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Identification of Intrinsic High-Level Resistance to Rare-Earth Oxides and Oxyanions in Members of the Class *Proteobacteria*: Characterization of Tellurite, Selenite, and Rhodium Sesquioxide Reduction in *Rhodobacter sphaeroides*

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We have identified intrinsic high-level resistance (HLR) to tellurite, selenite, and at least 15 other rare-earth oxides and oxyanions in the facultative photoheterotroph *Rhodobacter sphaeroides* grown either chemoheterotrophically or photoheterotrophically. Other members of the class *Proteobacteria*, including members of the α-2 and α-3 phylogenetic subgroups, were also shown to effect the reduction of many of these compounds, although genera from the α-1, β-1, and γ-3 subgroups did not express HLR to the oxyanions examined. Detailed analyses employing *R. sphaeroides* have shown that HLR to at least one class of these oxyanions, the tellurite class (e.g., tellurate, tellurite, selenate, selenite, and rhodium sesquioxide), occurred via intracellular oxyanion reduction and resulted in deposition of metal in the cytoplasmic membrane. The concomitant evolution of hydrogen gas from cells grown photoheterotrophically in the presence of these oxyanions was also observed. HLR to tellurite class oxyanions in *R. sphaeroides* was not affected by exogenous methionine or phosphate but was reduced 40-fold by the addition of cysteine to growth media. In contrast HLR to the periodate class oxyanions (e.g., periodate, siliconate, and siliconite) was inhibited by extracellular PO_4^{3-} but did not result in metal deposition or gas evolution. Finally, we observed that HLR to arsenate class oxyanions (e.g., arsenate, molybdate, and tungstate) occurred by a third, distinct mechanism, as evidenced by the lack of intracellular metal deposition and hydrogen gas evolution and an insensitivity to extracellular PO_4^{3-} or cysteine. Examination of a number of *R. sphaeroides* mutants has determined the obligate requirement for an intact CO_2 fixation pathway and the presence of a functional photosynthetic electron transport chain to effect HLR to K_2TeO_3 under photosynthetic growth conditions, whereas functional cytochromes bc_1 and c_2 were required under aerobic growth conditions to facilitate HLR. Finally, a purification scheme to recover metals from intact bacterial cells was developed.

Salts of the rare-earth oxyanions tellurite (TeO_3^{2-}) and selenite (SeO_3^{2-}) have long been employed as selective agents in bacteriological media to prevent the growth of gram-negative bacteria, most of which are highly sensitive to these oxidants. In fact, TeO_3^{2-}, like many rare-earth oxyanions, is toxic to most microorganisms at concentrations as low as 1 μg/ml (38, 39). Although intrinsic low-level resistance (LLR) to TeO_3^{2-} in a few gram-positive organisms (e.g., *Corynebacterium diphtheriae*, *Streptococcus faecalis*, and some *Staphylococcus aureus* strains) has previously been reported, little is known about the mechanism(s) responsible for this resistance (39).

In contrast, R-plasmid-mediated LLR to TeO_3^{2-} in gram-negative bacteria has been studied extensively in recent years (16, 38, 47–49). Summers and Jacoby first identified a TeO_3^{2-} resistance determinant on an IncP plasmid isolated from *Pseudomonas aeruginosa* (38), while Walter and Taylor have recently identified and cloned two genetically different TeO_3^{2-} resistance determinants from IncHII and IncPα broad-host-range plasmids (47). These determinants were expressed in *Escherichia coli* and several other gram-negative bacteria (47). The former encodes a reductase distinctly different from that of either the extreme thermophile *Thermus thermophilus* (2) or *Alcaligenes* sp. (17), while the latter appears to function in the reduced uptake or increased efflux of TeO_3^{2-} (48). Expression of these determinants in *E. coli* resulted in high-level resistance (HLR) to TeO_3^{2-}, with an MIC of 512 μg/ml (47).

Chiong et al. have purified a protein fraction from *T. thermophilus* which contained an NADH/NADPH-dependent tellurite- and selenite-reducing activity (2), while Terai et al. have demonstrated TeO_3^{2-} reduction in cell extracts of *Mycobacterium avium* (44). TeO_3^{2-} reductase activity has also been observed in *Alcaligenes* sp.; the activity is encoded by a large conjugative plasmid of the IncHI-2 incompatibility group (17).

In sharp contrast to previous work, we have identified photosynthetic members of the purple nonsulfur bacterial class *Proteobacteria* (36), including *Rhodobacter sphaeroides* (46), which demonstrated intrinsic, constitutive HLR to TeO_3^{2-} at concentrations approaching 1 mg/ml (4 mM), dependent upon growth conditions. We have also determined that in addition to TeO_3^{2-} and TeO_4^{2-}, *R. sphaeroides* is also resistent to at least 15 other toxic, rare-earth oxyanions including arsenate, periodate, stannate, tungstate, vanadate, and the oxoacids of molybdenum, rhodium, selenium, and silicon.

Biochemical and physiological analyses have determined that at least three distinct resistance mechanisms are responsible for HLR to these compounds. HLR to one group of these compounds, the tellurite class involves a membrane-localized, reduced flavin adenine dinucleotide ($FADH_2$)-

