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Membrane-Associated Chromate Reductase Activity from *Enterobacter cloacae*

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Washed cells of *Enterobacter cloacae* HO1 reduced hexavalent chromium (chromate: CrO_4^{2-}) anaerobically. Chromate reductase activity was preferentially associated with the membrane fraction of the cells. Right-side-out membrane vesicles prepared from *E. cloacae* cells showed high chromate reductase activities when ascorbate-reduced phenazine methosulfate was added as an electron donor.

Hexavalent chromium (chromate: CrO_4^{2-}) is a strong oxidant, and this property is likely related to its toxicity for most organisms. Bacterial resistance to CrO_4^{2-} has been found in *Pseudomonas* spp. (2, 4, 11), *Streptococcus lactis* (5), and *Alcaligenes eutrophus* (10). The chromate resistance in *Pseudomonas* spp. (4, 11) and in *A. eutrophus* (10) was related to the decreased uptake of CrO_4^{2-}. Recently we isolated from activated sludge an *Enterobacter cloacae* strain, HO1, that reduces CrO_4^{2-} anaerobically (13). *E. cloacae* HO1 was likely to utilize toxic CrO_4^{2-} as an electron acceptor anaerobically, because (i) anaerobic growth accompanied the decrease of CrO_4^{2-} in culture medium, (ii) the chromate-reducing activity of the growing cells was rapidly inhibited by oxygen, and (iii) the reduction occurred more rapidly in glycerol- or acetate-grown cells than in glucose-grown cells. However, the biochemistry of anaerobic reduction was not studied. In this study, we demonstrate that the chromate reductase activity is preferentially associated with the membrane fraction of *E. cloacae* HO1 cells. Right-side-out membrane vesicles prepared from *E. cloacae* HO1 showed high rates of chromate reduction anaerobically when ascorbate-reduced phenazine methosulfate (PMS) was added as an electron donor.

To determine whether chromate reduction requires the physical presence of *E. cloacae* cells, cells were grown anaerobically in KSC medium with 0.5 mM K_2CrO_4 and then removed from the culture by filtration through membrane filters (0.45-μm pore size). The composition of KSC medium has been previously described (13). Sodium acetate was added as a carbon source and electron donor for chromate reduction, and the pH of the medium was 7.4. The cell-free filtrate did not reduce CrO_4^{2-} even under anaerobic conditions (Fig. 1A). Cells were also removed by centrifugation at 13,000 × *g* for 10 min; no chromate reduction was observed in the supernatant. Adding fresh KSC medium to the supernatant did not cause chromate reduction. On the contrary, when washed cells were suspended in fresh KSC medium (about 2×10^7 cells per ml), chromate reduction was readily observed. They were able to reduce completely concentrations as high as 0.5 mM CrO_4^{2-} within 5 h; such a high rate of chromate reduction has never been reported.

This reductase activity was eliminated by aeration (Fig. 1B). No chromate reduction was observed at 4 or 60°C. Reduction occurred at pH 6.5 to 8.5 and was strongly inhibited at pH 5 and 9. Metabolic poisons including carbonyl cyanide *m*-chlorophenylhydrazone, sodium cyanide, formaldehyde, and 2,4-dinitrophenol inhibited chromate reduction by washed cells (Fig. 1C). Inhibition of cell growth by the addition of penicillin, cycloserine, or chloramphenicol resulted in a simultaneous loss of chromate reduction under anaerobic conditions (data not shown). The effect of chloramphenicol on the chromate-reducing activity also suggests that this activity may require de novo protein synthesis. In this respect, we have observed that nitrate-grown *E. cloacae* cells lost simultaneously their chromate-reducing ability and chromate resistance under anaerobic conditions H. Ohtake, K. Komori, C. Cervantes, and K. Toda FEMS Microbiol. Lett., in press).

In our previous study (13), we observed that as the CrO_4^{2-} concentration decreased, the turbidity of the *E. cloacae* culture rapidly increased, and the medium color changed from yellow to white. The initial turbidity increase was not accompanied by cell growth, which was delayed depending on the amount of added chromate. We assumed that the white substance of the external medium was a reduction product of CrO_4^{2-}, perhaps identical to that resulting from chromate reduction by strong chemical reductants.

