LB303	LB300(pLHB1)
Sulfate transport		
V_{max} (μmol of SO_4^{2-} min^{-1} g^{-1})	0.84 ± 0.11 (3)	0.91 ± 0.06 (4)
K_m (μM SO_4^{2-})	6.4 ± 0.5 (3)	6.5 ± 1.2 (4)
K_i (μM CrO_4^{2-})	12.7 ± 1.4 (3)	14.1 ± 5.3 (4)
Chromate transport		
V_{max} (μmol of CrO_4^{2-} min^{-1} g^{-1})	0.17 ± 0.02 (4)	0.076 ± 0.014 (4)
K_m (μM CrO_4^{2-})	19 ± 10 (4)	18 ± 7 (4)
K_i (μM SO_4^{2-})	302 ± 183 (4)	137 ± 73 (3)

[a] Cells were grown in minimal medium with 0.15 mM SO_4^{2-}. LB303, Sensitive strain; LB300(pLHB1), resistant strain.

However, the K_m for $^{35}SO_4^{2-}$ transport was clearly less than from the K_i for SO_4^{2-} as an inhibitor of $^{51}CrO_4^{2-}$ uptake. Furthermore, the K_i for SO_4^{2-} inhibiting chromate transport was 2.2 times greater in the resistant than in the sensitive cells.

CrO_4^{2-} resistance in *P. fluorescens* was related to reduced uptake of $^{51}CrO_4^{2-}$. Bopp and Ehrlich (manuscript in preparation) also concluded that reduced uptake was responsible for the chromate resistance of *P. fluorescens* LB300 (pLHB1). Kinetics of $^{51}CrO_4^{2-}$ uptake by *P. fluorescens* strains showed that the V_{max} for $^{51}CrO_4^{2-}$ uptake with the resistant strain was 2.2 times less than the V_{max} for the sensitive strain. $^{51}CrO_4^{2-}$ was mainly (but apparently not exclusively) accumulated by the SO_4^{2-} active transport system in *P. fluorescens*. The ability of SO_4^{2-} to protect growing cells from the inhibitory effects of CrO_4^{2-} (Fig. 1) suggests a direct competition between SO_4^{2-} and CrO_4^{2-} for the same transport carrier. $^{35}SO_4^{2-}$ and $^{51}CrO_4^{2-}$ uptake followed Michaelis-Menten kinetics (Fig. 3), and Lineweaver-Burk plots showed that SO_4^{2-} and CrO_4^{2-} were competitive inhibitors of $^{51}CrO_4^{2-}$ and $^{35}SO_4^{2-}$ uptake, respectively. However, the K_i for SO_4^{2-} as an inhibitor of $^{51}CrO_4^{2-}$ uptake was much larger than the K_m for $^{35}SO_4^{2-}$ uptake (in both sensitive and resistant *P. fluorescens* strains; Table 1). These results suggest that $^{51}CrO_4^{2-}$ was transported partially via a second transport system without involvement of the SO_4^{2-} carrier.

Microorganisms take up normal nutrient cations and anions via specific, energy-dependent transport systems (10). Toxic ions are generally thought to enter cells by the same transport system as used for structurally related nutrient ions (11, 13, 15).Since CrO_4^{2-} is toxic for *Pseudomonas* strains, one does not expect these bacterial strains to have developed a transport system specific for CrO_4^{2-}. It is still unclear which additional transport systems might be responsible for $^{51}CrO_4^{2-}$ transport without involvement of SO_4^{2-} carriers.

We do not know the molecular basis of the reduced uptake of $^{51}CrO_4^{2-}$ in the resistant strain. Accumulation of toxic ions may be impaired either by modification of specific carriers (11) or by possession of efflux systems (6, 12, 16).

To determine whether a CrO_4^{2-} efflux system is operative in the resistant cells, the release of $^{51}CrO_4^{2-}$ from preloaded cells was tested. Although $^{51}CrO_4^{2-}$ was released to some extent from preloaded cells, there was no relationship between the rate or extent of $^{51}CrO_4^{2-}$ release and the presence of a CrO_4^{2-} resistance plasmid (data not shown). Resistant cells took up significant amounts of $^{51}CrO_4^{2-}$ within the short period of time (Fig. 2). In this respect, $^{51}CrO_4^{2-}$ transport by CrO_4^{2-}-resistant *P. fluorescens* seems to differ from the lack of net $^{109}Cd^{2+}$ and $^{74}AsO_4^{3-}$ accumulation by resistant strains (13, 15).