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TABLE 1. Bacterial strains

Species and strain	Relevant genotype or phenotype[a]	Source	Reference
Escherichia coli			
JM83	*ara* Δ(*lac-proAB*) *rpsL thi* φ80d*lacZ*Δ*M15*	J. Messing	24
S17-1	C600::RP-4 2-Tc::Mu::Km::Tn*7* *hsdR hsdM*$^+$ *recA*	A. Puhler	31
Rhodobacter capsulatus B10	Wild type	J. Wall	50
Rhodobacter sphaeroides 2.4.1	Wild type, 5 endogenous plasmids	W. Sistrom	46
2.4.1ΔS	2.4.1 Δ(42-kb plasmid)	A. Suwanto	40
2.4.1-Ga	Car$^-$	W. Sistrom	3
2.4.7	Wild type, 2 endogenous plasmids	W. Sistrom	46
BC17	2.4.1-Ga *fbcBC* Car$^-$ Km^r	C.-H. Yun	56
CFXA$^-$	*cfxA* Km^r	P. Hallenbeck	13
CFXB$^-$	*cfxB* Sp^r Sm^r	P. Hallenbeck	13
CFXA$^-$B$^-$	*cfxA cfxB* Km^r Sp^r Sm^r	P. Hallenbeck	13
CYCA1	*cycA* Km^r	T. Donohue	7
MM1004	2.4.1::Tn*phoA* DORase$^-$ Km^r	This laboratory	25
MM1006	2.4.1::Tn*phoA* Bchl$^-$ Km^r	This laboratory	25
PRKA$^-$	*prkA* Km^r	P. Hallenbeck	14
PRKB$^-$	*prkB* Sp^r Sm^r	P. Hallenbeck	14
PRKA$^-$B$^-$	*prkA prkB* Km^r Sp^r Sm^r	P. Hallenbeck	14
PUC705-BA	*pucBA* Km^r	J. Lee	19
PUFB1	*pufBALMX* Km^r	J. Davis	4
PUHA1	*puhA* Km^r	T. Donohue	34
RS2	Wild type	S. Harayama	23
WS8	Wild type, 1 endogenous plasmid	W. Sistrom	33
Rhodocyclus gelatinosus str-1	Wild type	R. Uffen	45
Rhodopseudomonas palustris 1e5	Wild type	G. Drews	9
Rhodopseudomonas viridis F	Wild type	G. Drews	8
Rhodospirillum rubrum Ha	Wild type	G. Drews	46

[a] Km^r, Sp^r, and Sm^r denote resistance to kanamycin, spectinomycin, and streptomycin, respectively.

dependent enzymatic reduction of the oxyanion to its metallic ground state, deposition of the metal in the cytoplasmic membrane, and concomitant evolution of hydrogen gas from photoheterotrophically grown cells.

Two additional groups of oxyanions, the periodate class and the arsenate class were also examined. Unlike the tellurite class oxyanions, which *R. sphaeroides* intrinsically reduced, HLR to these compounds occurred in the absence of oxyanion reduction, metal deposition, or gas evolution. These classes could be distinguished, however, by their differing sensitivities to oxyanions in the presence of extracellular PO_4^{3-}. Both of these classes appeared to facilitate resistance to oxyanions by a mechanism similar to that encoded by the *telA* and *telB* determinants of the IncPα plasmid RK2 (48, 49).

In this report we characterize rare-earth oxide and oxyanion resistance in members of the *Proteobacteria* and present evidence suggesting the utility of *R. sphaeroides* as an agent for the bioremediation of oxyanion-contaminated environments. The enormous metabolic diversity exhibited by this organism with respect to its nutritional requirements, its ability to thrive in ambient environmental conditions, and its ability to effect oxyanion reduction and intracellular metal sequestration offers evidence of the enormous commercial potential for the exploitation of *R. sphaeroides* as a biocatalyst for the reclamation of rare-earth metals.

(A preliminary report of this work has been presented [26].)

MATERIALS AND METHODS

Bacterial strains, growth conditions, and media. Bacterial strains used in this study are listed in Table 1. All members of the *Proteobacteria* were grown at 30°C, with the exception of *E. coli*, which was cultured at 37°C on a Gyrotory shaker. Cultures of *R. sphaeroides* and *Rhodocyclus gelatinosus* were grown in LB (21), YP (55), or Sistrom's minimal medium A (SMM) containing either 0.4% succinate, 0.4% malate, or 0.4% butyrate as a carbon source (5, 10). Cultures of *Rhodobacter capsulatus* were grown in RCVB minimal medium containing 0.4% malate as a carbon source (50); *Rhodospirillum rubrum* was grown in SMM containing 0.4% malate and 0.1% yeast extract. *Rhodopseudomonas palustris* and *Rhodopseudomonas viridis* were grown in SMM containing 0.4% malate, 0.1% yeast extract, and 50 μg each of *p*-aminobenzoic acid and cyanocobalamin per ml. When necessary, antibiotics were added to growth media at the following final concentrations: kanamycin, 25 μg/ml; spectinomycin, 50 μg/ml; and streptomycin, 50 μg/ml. Anaerobic growth of *R. sphaeroides* in the dark on SSM medium containing dimethyl sulfoxide (DMSO) and photoheterotrophic growth conditions have been previously reported (6).

MICs. Quantitative susceptibility tests were performed by the tube dilution method in medium containing graded concentrations of inhibitor inoculated with approximately 5×10^7 organisms. The MIC was defined as the lowest concentration of inhibitor preventing growth at 30°C after 48 h of incubation for cultures grown chemoheterotrophically or photoheterotrophically (at incident light intensities of 100 and 10 W/m^2), after 96 h of incubation for cultures grown photoheterotrophically at an incident light intensity of 3 W/m^2 or after 120 h of incubation for cultures grown anaerobically in the dark in SSM medium containing 60 mM DMSO. These conditions allowed for the longer generation times of the purple nonsulfur bacteria under low-light photoheterotrophic and anaerobic-dark-DMSO growth conditions.

Cell fractionation and enzyme assays. Subcellular fractions were prepared by the method of Weiss (51), as previously reported (42). Protein content was determined by using the Lowry method as modified by Markwell et al. (20, 22); bovine serum albumin was the standard. The TeO_3^{2-}-dependent oxidation of NADH, NADPH, and $FADH_2$ was assayed with a Lambda 4C UV/VIS spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.). $FADH_2$ was prepared from FAD immediately prior to assay by reduction with excess $Na_2S_2O_4$.

Isolation and recovery of Te^0 from whole cells. After spheroplast formation (51), the total membrane fraction of a 1-liter culture of *R. sphaeroides* 2.4.1 grown in the presence of TeO_3^{2-} was suspended in solubilization buffer containing 50 mM EDTA, 1 N NaOH, and 0.1% sodium dodecyl sulfate and then sonicated at room temperature for 10 min (50% duty cycle) in a Sonifier Cell Disruptor, Model W-350, fitted with a microtip (Branson Sonic Power Co., Danbury, Conn.). Dense membrane-metal complexes which pelleted after low-speed centrifugation (10,000 × *g*, 10 min, 4°C), were subsequently layered onto a discontinuous (20:40:60 [wt/vol]) sucrose gradient (130,000 × *g*, 2 h, 4°C) prepared in ICM buffer (0.1 M Tris-HCl, 10 mM EDTA, pH 8.8). Following centrifugation, a coarse, black, metallic pellet was recovered and then washed with ICM buffer to remove excess sucrose. After sequential extraction with acetone-methanol (5:2) and 95% ethanol and drying at 100°C for 8 h, a finely divided black metallic powder was collected and weighed. Identification of this powder as Te^0 was consistent with the physical properties of elemental Te (black metal, insoluble in aqueous and organic solvents); the known oxoacids of Te are colorless and soluble in aqueous and polar organic solvents.