A direct demonstration of chromate reduction by washed cells was provided by means of electron paramagnetic resonance (EPR) spectroscopy (Fig. 2). Preparation of samples and recording of EPR spectra were as described by Lowe et al. (8). Washed cells were suspended in fresh KSC medium with 0.5 mM CrO_4^{2-} and incubated anaerobically at 30°C for 6 h. Samples were taken before and after the 6-h incubation and frozen in liquid nitrogen for EPR spectroscopy. The EPR spectra were obtained with a JEOL spectrometer (model JES RE2X). In Fig. 2, the EPR signal was plotted in an arbitrary intensity unit against the intensity of the magnetic field (milliteslas). EPR signals of trivalent chromium and its *g* values were obtained from an aqueous solution of $Cr(H_2O)_6Cl_3$ with diphenylpicryl-hydrazyl in benzene solution as the *g* standard (*g* 2.0036) (1). After the incubation, the culture originated a signal that could be attributed to a paramagnetic species, trivalent chromium. The intensity of the signal, calculated from the height of the maximum peak of absorption, increased as the incubation proceeded. The EPR signal (*g* 1.98) was observed only when cells were grown with CrO_4^{2-} anaerobically. We have not tested whether the EPR signal also originates from chromate-sensitive bacterial cells. However, since CrO_4^{2-} easily passes through the cellular membrane (11), and is reduced to

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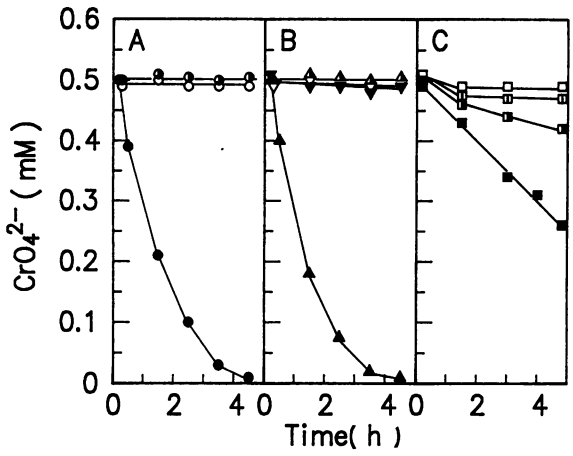

FIG. 1. Chromate reduction by *E. cloacae* HO1 under various conditions. Reduction experiments were performed with washed cells in KSC medium at pH 7 and 30°C under anaerobic conditions, unless otherwise noted. (A) Chromate reduction by washed cells (●), the filtrate of an anaerobic culture of HO1 (◑), and the supernatant of anaerobic culture of HO1 prepared by centrifugation at 13,000 × *g* for 10 min (○). (B) Chromate reduction by washed cells at 37°C (▲), 60°C (▽), and pH 9 (▼) under anaerobic conditions and at 30°C and pH 7 under aerobic conditions (△). (C) Inhibition of chromate reduction by 50 μM carbonyl cyanide *m*-chlorophenylhydrazone (□), 0.01% formaldehyde (▨), 200 μM sodium cyanide (■), and 500 μM 2,4-dinitrophenol (◨).

trivalent chromium in the cytoplasm, it is possible that the EPR signal may originate from the sensitive cells. Arslan et al. (1) demonstrated intracellular chromate reduction in mammalian cells by using EPR spectroscopy.

We believe that the reduced chromium existed mainly in the external medium. After CrO_4^{2-} disappeared, we attempted to remove the reduced chromium, together with bacterial cells, by centrifugation. Although as much as 30% of the total chromium was removed by centrifugation at 13,000 × *g* for 10 min, about 70% remained in the supernatant. Furthermore, transmission electron microscopic examination of the pellets revealed that electron-scattering particles were deposited at the outside of bacterial cells, and energy-dispersive X-ray analyses showed that these precipitates contained exclusively chromium (data not shown).

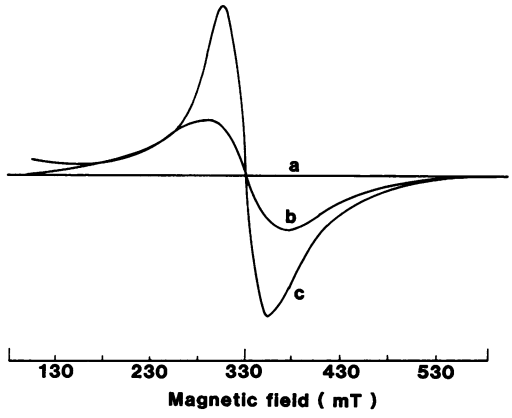

FIG. 2. EPR spectra of *E. cloacae* culture before (a) and after (b) 6 h of incubation with CrO_4^{2-} at 30°C. The spectrum from a standard solution of $Cr(H_2O)_6Cl_3$ (c) is also given as a control. The EPR signals were plotted in an arbitrary intensity unit against the intensity of the magnetic field (mT). The conditions of EPR running were as follows: temperature, 123 K; microwave power, 4 mW; frequency, 9.4 GHz; field, 330 ± 250 mT; modulation amplitude, 0.63 mT; receiver gain, 1. Diphenylpicrylhydrazyl was used for the *g* standard.

