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Reduction of hexavalent chromium by *Pseudomonas aeruginosa* HP014

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Microbial reduction of hexavalent(VI) to trivalent(III) chromium decreases its toxicity by two orders of magnitude. In order to investigate the nature of Cr-reduction, Cr-resistant *Pseudomonas aeruginosa* HP014 was isolated and tested for its reduction capability. At the concentration of 0.5 mM Cr(VI), cell growth was not inhibited by the presence of Cr(VI) in a liquid medium, and Cr(VI) reduction was accompanied by cell growth. When cell-free extract was tested, the reduction of Cr(VI) showed a saturation kinetics with the maximum specific activity of 0.33 μmol min^{-1} mg^{-1} cell protein, and an apparent K_m of 1.73 mM Cr(VI). The activity required either NADH or NADPH as an electron donor. However, NADPH gave 50% as much activity as NADH. To locate the reductase activity, cell-free extract was centrifuged at 150,000×*g*, and subsequently the supernatant and pelleted membrane fractions were tested for Cr(VI) reduction activity. The supernatant of the centrifugation showed almost the same Cr(VI) reduction activity as compared with that of the cell-free extract, indicating that the Cr(VI)-reducing activity of *P. aeruginosa* HP014 is due to a soluble enzyme. Moreover, the activity appeared to be the highest among the known activities, suggesting that the strain might be useful for remediation of Cr(VI)-contaminated sites.

Key words: chromium, reduction, bioremediation

Chromium is considered to be an essential trace mineral that helps to maintain normal metabolism of glucose, cholesterol, and fat in humans (17, 19). Despite such biological requirements, high concentrations of chromium are proved to be toxic (7), mutagenic, and carcinogenic (8, 26). High concentrations of chromium found in the environment mainly originated from anthropogenic pollution (3, 18). Chromium is an important industrial metal used in the manufacture of various products including alloys, corrosion-inhibitory paints, wood preservatives, photographic sensitizers, mordants and fixatives for dyes and tanning, and pigments for rubber and ceramics (9). Similar to other transition metals, chromium can exist in various oxidation states from -2 to +6, with +3 and +6 being the most stable forms (12, 21). In biological systems, chromium is naturally found in its trivalent state at variable levels, whereas the hexavalent form is generally a derivative of man's activities.

Change in the oxidation state of chromium has a profound effect on toxicity and bioavailability (13). It is proposed that bacterially mediated reduction of Cr(VI) to Cr(III) significantly reduced its toxicity (15). This phenomenon has been reported for a very limited number of species of gram-negative environmental isolates. Several *Pseudomonas* species appear to mediate the reduction aerobically, and an *Enterobacter cloacae* which is facultative, was reported to reduce Cr(VI) only under anaerobic conditions (14). Recently, *Desulfovibrio vulgaris* was found to mediate reduction of Cr(VI) to Cr(III) via cytochrome c_3 (16). The ability to reduce chromium appear to be a mechanism by which these organisms are able to resist high concentrations of chromium. For the pseudomonads it was proposed that Cr(VI) reduction may be a secondary activity of a soluble reductase for which the physiological role is different. The primary function of the reductase was not determined as yet.

In this study, we propose *Pseudomonas aeruginosa* HP014, isolated from a local industrial sewage as an useful strain to investigate the mechanism of aerobic Cr(VI) reduction based on the subcellular location and kinetic parameters of NADH-dependent Cr(VI) reductase activity in this microorganism.

Materials and Methods

Strain

The strain used in this study was isolated from an industrial sewage discharged from Hupyong In-

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dustrial Complex near Chunchon, Kangwon. Based on its morphological, biochemical characteristics and fatty acid methyl ester(FAME) analysis (4), the strain was identified as a species of *Pseudomonas aeruginosa* with a similarity index of 0.865.

Cell growth and extract preparation

Cells of *P. aeruginosa* were grown at 37°C for 8 hrs (late log phase) in 2 liter of mPlate Count Broth (Difco), and harvested by centrifugation. Cell pellet was suspended into 10 ml of 30% glycerol-containing 50 mM potassium phosphate buffer, pH 7.0. Disruption of the cells were performed with a French press at 16 kpsi, and unbroken cells were removed by centrifugation at 12,000×*g* for 20 min. The supernate (S_{12}), designated as the cell-free extract, was centrifuged at 150,000×*g* for 60 min. The membrane fraction (pellet) was resuspended in the disruption phosphate buffer at the original volume. The supernatant (S_{150}) was designated as soluble protein fraction. When tested for the presence of viable cells on Plate Count Agar (Difco), less than 100 colony forming units per ml was detected from cell extract, membrane, and soluble protein fractions. Protein was quantitatively determined by dye-binding method of Bradford (5).