Chromate-resistant chromosomal mutants were isolated in *Salmonella typhimurium* (7). These mutants were defective in SO_4^{2-} transport, and most mapped to a single chromosomal locus. This locus was different (7) from that determining the sulfate-binding protein, the periplasmic protein involved in SO_4^{2-} transport (9). The *Pseudomonas* SO_4^{2-} and CrO_4^{2-} transport system was derepressed by growth on djenkolic acid and repressed by growth on cysteine, similarly to *S. typhimurium* (7). However, we do not know whether a periplasmic binding protein is involved in the *Pseudomonas* system or how the plasmid resistance determinant interacts with the chromosomally determined SO_4^{2-} and CrO_4^{2-} transport system.

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LITERATURE CITED

1. **Bopp, L. H., A. M. Chakrabarty, and H. L. Ehrlich.** 1983. Chromate resistance plasmid in *Pseudomonas fluorescens*. J. Bacteriol. **155**:1105–1109.
2. **Durham, D. R., and P. V. Phibbs, Jr.** 1982. Fractionation and characterization of the phosphoenolpyruvate-fructose 1-phosphotransferase system from *Pseudomonas aeruginosa*. J. Bacteriol. **149**:534–541.
3. **Horitsu, H., S. Futo, K. Ozawa, and K. Kawai.** 1983. Comparison of characteristics of hexavalent chromium-tolerant bacterium, *Pseudomonas ambigua* G-1, and its hexavalent chromium-sensitive mutant. Agric. Biol. Chem. **47**:2907–2908.
4. **Kvasnikov, E. I., V. V. Stepanyuk, T. M. Klyushnikova, N. S. Serpokrylov, G. A. Simonova, T. P. Kasatkina, and L. P. Panchenko.** 1985. A new chromium-reducing Gram-variable bacterium with mixed type flagellation. Mikrobiologiya **54**:83–88.
5. **Lebedeva, E. V., and N. N. Lyalikova.** 1979. Reduction of crocoite by *Pseudomonas chromatophila* sp. nov. Mikrobiologiya **48**:517–522.
6. **Mobley, H. L. T., and B. P. Rosen.** 1982. Energetics of plasmid-mediated arsenate resistance in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **79**:6119–6122.
7. **Ohta, N., P. R. Galsworthy, and A. B. Pardee.** 1971. Genetics of sulfate transport by *Salmonella typhimurium*. J. Bacteriol. **105**:1053–1062.
8. **Petrilli, F. L., and S. DeFlora.** 1977. Toxicity and mutagenicity of hexavalent chromium on *Salmonella typhimurium*. Appl. Environ. Microbiol. **33**:805–809.
9. **Pfugrath, J. W., and F. A. Quiocho.** 1985. Sulphate sequestered in the sulphate-binding protein of *Salmonella typhimurium* is bound solely by hydrogen bonds. Nature (London) **314**:257–260.

10. **Silver, S.** 1978. Transport of cations and anions, p. 221–324. *In* B. P. Rosen (ed.), Bacterial transport. Marcel Dekker, Inc., New York.
11. **Silver, S., K. Budd, K. M. Leahy, W. V. Shaw, D. Hammond, R. P. Novick, G. R. Willsky, M. H. Malamy, and H. Rosenberg.** 1981. Inducible plasmid-determined resistance to arsenate, arsenite and antimony(III) in *Escherichia coli* and *Staphylococcus aureus*. J. Bacteriol. **146**:983–996.
12. **Silver, S., and D. Keach.** 1982. Energy-dependent arsenate efflux: the mechanism of plasmid-mediated resistance. Proc. Natl. Acad. Sci. USA **79**:6114–6118.
13. **Silver, S., and T. K. Misra.** 1984. Bacterial transformations of and resistances to heavy metals, p. 23–46. *In* G. S. Omenn and A. Hollaender (ed.), Genetic control of environmental pollutants. Plenum Publishing Corp., New York.
14. **Summers, A. O., and G. A. Jacoby.** 1978. Plasmid-determined resistance to boron and chromium compounds in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **13**:637–640.
15. **Tynecka, Z., Z. Gos, and J. Zajac.** 1981. Reduced cadmium transport determined by a resistance plasmid in *Staphylococcus aureus*. J. Bacteriol. **147**:305–312.
16. **Tynecka, Z., Z. Gos, and J. Zajac.** 1981. Energy-dependent efflux of cadmium coded by a plasmid resistance determinant in *Staphylococcus aureus*. J. Bacteriol. **147**:313–319.