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Inducible Periplasmic Chromate Reducing Activity in *Pseudomonas aeruginosa* Isolated from a Leather Tannery Effluent

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Abstract A chromate tolerant strain of *Pseudomonas aeruginosa* isolated from the effluent of a tannery showed significant enzymatic activity of chromate reduction. Cells grown in chromate-supplemented medium reduced 8 μg chromate/mg protein/h in the presence of NADH/NADPH. The chromate reducing activity was inducible as cells pregrown in chromate showed higher chromate reduction. In contrast, the periplasmic fraction of cells grown in chromate reduced 75% chromate in 4 h and the spheroplast fraction failed to do so, indicating that chromate reductase may be located in the periplasm. The presence of a 30 kDa protein in the periplasmic extracts of cells grown in the presence of chromate, but its absence of the protein in cells grown without chromate, points out a possible role of this protein in chromate reduction.

Key words: *Pseudomonas aeruginosa*, chromate reductase, inducibility, periplasmic enzyme

Hexavalent chromium (chromate: CrO_4) is toxic and mutagenic for most organisms [1, 19] and causes skin irritation, respiratory tract corrosion, and lung carcinoma [2, 4, 23]. It is released in waste-waters from such industrial processes as chrome leather tanning, chrome plating, metal cleaning and processing, alloy preparation, and wood preservation [14]. Conventional methods to treat toxic chromate require large amounts of chemicals and energy, and hence are unsuitable for small-scale leather, dye, and electroplating units. Biotransformation of hexavalent chromate to the non-toxic trivalent form under natural conditions, therefore, offers an eco-friendly option for its detoxification and bioremediation.

Chromate reduction is a redox reaction that requires a supply of electrons. Chromate reducing activity is widespread in microorganisms which may directly reduce chromate enzymatically or indirectly by producing hydrogen sulfide that precipitates chromium(VI) to chromium sulfide (Cr_2S_3). Direct chromate reduction may occur aerobically or anaerobically. A *Desulphovibrio* strain can reduce Cr(VI) to Cr(III) by using C_3 cytochrome as a Cr(VI) reductase and H_2 as electron donor [17]. In anaerobic reduction, chromate acts as the final electron acceptor [5, 17, 26] to be reduced. Indirect reduction *via* H_2S has been reported in a consortium of sulfate-reducing bacteria (SRB III) [11]. Many compounds of biological origin such as amino acids, ascorbic acids, glutathione, reducing sugars, humic and fulvic acids, diols, cytochromes - C_3 and P-450 [13, 15, 21] may take part in such a process directly or indirectly by donating electrons. Enzymes such as aldehyde oxidase [3], glutathione reductase [24], and DT- diaphorase [9] can also act as electron donors for chromate reduction. However, at present, the enzyme(s) responsible for the direct reduction of chromate were not well characterized. Studies with a partially purified chromate reductase from *P. ambigua* G-I [25] suggested that Cr(VI) is reduced to Cr(III), via Cr(V) as an intermediate with NAD(P)H acting as a source of electrons. The purification of chromate reductase to homogeneity from *P. putida* MK1 has recently been reported [23]. In this study, we have attempted to characterize the chromate reducing activity of *Pseudomonas aeruginosa* strain A2Chr isolated from a leather tannery effluent.

Materials and Methods

Bacterial Strain and Culture Conditions

Pseudomonas aeruginosa A2Chr is a chromate tolerant, chromate-reducing strain, which we isolated from the effluent of a leather-tanning unit located in Kanpur, India [18]. The ability of this strain to survive and reduce hexavalent chromium in the effluents of tanning and electroplating

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units was reported in a previous study [12]. It was grown in minimal medium containing succinate as the sole carbon source [17]. Sterile stock solution of potassium dichromate was added to achieve the desired final concentrations of chromate. All cultures were grown with shaking at 150 rpm at 37°C for 10–12 h.