Analytical chemistry. The metallic material isolated was subjected to a quantitative determination for tellurium, using the phosphinate reduction assay (15). Samples were treated at 100°C for 2 h in the presence of 3 M H_2SO_4; K_2TeO_3 was independently assayed as the standard. Headspace gases from 1-liter photoheterotrophic cultures of *R. sphaeroides* 2.4.1 grown either in the presence or absence of 250 μg of K_2TeO_3 per ml were collected over water, transferred to evacuated collection funnels, and dried at −70°C for 12 h prior to mass spectrographic analysis.

Mass spectroscopy. Gas samples were analyzed in the Analytical Chemistry Center of The University of Texas Medical School by direct injection, using an INCOS 50 mass spectrometer (Finnigan MAT, Bremen, Germany); mass spectra were obtained by electron impact. Prior to analysis and between samples, the instrument was purged with Ar (ultrahigh purity grade [UHP of 5.0]); H_2 (UHP of 5.0) was subsequently analyzed as a positive control.

Materials. Amino acids, antibiotics, FAD, K_2TeO_4, NADH, NADPH, $NaH_2PO_2 \cdot H_2O$, Na_2MoO_4, $Na_2S_2O_4$, Na_2SeO_3, Na_2SeO_4, Na_2SiO_4, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, *N*-tosyl-L-phenylalanine chloromethyl ketone, and vitamins were all obtained from Sigma Chemical Co. (St. Louis, Mo.). $Na_2HAsO_4 \cdot 7H_2O$ was the product of Allied Chemical Co. (Morristown, N.J.), and $NaIO_4$ was the product of the G. F. Smith Chemical Co., (Columbus, Ohio). K_2TeO_3 and $NaCO_2CH_2SH$ were obtained from Difco Laboratories (Detroit, Mich.); CrO_3, $KMnO_4$, MoO_3, Na_2CrO_4, Na_2SiO_3, $NaSnO_3 \cdot 3H_2O$, Na_2SO_3, $Na_2WO_4 \cdot 2H_2O$, NH_4VO_3, $Rh_2O_3 \cdot 5H_2O$, Sb_2O_3, and SnO_2 were all obtained from Aldrich Chemical Co. (Milwaukee, Wis.). All other chemicals were of reagent grade purity and were used without further purification.

TABLE 2. Determination of intrinsic HLR to TeO_3^{2-} by some representative members of the *Proteobacteria*

Species	Strain	Phylogenetic subgroup[a]	MIC of K_2TeO_3 (μg/ml)[b]	
			Aerobic	Photosynthetic[c]
Rhodospirillum rubrum	Ha	α-1	20	10
Rhodopseudomonas palustris	1e5	α-2	200	100
Rhodopseudomonas viridis	F	α-2	80	50
Rhodobacter sphaeroides	2.4.1	α-3	900	600
	WS8	α-3	800	600
	2.4.7	α-3	800	500
	RS2	α-3	400	250
Rhodobacter capsulatus	B10	α-3	800	500
Rhodocyclus gelatinosus	str-1	β-1	5	10
Escherichia coli	JM83	γ-3	<5	NA[d]
	S17-1	γ-3	<5	NA

[a] Based on the classification of Woese et al. (52–54).
[b] MICs were determined in the appropriate minimal synthetic medium at 30°C.
[c] Incident light intensity, 10 W/m².
[d] NA, not applicable.

RESULTS

TeO_3^{2-} resistance in members of the *Proteobacteria*. While most gram-negative bacteria are unable to grow in media containing as little as 1 μg of TeO_3^{2-} per ml, we observed some years ago that *R. sphaeroides* 2.4.1 grew normally in Sistrom's minimal succinate medium containing 100 μg of K_2TeO_3 per ml (0.4 mM) under photosynthetic (incident light intensity of 10 W/m²) (30) and anaerobic-dark-DMSO (27) growth conditions. We extended this observation by demonstrating that the MICs of K_2TeO_3 for this strain grown in SMM under chemoheterotrophic and photoheterotrophic growth conditions were 900 and 600 μg/ml (3.5 and 2.4 mM), respectively (Table 2). To determine if this was a strain- or genus-specific phenomenon, we assayed HLR to TeO_3^{2-} in a number of wild-type *R. sphaeroides* strains as well as in other phylogenetically related purple nonsulfur species.

Table 2 summarizes the susceptibility data for cultures grown either aerobically or photosynthetically in minimal media in the presence of TeO_3^{2-}. Under aerobic conditions, both *R. sphaeroides* and *R. capsulatus* expressed intrinsic HLR to TeO_3^{2-}, with MICs at least 80 times higher than the MIC previously reported for *E. coli*, an enteric member of the γ-3 phylogenetic subgroup (53). *R. palustris*, an α-2 species (52), expressed intrinsic resistance to TeO_3^{2-} 40 times greater than that expressed by *E. coli*, while *R. viridis* expressed LLR to TeO_3^{2-} at concentrations <75 μg/ml. In contrast, two photosynthetic members of the α-1 (52) and β-1 (54) subgroups (*R. rubrum* and *R. gelatinosus*, respectively) were able to effect only LLR to TeO_3^{2-}.

These data demonstrated that intrinsic HLR to TeO_3^{2-} was expressed by only a few species of purple nonsulfur bacteria under chemoheterotrophic and photoheterotrophic growth conditions. Moreover, the level of TeO_3^{2-} resistance was strain dependent: the MIC of K_2TeO_3 for *R. sphaeroides* RS2 was approximately two- to threefold lower than the MIC for strain 2.4.1, 2.4.7, or WS8. With the exception of *R. gelatinosus*, which exhibited no growth-dependent difference in inhibitory TeO_3^{2-} concentration, MICs in minimal media were approximately 50% higher when cells were grown aerobically, regardless of the strain or species.