TABLE 1. Chromate reductase activity in various fractions prepared from *E. cloacae* HO1 and IAM1615 and *E. coli* HB101 cells[a]

Strain	Electron donor	Sp act (U/mg of protein)[b]		
		Periplasmic components and outer membrane	Cytoplasmic components	Right-side-out membrane vesicles
E. cloacae				
HO1	None	0.00	0.00	1.64
HO1	Ascorbate-PMS[c]	1.40	1.22	5.47
IAM1615	None	0.00	0.00	0.17
IAM1615	Ascorbate-PMS	0.52	1.05	0.82
E. coli				
HB101	None	0.00	0.00	0.00
HB101	Ascorbate-PMS	0.68	0.98	0.53

[a] See the text for details on the chromate reductase assay.
[b] One unit is defined as 1 μg of CrO_4^{2-} reduced per min. Protein was estimated spectrophotometrically with a Coomassie brilliant blue R-250 solution (3).
[c] Donor was 100 μM ascorbate plus 5 μM PMS.

To determine localization of chromate reductase activity, cells were disrupted and separated into various fractions. Right-side-out membrane vesicles were prepared essentially as described by Kaback (6). Spheroplast suspensions were prepared by the lysozyme-EDTA method and then centrifuged at 10,000 × *g* for 10 min. The supernatant contained periplasmic and outer membrane components. The spheroplast pellet was suspended in a solution of 1 mM $MgSO_4$ and 0.1 mg of DNase I and centrifuged at 100,000 × *g* for 1 h. The supernatant contained cytoplasmic components. Finally, the membrane-containing pellet was homogenized in 100 mM sodium phosphate buffer (pH 7) to a final concentration of 5 to 10 mg of protein per ml. Chromate reductase assays were performed in 100 mM sodium phosphate buffer (pH 7) containing about 1 mg of protein and 0.5 mM K_2CrO_4. The reaction mixture was incubated for 30 min at 30°C; after centrifugation, the residual concentration of CrO_4^{2-} was determined by using diphenylcarbazide (12). One unit of enzymatic activity was defined as 1 μg of CrO_4^{2-} reduced per min at 30°C. By this procedure, about 80% of the chromate-reducing activity in the intact cells was recovered in the subcellular fractions at the final stage. The chromate reductase activity was preferentially associated with the membrane fraction of *E. cloacae* HO1 cells prepared as right-side-out membrane vesicles (Table 1). Ascorbate-PMS remarkably increased the reductase activity of right-side-out membrane vesicles of *E. cloacae* HO1 cells. In the absence of added electron donors, a low but significant reductase activity was detected in *E. cloacae* HO1 membrane vesicles, suggesting that these preparations were not depleted of endogenous reserves of electron donors. Reductase activity was lost when the membrane vesicles were heated at 100°C for 1 min. The addition of NADH did not increase the reductase activity, probably because the inside of right-side-out membrane vesicles is not accessible to NADH (9). Right-side-out membrane vesicles were also prepared from *E. cloacae* IAM1615 and *Escherichia coli* HB101, which were sensitive to CrO_4^{2-} and incapable of reducing CrO_4^{2-}.

E. cloacae IAM1615 was obtained from the culture collection of the Institute of Applied Microbiology, University of Tokyo. These membrane vesicles showed very low activities, even when ascorbate-PMS was added. Lower activities also appeared in periplasmic and cytoplasmic fractions of each strain in the presence of ascorbate-PMS. However, as to these activities, no significant difference was observed among the three strains, and the activities were also stimulated by NADH (data not shown).

This is the first report of membrane-associated chromate reductase activity from bacteria. This result supports our previous speculation that *E. cloacae* HO1 was likely to utilize toxic chromate as an electron acceptor anaerobically (13). We tentatively conclude that the reduction takes place on the cell surface, forming insoluble chromium hydroxide in the external medium. Chromate is a strong oxidant, and intracellular CrO_4^{2-} is undoubtedly toxic for bacterial cells. Chromate reduction on the cell surface and formation of insoluble chromium hydroxide favor protecting cells from the toxicity of CrO_4^{2-}. Lebedeva and Lyalikova (7) also isolated a chromate-reducing *Pseudomonas* strain from industrial sewage; they observed that the pseudomonads adhered to form chromium hydroxide aggregates and precipitated as chromate reduction proceeded.

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