Chromate Reduction by Whole Cells

Chromate reducing ability of the cells was measured by estimating the residual hexavalent chromium in the culture supernatant up to 10 h after inoculation in the chromate-supplemented culture medium. Hexavalent chromium was estimated by its ability to specifically react with s-diphenylcarbazide (DPC) reagent [12]. The log-phase cells were inoculated in two sets of succinate minimal medium (SMM) containing 50 μg chromate/ml. One of the two sets was placed in a boiling water bath for 10 min. Both sets were then incubated with shaking at 37°C. Supernatants were obtained at different time intervals, by harvesting cells at 12,000 rpm (4°C, 2 min). DPC reagent (200 μl) was added to the appropriately diluted supernatant (1.6 ml) from each of the two sets. The samples were incubated at room temperature for 20 min and the absorbance of the magenta color developed was read at 540 nm in a UV-visible spectrophotometer (Spectronic 1201, Milton Roy Company, U.S.A.). Concentrations of residual dichromate were deduced from a calibration curve plotted with dichromate concentrations ranging from 5 to 100 μg/ml. Total chromium in the supernatants was measured in an atomic absorption spectrophotometer (Perkin-Elmer Model 2380, U.S.A.) using suitable standards (E. Merck, Darmstadt, Germany).

Evaluation of the Extracellular Chromate Reducing Activity

Ten ml of an overnight culture suspension was inoculated into 1 l of SMM containing 10 μg chromate/ml, incubated with shaking at 37°C to mid-log phase, harvested by centrifugation at 10,000 rpm (4°C, 10 min), and the supernatant concentrated to 5 ml in a lyophilizer (SpeedVac, Savant Instruments, U.S.A.). The concentrated supernatant was dialyzed for 24 h at 4°C against 10 mM Tris-HCl buffer (pH 7.2) with repeated changes of the latter to concentrate it further. Protein content of the concentrated supernatant was estimated by the Bradford method [7]. For estimating chromate reducing activity, the concentrated supernatant containing 1 mg protein/ml was added to 2 ml of reaction mixture containing 10 mM Tris-HCl buffer (pH 7.2) and 10 μg/ml chromate. NADH was added at 10 μg/ml and the mixture incubated with shaking at 37°C; blanks contained the same reaction mixture without the supernatant concentrate. Aliquots (0.5 ml) were withdrawn at intervals of 2 h, heated for 2 min, centrifuged, and the supernatants used for determining residual chromate by the DPC method [12].

Chromate Reduction by Cell-Free Extracts

To estimate chromate reduction by cell-free extracts (CFE), cells grown as described above were harvested by centrifugation (10,000 rpm, 10 min, 4°C) and resuspended in 10 mM Tris-HCl buffer (pH 7.2) after washing twice with the same buffer. Cells were then disrupted by sonication under ice cooling. The broken cells were clarified by centrifugation at 15,000 rpm at 4°C for 20 min, and the clear supernatant was used immediately for assaying chromate reductase activity as follows: In a 2 ml reaction mixture containing 10 mM Tris-HCl buffer (pH 7.2), 10 μg chromate/ml and 10 μg NADH/ml, chromate reduction was initiated by adding cell-free extract containing 1 mg protein/ml and incubated at 37°C for 6 h. Aliquots (300 μl) were withdrawn and centrifuged at 15,000 rpm for 10 min at 4°C. The supernatants were used to estimate residual chromate by the DPC method. In parallel, a 500 μl aliquot of the supernatant was examined for total chromium in an atomic absorption spectrophotometer (Perkin-Elmer Model 2380, U.S.A.). The nature of the reducing principle was ascertained by boiling an aliquot of the cell-free extract for 10 min and assaying for chromate reductase activity by the DPC method.