Two strains of *E. coli*, JM83 and S17-1, were also exam-

TABLE 3. Effects of medium composition and growth conditions on HLR to TeO_3^{2-} in *R. sphaeroides* 2.4.1

Medium[a]	Supplement[b]	MIC of K_2TeO_3 (μg/ml)			
		Aerobic	Photosynthetic		Anaerobic-dark[c]
			10 W/m²[d]	3 W/m²	
Complex					
LB		20	80	40	<10
YP		20	20	10	<10
Proteose-Peptone		10	20	10	10
Defined					
SMM + butyrate		1,000	700	400	200
SMM + succinate		900	600	300	150
SMM + malate		800	400	150	100
SMM + succinate	30 mM TMAO	850	650	300	150
	1 mM L-methionine	800	500	200	100
	1 mM cystine	500	550	200	100
	1 mM glutathione	550	550	250	150
	1 mM thioglycolate	500	500	250	100
	0.3% peptone	30	20	20	20
	0.3% yeast extract	30	20	10	10
	0.3% tryptone	20	30	10	20
	0.3% CAA	20	30	10	20
	1 mM L-cysteine	20	40	20	10

[a] SMM contained the carbon source listed at 0.4%.
[b] Supplement was added to culture medium to the final concentration listed. TMAO, trimethylamine-*N*-oxide; CAA, Casamino Acids.
[c] Supplemented with 60 mM DMSO.
[d] Incident light intensity.

ined as controls. Both failed to grow in minimal medium containing 5 μg of K_2TeO_3 per ml. This observation was consistent with previous studies which reported that the MIC of K_2TeO_3 for *E. coli* was ≈1 μg/ml (16, 38).

Growth of members of the *Proteobacteria* in the presence of Te-, Se-, and Rh-containing oxyanions. Cells grown in liquid medium containing TeO_3^{2-} or TeO_4^{2-} settled to the bottom of culture tubes over the course of the growth phase due to the intracellular accumulation of a dense metallic deposit. Copious gas evolution was observed concomitant with cell growth. Centrifugation of broth cultures resulted in a black cell pellet and a clear supernatant. Colonies of *R. sphaeroides* which formed on agar medium containing TeO_3^{2-} produced a black deposit which did not diffuse into the medium. Cells remained viable despite the accumulation of intracellular deposits: black colonies streaked onto agar medium containing no TeO_3^{2-} gave rise to normally pigmented colonies apparently through the dilution of metal complexes in the membranes of progeny cells.

Similar results were obtained for Se- and Rh-containing compounds: when culture media contained SeO_3^{2-} or SeO_4^{2-}, the cells became bright red in color; in rhodium sesquioxide-containing media, the cells appeared greyish bronze. The relative toxicity of these five compounds to *R. sphaeroides*, $SeO_4^{2-} > TeO_4^{2-} > TeO_3^{2-} > SeO_3^{2-} > Rh_2O_3 \cdot 5H_2O$, was significantly different from data published for *E. coli*, in which the +VI valence oxoacids of Te and Se were substantially less toxic than their +IV valence counterparts (29).

Effects of culture conditions and medium composition on HLR to TeO_3^{2-}. While cultures of *R. sphaeroides* 2.4.1 grown in SMM expressed HLR to TeO_3^{2-}, cells grown in rich medium such as LB, YP, or Proteose-Peptone were sensitive to very low levels of the oxyanion (Table 3). This was true for cultures grown aerobically or anaerobically. Likewise, a 30- to 40-fold reduction in TeO_3^{2-} resistance was observed when SMM was supplemented with peptone, Casamino Acids, tryptone, or yeast extract. To determine if there was a single common component present in these supplements which was affecting HLR, SMM containing 0.4% succinate was supplemented individually with each of the 20 amino acids (27). This analysis confirmed a single amino acid, cysteine, could be solely responsible for the increased sensitivity to TeO_3^{2-}. Neither cystine, glutathione, nor thioglycolate, however, decreased HLR to TeO_3^{2-} when added to SMM, nor did the presence of alternate electron acceptors, such as trimethylamine-*N*-oxide or DMSO (Table 3). In separate analyses, we observed a similar cysteine-dependent inhibition of HLR to TeO_4^{2-}, SeO_3^{2-}, and SeO_4^{2-} (27). The addition of exogenous L-methionine, however, did not effect HLR to any of these compounds (27).

The MIC of K_2TeO_3 for *R. sphaeroides* 2.4.1 in minimal medium was at least 50% higher in aerobically grown cultures than in photosynthetically grown ones, regardless of the carbon source provided. This was consistent with results obtained earlier for cells grown in succinate-containing SMM (Table 2). Our analyses also demonstrated that the HLR to TeO_3^{2-} in photosynthetically grown cultures was directly proportional to incident light intensity; in minimal medium, MICs were at least twofold higher for cultures grown at 10 W/m² than for those grown at 3 W/m² (Table 3).

A final observation with respect to medium composition was made concerning the effect the oxidation state of carbon sources had on the level of TeO_3^{2-} resistance in *R. sphaeroides* 2.4.1. While the MIC of K_2TeO_3 for cells grown aerobically in SMM containing malate as the carbon source was 800 μg/ml, when more reduced carbon sources such as succinate or butyrate were substituted, the MICs increased to 900 and 1,000 μg/ml, respectively. Similar results were also observed when cells were grown photosynthetically or anaerobically in the dark in SMM containing DMSO (Table 3). These data suggested that the toxicity of TeO_3^{2-} was inversely related to the oxidation state of the carbon source:

TABLE 4. Quantitation of rare-earth oxide and oxyanion resistance in *R. sphaeroides* 2.4.1

Compound	Intracellular deposition	Gas evolution	MIC, μg/ml (mM)[a]	
			Standard medium	Low-PO_4^{3-} medium[b]
MoO_3	−	−	SS[c]	SS
NH_4VO_3	−	−	SS	SS
Sb_2O_3	−	−	SS	SS
SnO_2	−	−	SS	SS
$Rh_2O_3 \cdot 5H_2O$	+++	+++	SS	SS
Na_2SeO_4	++	+	150 (0.81)	100 (0.53)
Na_2SeO_3	++++	+++	800 (4.6)	500 (2.9)
K_2TeO_4	+++	++	500 (1.9)	500 (1.9)
K_2TeO_3	++++	++++	600 (2.4)	600 (2.4)
$NaIO_4$	−	−	400 (1.9)	150 (0.70)
Na_2SiO_3	−	−	400 (3.3)	100 (0.81)
Na_2SiO_4	−	−	300 (2.2)	100 (0.73)
Na_2HAsO_4	−	−	1,500 (8.0)	1,600 (8.6)
Na_2MoO_4	−	−	1,400 (6.8)	1,500 (7.3)
Na_2WO_4	−	−	1,600 (5.4)	1,600 (5.4)
Na_2SnO_4	−	−	800 (3.5)	800 (3.5)
Na_2SO_3	−	−	600 (4.8)	500 (4.0)
Na_2CrO_4	−	−	10 (0.06)	20 (0.12)
$KMnO_4$	−	−	20 (0.12)	<10 (<0.06)
CrO_3	−	−	20 (0.21)	<10 (<0.10)

[a] Photoheterotrophic growth in SMM containing succinate (10-W/m^2 incident light intensity).