For investigating the role of electron donors (NADH/NAD(P)H) in chromate reduction by the CFE, 5 ml of the latter was dialyzed against 10 mM Tris-HCl buffer (pH 7.2) for 12 h at 4°C. The dialyzate (concentrated by lyophilization) and the dialyzed cell-free extract were assayed for chromate reduction separately and in combination. The ability of NADH or NAD(P)H to act as an electron donor was also studied by adding NADH or NAD(P)H to the cell-free extract. After 6 h, residual chromate and total chromium were estimated in the supernatants as described above.

Subcellular Localization of Chromate Reductase Activity

Cells grown in succinate medium (with and without chromate) to log phase, were subjected to a modified cold osmotic shock procedure specially suited to extract relatively pure periplasmic fraction from *P. aeruginosa* [22, 10]. The supernatants containing periplasmic fraction were lyophilized and stored at −20°C until use. Their protein content was estimated by the Coomassie blue dye-binding assay using bovine serum albumin as a standard [7]. Authenticity and purity of the periplasmic fraction was confirmed by assaying for the cytosolic (malic enzyme) and periplasmic (acid phosphatase and alkaline phosphatase) marker enzymes with the help of kits (Bio-Meriux, France). Periplasmic fraction was assayed for chromate reducing activity in a 2 ml reaction volume with 1 mg protein/ml, 10 μg chromate/ml, and 10 μg NADH/ml in 10 mM Tris-HCl buffer (pH 7.2). Incubation conditions were essentially the same as those used for CFE. Heat susceptibility of the chromate reducing activity was tested by boiling an aliquot (1 mg protein/ml) of periplasmic fraction for 5 min before estimating chromate reduction. Chromate reducing activity

in the spheroplasts was evaluated by gently resuspending the spheroplasts, washing once in chilled 10 mM Tris-HCl buffer (pH 7.2), and assaying for chromate reductase activity.

Determination of Inducibility of Chromate Reductase

Cells of *P. aeruginosa* A2Chr were grown to log phase, harvested by centrifugation, washed twice with SMM, and inoculated at equal cell densities in succinate medium without or with 10 μg chromate/ml. The culture suspension with 10 μg chromate/ml was divided into two sets, to one of which was added rifampicin to a concentration of 25 μg/ml so as to block the induction of genes caused by chromate. Suspensions were incubated with shaking at 37°C. Aliquots (1.5 ml) withdrawn at intervals of 2 h were centrifuged and the pellets processed for the estimation of total protein. The supernatants were analyzed for residual chromate by the DPC method.

In order to compare the protein profiles of the periplasmic fractions of the cells grown with and without chromate, the concentrated periplasmic fractions were suspended in 100 μl of 1× cracking buffer (0.0625 M Tris, pH 6.8; 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromophenol blue) and mixed thoroughly by vortexing for 1–2 min. The lyzed suspensions were then boiled for 3–4 min in a boiling water bath and placed on ice immediately. Aliquots (20–25 μl) corresponding to a protein concentration of about 200 μg were loaded onto a 10% SDS-polyacrylamide linear resolving gel overlaid with a stacking gel [6]. Proteins were resolved by electrophoresis at 100 V for 2 h (stacking) and 200 V for 4 h (resolving). Gels were stained with Coomassie brilliant blue R-250.

RESULTS AND DISCUSSION

The ability of the whole cells to reduce Cr(VI) was estimated by measuring Cr(VI) and total chromium in the culture supernatant at different time intervals (Fig. 1). Whereas the total chromium content remained almost constant throughout, the Cr(VI) content decreased in the culture supernatant with the growth of the cells. Estimation of total chromium associated with the cell pellet showed that only negligible amounts were associated with the cells, either within or on the cell surface. This indicates that reduction in Cr(VI) levels was not due to bio-accumulation within the cells. The inability of the heat-killed cells to reduce Cr(VI) (data not shown) further suggests that decrease in Cr(VI) content of the culture supernatant was not due to biosorption on the cell surface but due to a probable change in the valency of the chromium. To find out the optimum concentration of chromate in the growth medium for chromate reduction, the effects of 10, 25, 50, and 100 μg chromate/ml on chromate reduction were tested. Chromate was completely reduced in the case of