[b] Medium contains 2 mM PO_4^{3-}, 10-fold lower than that of the standard formulation.

[c] Compounds with solubilities of <10 μg/ml did not inhibit growth in saturated solution (SS).

viz., the more reduced the carbon source, the higher the MIC of TeO_3^{2-}.

Multiple-oxyanion HLR in *R. sphaeroides* 2.4.1. A total of 20 rare-earth oxides and oxyanions were assayed for toxicity to *R. sphaeroides* 2.4.1 (Table 4). Of those examined, only CrO_3, CrO_4^{2-}, and MnO_4^- had MICs <20 μg/ml; the others had MICs in SMM > 100 μg/ml under all growth conditions examined. Oxides having limited solubilities in SMM (e.g., MoO_3, NH_4VO_3, $Rh_2O_3 \cdot 5H_2O$, Sb_2O_3, and SnO_2) did not affect cell growth when present in growth media as saturated solutions. Only cultures grown in the presence of Te-, Se-, or Rh-containing oxyanions evolved gas and accumulated intracellular deposits (Table 4). HLR to these five compounds was unaffected by extracellular PO_4^{3-}, which suggested that HLR to these compounds in *R. sphaeroides* 2.4.1 did not involve components of the phosphate transport system. This would preclude any similarity between the mechanism of intrinsic HLR in *R. sphaeroides* and that encoded by the IncPα plasmid determinants *telA* and *telB* (48).

R. sphaeroides was also highly resistant to a second class of oxyanions, the periodate class, but the resistance mechanism to this class differed significantly from that to the tellurite class. Neither IO_4^-, SiO_3^{2-}, nor SiO_4^{2-} was reduced to its elemental state, and no gas evolution was observed. In sharp contrast to resistance to the tellurite class, resistance to these oxyanions decreased three- to fourfold when the extracellular [PO_4^{3-}] was reduced 10-fold. This suggested that reduced transport or increased efflux via a phosphate transport system-mediated mechanism may facilitate HLR to periodate-class oxyanions in *R. sphaeroides*, although we have no direct evidence bearing on this point. It is interesting, however, that intrinsic resistance to these compounds in *R. sphaeroides* was still some 20-fold greater than that demonstrated by members of the γ−3 *Proteobacteria* (39).

A third class of oxyanions to which *R. sphaeroides* was highly resistant, the arsenate class, was also examined. This group included arsenate, molybdate, stannate, sulfite, and tungstate. Similar to resistance to the tellurite class oxyanions, resistance to these compounds was unaffected by extracellular PO_4^{3-} levels. In contrast, however, HLR to arsenate class compounds did not result in oxyanion reduction or intracellular metal sequestration. Like the periodate class oxyanions, these compounds were not reduced to their elemental states and no gas was evolved. These data supported the existence of a third and distinctly different mechanism to effect HLR to arsenate class oxyanions.

Purification of Te from membrane fractions of *R. sphaeroides* 2.4.1. Two 1-liter cultures of *R. sphaeroides* 2.4.1 were grown photoheterotrophically (incident light intensity of W/m^2) in SMM containing 0.4% succinate to a cell density of approximately 1.5 × 10^9 cells per ml. Prior to inoculation one flask was supplemented with 275 mg of K_2TeO_3 (equivalent to 138.3 mg of Te^{IV}) to give a final medium concentration of 250 μg of K_2TeO_3 per ml. Following subcellular fractionation, the dense black deposit which accumulated within cells grown in TeO_3^{2-}-containing medium was localized to the cytoplasmic membrane via centrifugation through a discontinuous sucrose gradient. No metallic material was observed in the enriched chromatophore fraction (consisting of photosynthetic or intracytoplasmic membrane) at the 20%/40% interface.

We isolated 850 mg of crude membrane-metal complex. After purification and extraction with acetone-methanol and ethanol, 203 mg of a finely divided metallic material was obtained. Analysis of a 50.4-mg sample of this material identified 23.2 mg of Te^0 (a minimum net purity of 46%). The minimum amount of Te^0 deposited in the 1-liter culture, therefore, was 93 mg (203 mg × 0.46) or 0.733 mmol. Since the growth medium initially contained 138.3 mg of Te^{IV}, a minimum Te^{IV}-to-Te^0 conversion of 67% was obtained.

Assay of the membrane fraction of cells grown in the absence of TeO_3^{2-} revealed no Te^0, nor was any detected in the cytoplasmic or periplasmic fractions of either culture by this method (27). These results demonstrated conclusively that *R. sphaeroides* 2.4.1 could effect the intracellular reduction of Te^{IV}, which resulted in the deposition of metallic Te^0 in the cytoplasmic, but not intracytoplasmic, membrane.

Hydrogen evolution from tellurite class oxyanion reduction. Although no gas was evolved from cells grown in the absence of TeO_3^{2-}, 208 ml of water was displaced from the gas collection vessel over the culture grown in the presence of TeO_3^{2-}. This corresponded to 8.37 mmol of gas (1 atm, 101.3 kPa, 303 K), the major component of which was subsequently identified as H_2 by mass spectroscopy (Fig. 1B). While ionization products of H_2O, N_2, and CO_2 were detected in both samples, no H_2 was detected in the headspace over the control culture (Fig. 1A). The trace amounts of Ar recorded in each spectra resulted from its use as a carrier in the analyses. Combined with earlier results, these data suggested that approximately 11.5 mmol of H_2 was evolved per mmol of Te^0 deposited.

Mechanism of TeO_3^{2-} HLR in *R. sphaeroides* 2.4.1. To determine the requirements for TeO_3^{2-} reduction in *R. sphaeroides* in vivo, we assayed tellurite resistance in a number of mutant strains. Our results indicated that neither the DMSO reductase, the B800-850 spectral complex, nor the B875 spectral complex was required to effect HLR to TeO_3^{2-} under any growth condition examined (Table 5).

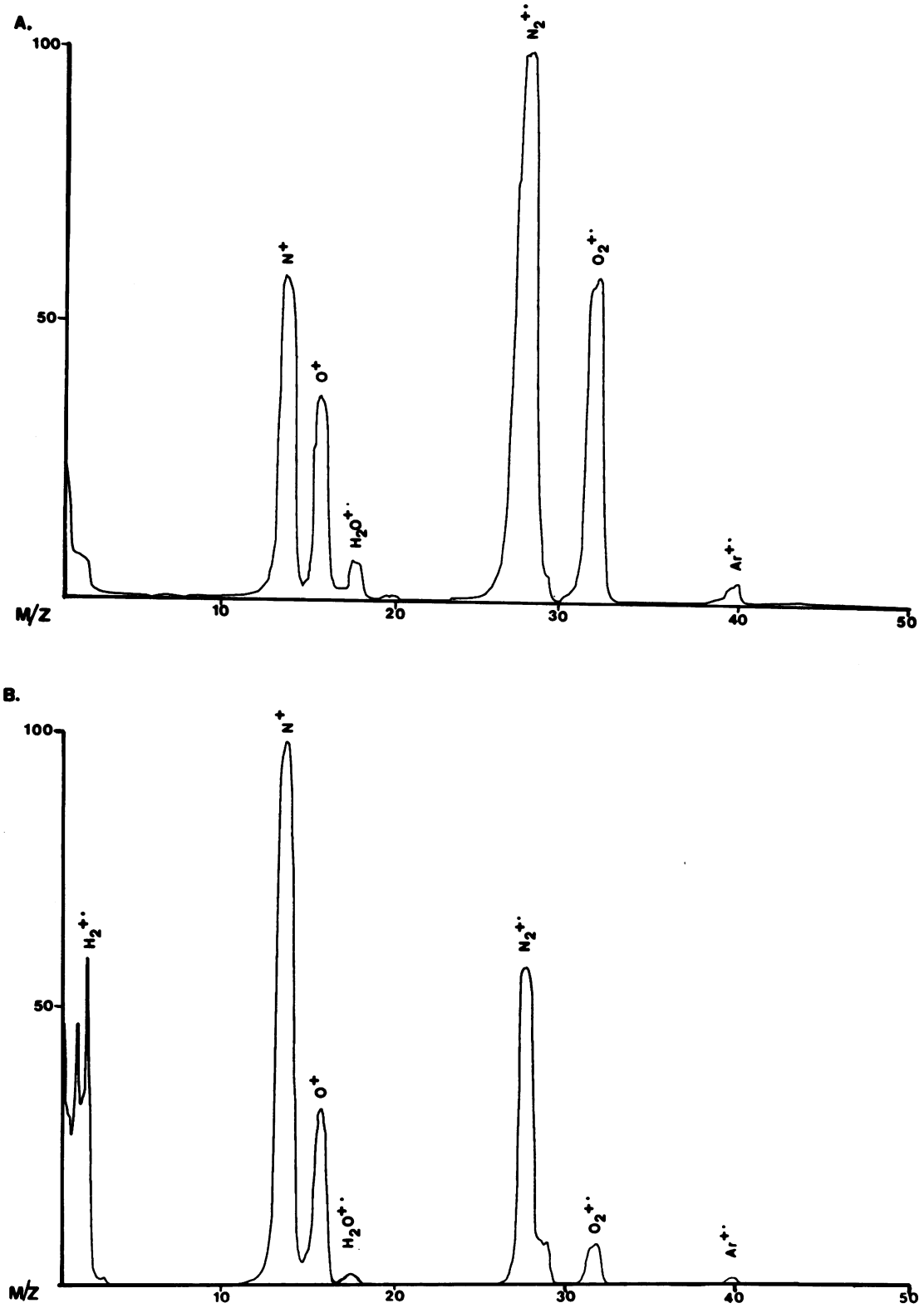

FIG. 1. Mass spectra of the headspace gas collected above photosynthetic (10-W/m^2 incident light intensity) cultures of *R. sphaeroides* 2.4.1 grown either in the absence (A) or presence (B) of 250 μg of K_2TeO_3 per ml. The *y* axis shows relative intensity.

TABLE 5. Analysis of intrinsic HLR to tellurite in *R. sphaeroides* mutants

Strain	Relevant genotype or phenotype	Photosynthetic competence	MIC of K_2TeO_3 (μg/ml)[a]		
			Aerobic	Photosynthetic (10 W/m^2)[b]	Anaerobic-dark[c]
2.4.1	Wild type	+	900	600	150
2.4.1ΔS	Δ(42-kb plasmid)	+	850	600	150
2.4.1-Ga	Car$^-$	+	600	450	100
MM1004	DORase$^-$	+	800	550	NG
PUC705-BA	B800-850$^-$	+	850	500	150
PRKB$^-$	PrkB$^-$	+	800	500	150
CFXB$^-$	CfxB$^-$	+	750	650	150
CFXA$^-$	CfxA$^-$	+	400	100	50
PRKA$^-$	PrkA$^-$	+	350	100	60
MM1006	Bchl$^-$	−	(400)[d]	NG[e]	<10
CYCA1	Cyt c_2^-	−	(400)	NG	<10
PUFB1	Puf$^-$	−	(200)	NG	<10
CFXA$^-$B$^-$	CfxA$^-$ CfxB$^-$	−	(200)	NG	<10
PRKA$^-$B$^-$	PrkA$^-$ PrkB$^-$	−	(150)	NG	<10
PUHA1	RC-H$^-$	−	<10	NG	<10
BC17	Car$^-$ Cyt bc_1^-	−	<10	NG	<10

[a] MICs were determined in SMM containing succinate at 30°C.
[b] Incident light intensity.
[c] Supplemented with 60 mM DMSO.
[d] Parentheses indicate resistance to TeO_3^{2-} but no deposition of Te^0.
[e] NG, no growth.