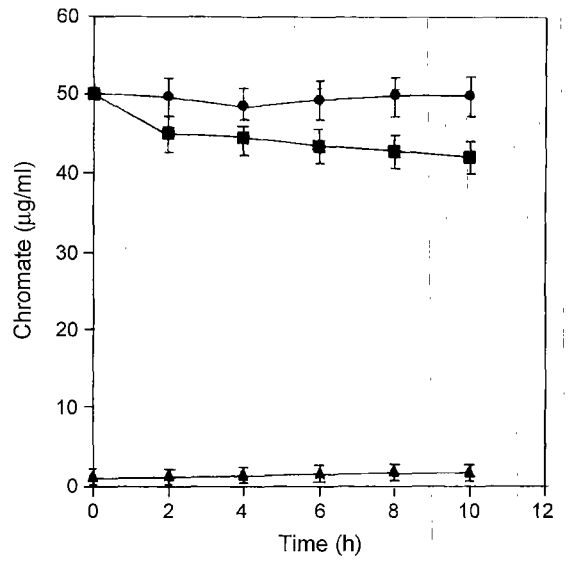

Fig. 1. Kinetics of chromate reduction by *Pseudomonas aeruginosa* A2Chr and localization of reduced chromium.
Symbols: (●) total chromium in the supernatant, (■) chromium(VI) in the supernatant, (▲) total chromium inside the cells.

10 μg chromate/ml by cells equivalent to 1 mg total protein/ml within 2 h. In case of 25, 50, and 100 μg chromate/ml, 8.0, 5.0, and 3.5 μg chromate/ml, respectively, was reduced by the same amount of cells within the same time. Therefore, 10 μg chromate/ml concentration was considered as the optimum concentration for further studies on chromate reduction.

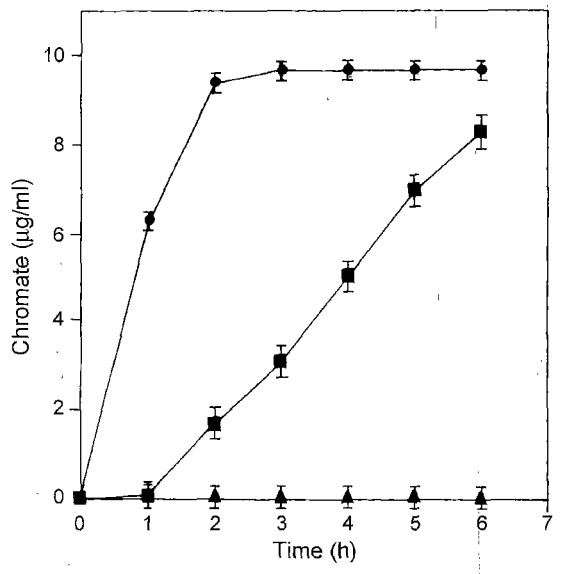

Fig. 2. Kinetics of chromate reduction by *P. aeruginosa* A2Chr, pregrown with and without chromate, after shifting into succinate minimal medium.
Symbols: (●) cells pregrown in chromate containing medium, (■) cells pregrown in chromate-free medium, (▲) cells pre-grown in chromate-free medium and shifted to medium containing rifampicin.

In order to determine whether chromate reductase is constitutively synthesized or is induced by the presence of chromium, chromate reductase activity was measured in the cells pregrown with or without 10 μg chromate/ml (Fig. 2). Chromate reduction, measured 2 h after the transfer of cells in medium containing 10 μg chromate/ml-containing medium showed only 1.75 μg Cr(VI)/ml reduction if the cells were pregrown without chromate. Nearly complete reduction occurred in the cells pregrown with 10 μg chromate/ml. Chromate reduction started slowly in the cells pregrown without chromate, suggesting an induction of the chromate reductase in response to chromate. Rifampicin was used for induction of new proteins by inhibiting transcription. Cells pregrown without chromate did not show any chromate reduction up to 6 h of incubation when shifted to SMM containing 10 μg chromate/ml plus 25 μg rifampicin/ml.