Deletion of the 42-kb endogenous plasmid of *R. sphaeroides* 2.4.1 did not diminish HLR to TeO_3^{2-}, although we did observe a 20% increase in TeO_3^{2-} sensitivity in the carotenoid-deficient strain 2.4.1-Ga.

A single mutation in either the form II region phosphoribulokinase gene, *prkB* (14), or the downstream aldolase gene, *cfxB* (1), diminished HLR to TeO_3^{2-} 10 to 20%, whereas strains with deletions in either of the genes' form I region homologs, *prkA* (14) or *cfxA* (12), were twofold more sensitive to TeO_3^{2-} under aerobic growth conditions and at least three- to fivefold more sensitive under photosynthetic and anaerobic-dark-DMSO growth conditions.

Analyses of additional *R. sphaeroides* mutants determined the obligate requirement for bacteriochlorophyll synthesis, an intact photosynthetic reaction center (RC), and a functional electron transport system for HLR to TeO_3^{2-}. These analyses also demonstrated that certain mutants, while unable to facilitate tellurite reduction, were resistant to intermediate concentrations of tellurite: viz., a Bchl$^-$ mutant (MM1006), a Puf$^-$ mutant (PUFB1), and a strain deleted for cytochrome c_2 (CYCA1) were inhibited by 10 μg of K_2TeO_3 per ml under anaerobic-dark-DMSO growth conditions but were unaffected by the addition of tellurite under aerobic growth conditions. Likewise, the photosynthetically incompetent double-deletion strains CFXA$^-$B$^-$ and PRKA$^-$B$^-$, while unable to effect TeO_3^{2-} reduction either aerobically or anaerobically in the dark in the presence of DMSO (Table 5), were resistant to tellurite at concentrations of <200 μg/ml under aerobic conditions. In contrast, strains lacking either the RC-H polypeptide (PUHA1) or the cytochrome bc_1 complex (BC17) were sensitive to 10 μg of K_2TeO_3 per ml under all growth conditions.

TeO_3^{2-} reductase activity in cell extracts. To determine the intracellular localization of TeO_3^{2-} reductase activity, subcellular fractions of aerobically grown cells were prepared from the wild type and three mutant strains unable to reduce TeO_3^{2-}. These cells were grown in the absence of TeO_3^{2-} and were harvested during the mid-exponential phase of growth. This analysis (Table 6) identified an $FADH_2$-dependent TeO_3^{2-} reductase activity in the membrane fraction of wild-type *R. sphaeroides* 2.4.1. Cells cultured in the presence of TeO_3^{2-} also expressed similar TeO_3^{2-}-dependent $FADH_2$ oxidation activity in vitro (27). A specific activity of 300 nmol of $FADH_2$ per min per mg of protein was detected in the membrane fraction of wild-type cells.

An $FADH_2$-dependent TeO_3^{2-} reductase activity was also observed in the photosynthetically incompetent strain PRKA$^-$B$^-$, despite this strain's inability to reduce TeO_3^{2-} in vivo (Table 5). This suggested that in addition to an $FADH_2$-dependent reductase, at least one other component was required to facilitate complete reduction to Te^0 in vivo.

Neither BC17 nor PUHA1, two mutants which were previously shown to be tellurite sensitive under both aerobic and anaerobic-dark-DMSO growth conditions, expressed significant levels of a TeO_3^{2-}-dependent $FADH_2$ oxidase

TABLE 6. TeO_3^{2-}-dependent $FADH_2$ oxidation in *R. sphaeroides* 2.4.1

Strain[a]	Subcellular fraction	$FADH_2$ oxidation[b] (nmol min^{-1} mg^{-1})
2.4.1	Periplasm	1
	Membrane	300
	Cytoplasm	60
BC17	Periplasm	2
	Membrane	51
	Cytoplasm	20
PRKA$^-$B$^-$	Periplasm	1
	Membrane	200
	Cytoplasm	20
PUHA1	Periplasm	3
	Membrane	28
	Cytoplasm	37

[a] Cells were grown aerobically in SMM containing 0.4% succinate.
[b] 100 μg of K_2TeO_3 per ml was used in all assays.

activity in vitro. This could explain the inability of either to effect oxyanion reduction and metal sequestration in vivo.

Negligible reductase activity was observed in the periplasmic and cytoplasmic fractions of all strains, and in separate analyses we did not detect a TeO_3^{2-}-dependent oxidation of NADH or NADPH in subcellular fractions from any of these strains (27). This would not preclude, however, the participation of an NADH- or NADPH-dependent oxidation step in the reduction of an intermediate in the reduction of Te^{IV} to Te^0.

DISCUSSION

We have demonstrated intrinsic resistance to tellurite, selenite, and 15 other rare-earth oxides and oxyanions in several representatives of the photosynthetic *Proteobacteria* including *R. sphaeroides* 2.4.1, which expressed intrinsic resistance to metal oxides and oxyanions under aerobic, photosynthetic, and anaerobic-dark-DMSO growth conditions. That HLR to Te- and Se-containing oxyanions in *R. sphaeroides* was inhibited 40-fold by the addition of cysteine to growth media contrasted sharply with previous results which showed that the addition of cysteine to growth media ameliorated the effects of tellurite and selenite toxicity in *E. coli* (35). As in *T. thermus* (2), and in contrast to results for *E. coli* (28, 29), the addition of L-methionine to culture media did not affect HLR to TeO_3^{2-} in *R. sphaeroides* 2.4.1.

Analytical methods demonstrated that HLR to TeO_3^{2-} was the result of reduction of Te^{IV} to Te^0, with deposits of black, metallic Te localized to the cytoplasmic, but not photosynthetic, membrane. That reduced metal was localized specifically in the cytoplasmic membrane of *R. sphaeroides* was consistent with electron microscopic observations of Te and Se deposits in the cytoplasmic membranes of *E. coli* (11, 43) and *M. avium* (44) cells grown in the presence of TeO_3^{2-}.

Mass spectrographic analysis confirmed that cultures of *R. sphaeroides* 2.4.1 grown photosynthetically in the presence of 250 μg of K_2TeO_3 per ml evolved H_2 at a ratio of approximately 11.5 mmol/mmol of Te^0 deposited. These data suggested that approximately 50 mol of electrons was required for the conversion of one mole of Te^{IV} to Te^0, reducing equivalents which ultimately must have been derived from oxidation of the carbon source. This significant expenditure of reducing power to facilitate oxyanion detoxification substantiates our finding that the extent of tellurite reduction in vivo was inversely related to the oxidation state of the carbon source present in the growth medium.

The obligate requirement for CO_2 fixation, an intact photosynthetic RC, and a functional electron transport system to effect TeO_3^{2-} reduction was demonstrated for both aerobically and anaerobically grown cultures. No photosynthetically incompetent mutant examined could effect reduction of Te^{IV} to Te^0 under any growth condition, although five strains were able to effect LLR to TeO_3^{2-} in the absence of reduction. In contrast, BC17 and PUHA1 were sensitive to tellurite under all growth conditions. The reason for this difference was initially unclear, although subcellular fractionation experiments provided some insight into this observation: the absence of a TeO_3^{2-}-dependent $FADH_2$ oxidation activity in cell extracts of BC17 and PUHA1 appears to account for the inability of these two strains to effect oxyanion resistance. In contrast, $PRKA^-B^-$, which contained a TeO_3^{2-}-dependent $FADH_2$ oxidation activity, expressed LLR to TeO_3^{2-} during aerobic growth. These data suggest that the reduction of Te^{IV} to Te^0 occurs as a result of at least two enzymatic steps, the first involving $FADH_2$, generating perhaps a nontoxic +II valence intermediate, followed by a second two-electron reduction which results in metal deposition. This mechanism would be consistent with one proposed for the biocatalytic detoxification of mercury compounds in vivo (39).

Further characterization of the mechanisms of oxyanion resistance, and particularly oxyanion reduction, will no doubt be integral to the study of electron transport, CO_2 fixation, and general photochemistry in *R. sphaeroides*. For example, the increased sensitivity to tellurite in the presence of the *puhA* mutational class, while useful as a selective tool, also raises additional questions regarding the role of the RC-H polypeptide in aerobic cells.

Likewise, the inability of two CO_2 fixation-deficient mutants, $PRKA^-B^-$ and $CFXA^-B^-$, to effect tellurite reduction during aerobic growth may define an important role for the expression of a Calvin cycle gene(s) in aerobic cells. While CO_2 fixation is implicit for aerobic chemolithotrophic growth, little has been elucidated about the specific role(s) a functional Calvin cycle (or CO_2 fixation in general) may play in chemoheterotrophic growth. In a recent report, however, Singer et al. have shown that conditional lethal mutations in the aldolase gene (*fda*) of *E. coli* preferentially inhibit stable RNA synthesis at the level of transcription initiation upon a shift to the nonpermissive temperature (32). Since stable *cfx* mRNA transcripts are not present in either the $CFXA^-B^-$ or the $PRKA^-B^-$ mutant (13, 14), it is possible that the absence of tellurite reduction in these strains is an indirect result of an overall change in the redox state of the cell (resulting from a global inhibition of RNA synthesis in a *cfx* background) and not a direct result of the absence of phosphoribulokinase or aldolase activity in vivo. Elucidation of the specific mechanism(s) of oxyanion reduction may greatly impact upon our understanding of the role(s) that *cfx* expression plays in the overall metabolism of this organism.

On a more practical level, our results have facilitated a direct and simple counterselection in interspecies conjugation protocols between *E. coli* and *R. sphaeroides*. While previous methods have relied on treatment of postincubation mating mixes with bacteriophage T4 to kill *E. coli* donor cells prior to plating on selective medium (4), recent experiments in our laboratory have shown that the inclusion of as little as 5 to 10 μg of K_2TeO_3 per ml in SMM agar plates obviates this need (27, 40).

Our results also suggest the use of TeO_3^{2-} and other oxyanions in photosynthetic enrichment techniques to facilitate the isolation of resistant organisms in practically pure culture from a variety of environmental inocula. Such enrichments would be useful for isolating new strains of known species or identifying as yet unknown species. Likewise, we anticipate the identification and characterization of new mutants unable to effect oxyanion resistance, and in the case of tellurite class compounds, oxyanion reduction.

It is interesting to note the tremendous shear forces produced during centrifugation of the membrane fraction of cells grown in the presence of tellurite. As a result of metal deposition in the cytoplasmic membrane, it may now be possible to isolate a highly purified cytoplasmic membrane fraction from photosynthetically grown cells by using sucrose density gradients. Previously, it has not been possible to isolate a pure cytoplasmic membrane fraction that was not contaminated by chromatophores by using discontinuous centrifugation.

The possible commercial implications of these findings also deserve attention. Our findings suggest a commercial

use of *R. sphaeroides* in the bioremediation of toxic rare-earth oxyanions in the environment. Sylvester et al. identified the threat to western United States water supplies by seleniferous contamination of agricultural wastewaters (41). In an effort to isolate microorganisms capable of effecting selenate reduction in situ, Steinberg and Oremland determined selenate levels in various surficial sediments, salinas, estuarial salterns, and littoral lake samples throughout the western United States (37). Although they were able to isolate bacteria from these environs which produced significant dissimilatory selenate reduction in vitro, the in situ SeO_4^{2-} reductase activity of the bacteria was inhibited by as much as 50% by the presence of other rare-earth oxyanions (e.g., tungstate, molybdate, and nitrate) in the contaminated waters, to which these organisms were sensitive.

That *R. sphaeroides* is able to effect multiple resistance to at least a dozen different oxyanions suggests its use in the cleanup of such multi-anion-contaminated waters. Its biochemical diversity, facultative photosynthetic capacity, and ability to flourish in habitats as diverse as farm drainage ponds, lakes, estuaries, and marine waters make *R. sphaeroides* an excellent candidate for exploitation in the bioremediated detoxification of rare-earth oxides and oxyanions.

Finally we note the considerable potential of *R. sphaeroides* as a biological means of heavy metal reclamation. Rare-earth metals exist in nature as trace contaminants of gold, platinum, and uranium ores, either in their elemental state or, more commonly, as oxides or oxoacids. Rhodium, for example, one of the rarest elements, is present in the earth's crust in concentrations of less than 1 ppb. Open-pit leaching of platinum ores during mining releases highly toxic precious-metal oxides and oxyanions into the environment, often at concentrations too low to be chemically reduced and recovered in elemental form. The adaptation of *R. sphaeroides* for use in this recovery process could undoubtedly result in increased rare-earth metal production and reduced environmental contamination.

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