The absence of Cr(VI) or its reduced form inside the cells or on the cell surface, and the presence of all the reduced chromium in the culture supernatant prompted us to investigate whether chromate reduction occurs extracellularly or within the cells. Culture supernatants of cells grown in the presence of chromate were extensively dialyzed with 10 mM Tris-HCl (pH 7.2) at 4°C to remove Cr(VI). The dialyzed supernatants were concentrated by lyophilization to obtain 1 mg protein/ml. The extracellular fractions were used to test their chromate reducing ability. The reaction mixture containing chromate as the substrate and NADH as an electron donor did not show any chromate reduction even after prolonged incubation at 37°C, with or without shaking (data not shown). This observation indicates that chromate reduction did not occur by any protein or enzyme secreted by the cells.

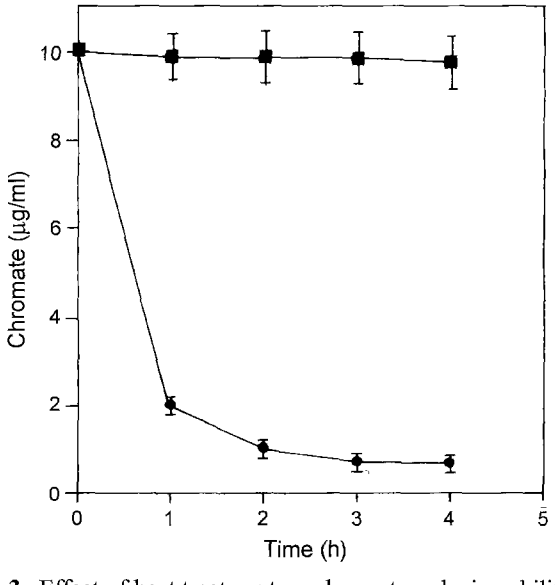

Fig. 3. Effect of heat treatment on chromate-reducing ability of the cell-free extract of *P. aeruginosa* A2Chr.
Symbols: (■) Boiled cell-free extract, (●) Native cell-free extract.

Table 1. Effect of dialysis and cofactors on *in vitro* chromate-reducing activity in cell-free extract of *P. aeruginosa* A2Chr.

Treatment	μg chromate reduced/mg protein/h
Undialyzed Cell-Free Extract (CFE)	8.0
Dialyzed CFE	0.46
Dialyzed CFE+Concentrated dialyzate	3.30
Dialyzed CFE+NADH	8.0
Dialyzed CFE+NADPH	8.3
NADPH	0.29

The cell-free extracts of *P. aeruginosa* A2Chr showed chromate reducing activity but the heat-denatured CFE did not (Fig. 3). The CFE of the exponential phase culture reduced 8 μg Cr(VI)/mg protein/h when incubated in 10 mM Tris-HCl (pH 7.2) plus NADH at 37°C with shaking. Decrease in Cr(VI) was accompanied by decrease in NADH levels measured spectrophotometrically at 340 nm. In order to determine the need of electron donor for chromate reduction, one set of CFE was extensively dialyzed against 10 mM Tris-HCl (pH 7.2) at 4°C and used to compare chromate reduction with undialyzed CFE. Dialysis was expected to remove the small molecular weight compounds including NADH/NAD(P)H from the cell-free extract. Table 1 shows that chromate reduction by the dialyzed CFE was much lower than that by the undialyzed CFE. Supplementation of NADH to the dialyzed CFE restored its chromate reducing ability. This clearly established a role of NADH in chromate reduction. Since, NAD(P)H has also been shown to act as an electron donor for chromate reduction [16], it was also used to test if NADH or NAD(P)H was a better electron donor. The data in Table 1 show that there was no significant difference in chromate reduction with NADH or NAD(P)H as electron donor, indicating that both may be used equally efficiently.

In order to investigate if chromate reducing activity was located within the cells or in the periplasmic fraction, a cold osmotic shock was given to the cells grown with or without chromate to dissociate the periplasmic fraction of proteins from the rest of the cells i.e. spheroplast. In view of the EDTA sensitivity of the cell wall of *Pseudomonas* species, a modified cold osmotic shock [10] procedure was used. Negligible malic enzyme activity but significant acid- and alkaline-phosphatase activities in the periplasmic preparation indicated that it was not contaminated with cytosolic proteins. The periplasmic fraction was concentrated by lyophilization. Equal amounts of periplasmic proteins and spheroplast proteins were used for chromate reduction with NADH as electron donors. Figure 4 shows that over a period of 4 h, the periplasmic fraction reduced Cr(VI) from 10 μg/ml to 2.5 μg/ml. In contrast, the spheroplast fraction showed only an insignificant amount of chromate reduction, i.e. 0.3 μg chromate/ml, which occurred within minutes

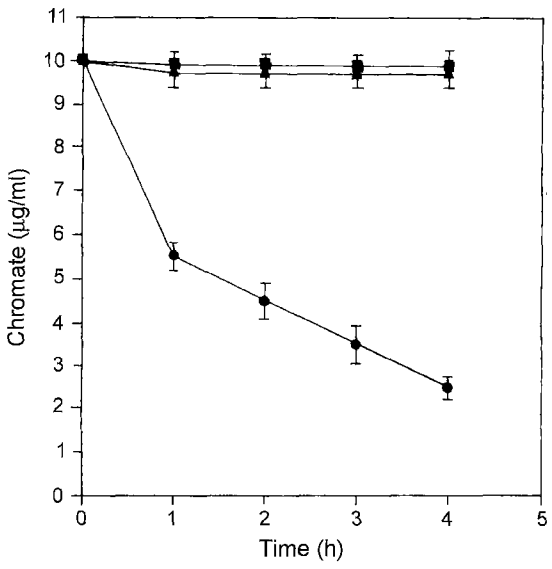

Fig. 4. Subcellular localization of chromate reducing activity in *P. aeruginosa* A2Chr.
Symbols: (▲) Spheroplast fraction, (●) Periplasmic extract, (■) Boiled periplasmic extract.

after addition of the spheroplast fraction, with no further decrease in Cr(VI) levels occurring with time. The chromate-reducing activity in the periplasmic fraction became inactivated by boiling for 2–3 min, suggesting that chromate reducing activity may be located in the periplasm.

Figure 5 shows the SDS-PAGE profiles of periplasmic proteins extracted from the cells grown with or without chromate. Comparison of the profiles shows a protein of 30 kDa being induced in the chromate grown cells. This protein may possibly be associated with either uptake or reduction of chromate. Earlier reports on chromate uptake suggest that chromate is transported inside the cells by sulfate transporters. Our work indicated that the chromate-induced periplasmic protein of about 30 kDa may be a likely candidate for chromate reductase. Our observations suggest that in *P. aeruginosa* A2Chr, chromate reductase is located in the periplasmic space and that its catalytic site is possibly exposed to the external environment, an arrangement that may allow the cells to detoxify chromate by transforming Cr(VI) into Cr(III); the Cr(III) can be excluded by complexation with exopolysaccharides.

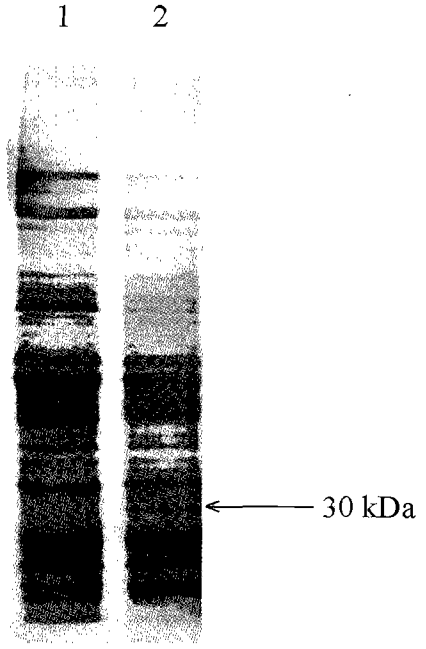

Fig. 5. Comparison of SDS-PAGE protein profiles of *P. aeruginosa* A2Chr grown with (1) and without (2) chromate.

Our observations on chromate reduction by *P. aeruginosa* A2Chr have shown that although after reduction of chromate, Cr(VI) accumulated in the culture supernatant, the reduction was not mediated by any extracellular enzyme. This is apparent from the observation that the supernatants fail to reduce chromate. The ability of the cell-free extracts to reduce chromate with time indicates that the reduction of hexavalent chromium was not due to sorption or chemical reaction but was enzymatic. The inability of boiled cell-free extracts to reduce chromate indicated the enzymatic nature of the reducing principle. A requirement of electron donors by the chromate reducing principle/protein, present in the cell-free extract as a concentrate, may be inferred from the observation that addition of the dialyzate to the protein concentrate and addition of exogenous NADH/NAD(P)H stimulated chromate reduction. The ability of the periplasmic fraction and the inability of the spheroplast fraction to reduce chromate suggests that the chromate reducing principle is probably located in the periplasm. Detection of a protein of about 30 kDa in the periplasmic fraction of the cells grown in chromate and its absence in the periplasmic extracts of cells grown without chromate points to a probable role of this protein in chromate reduction. Furthermore, the fact that the cells pregrown in chromate elicit higher chromate reductase activity, and that rifampicin (a transcription inhibitor) inhibits such reductase activity in cells shifted from chromium-free medium to chromium-supplemented medium, indicates that chromate reductase activity is inducible in nature.

A requirement of NADH as electron donor for chromate reductase activity has been reported in *P. ambigua* G-1 [16], *P. fluorescens* LB300 [5], *P. putida* [23], and *Commamonas testosteroni* [8]. Chromate reductase of these bacteria seems to share a common catalytic mechanism, viz., acceptance of an electron from one molecule of NADH, whereby at first a Cr(V) intermediate is generated and is then followed by acceptance of two electrons from two molecules of the same coenzyme [25]. As the reduction of Cr(VI) to Cr(III) involves transfer of three electrons, the mechanism of action of chromate reductases

may be similar to that involved in oxo-group transfers/ redox reactions catalyzed by such molybdoenzymes as nitrate/nitrite reductases, in which the substrate undergoes a two-valency change in the oxidation state. It appears that the chromate reducing enzyme present in our *P. aeruginosa* strain A2Chr may be similar in action to the enzyme and its mode of chromate reduction reported in *P. putida*, *P. aeruginosa*, and *P. fluorescens* [5, 17, 23, 25].

A soluble chromate reductase recently purified from *P. putida* MK1 has been shown to have a molecular mass of 20 kDa on SDS-PAGE. The molecular mass of the native protein, however, was about 50 kDa [23]. The *P. ambigua* chromate reductase was earlier shown to have a molecular mass of 25 kDa on SDS-PAGE but the native protein was 65 kDa [23]. Absence of nucleotide sequence homology between the *P. ambigua* chromate reductase gene and the *P. putida* genome [23] revealed that the chromate reducing enzyme of *P. putida* might be completely different from that of *P. ambigua*. The 30 kDa protein present in the SDS-PAGE of chromate-stressed *P. aeruginosa* cells, although higher in molecular weight than chromate reductases of *P. putida* or *P. ambigua*, may be yet another type of chromate reductase. The N-terminal amino acid sequence of the chromate reductase from *P. putida* MK1 [23] suggested a periplasmic location of this protein and shows similarity with the periplasmic location of chromate reducing activity in *P. aeruginosa* A2Chr.

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