Cr(VI) reduction assay

Cr(VI) reduction activity was assayed colorimetrically by measuring the decrease of Cr(VI) as determined by diphenylcarbazide method (2). This procedure measures only hexavalent chromium and the resulting red-violet color of unknown composition was quantitatively measured with spectrophotometer (Shimadzu UV-1601PC, Japan) at wavelength of 540 nm. The reaction mixture contained; 0.5 mM chromate, 0.4 mM NADH in 2 ml of 50 mM potassium phosphate buffer, pH 7.0. After 5 min of pre-incubation at 37°C, the reaction was initiated by the addition of 200 µl of the enzyme source. At various times of incubation, duplicate samples (100 µl aliquots) were taken and the remaining Cr(VI) was measured. Incubations without an enzyme source served as a control. For the determination of pyridine nucleotide specificity, NADPH substituted for NADH at the same concentration.

Results and Discussion

Cell growth and Cr(VI) reduction

The effect of Cr(VI) on the growth of *P. aeruginosa* HP014 is shown in Fig. 1. Cell growth was not inhibited by the presence of 0.5 mM Cr(VI) and a comparable growth was observed up to 5 mM Cr (VI), although with an increased lag phase. This phenomenon might be explained as an increased time period for adaptation or DNA repair during the exposure to high levels of Cr(VI) in the medium. When bacterial cells are exposed to highly soluble Cr(VI) compounds, they induce frameshift errors and, to a greater extent, base-pair substitutions both at G-C and A-T base pairs (8). It has been also proposed that bacterial SOS functions can repair the DNA damage produced by Cr (VI) (8). In our experiments, the lag phase observed was proportionally increased as the concentration of Cr(VI) increased.

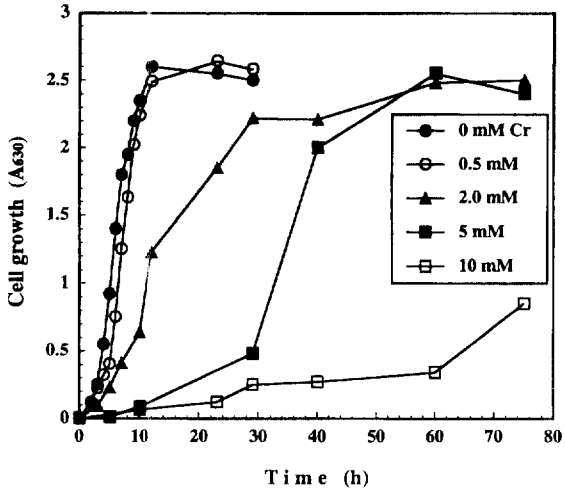

Fig. 1. Effect of chromate on the growth of *P. aeruginosa* HP014. Cell growth was not inhibited by the presence of 0.5 mM Cr(VI) in mPlate Count Broth, and a comparable growth was observed up to 5 mM Cr(VI), although with an increased lag phase.

At a moderate initial concentration of Cr(VI) (0.5 mM), *P. aeruginosa* HP014 actively reduces Cr(VI) to Cr(III) while it was growing on mPlate Count Broth (mPCB) medium under aerobic growth conditions (Fig. 2). The reduction also occurred when 20 mM of either glucose or benzoate were added as a sole carbon source (data not shown). When 1:1 mixture of fresh mPCB medium and autoclaved cells were tested, no measurable reduction of Cr(VI) occurred over the same period of incubation time, indicating that there was no significant chemical Cr(VI) reduction or adsorption onto the cells.

Although we observed the sharp decrease of Cr (VI) until the strain ceased to grow, the final concentration never reached to zero (Fig. 2). This result may reflect the fact that the reduction of Cr (VI) requires cellular reductant for the reduction. It is assumed that no further reduction of Cr(VI) after 24 hrs of cultivation is due to the depletion of cellular reductant pool.

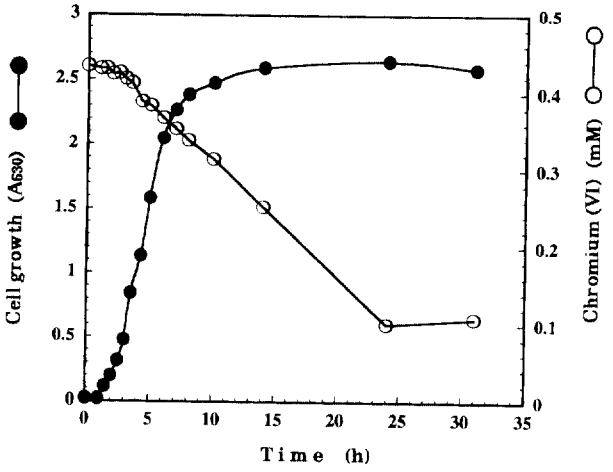

Fig. 2. Reduction of Cr(VI) by *P. aeruginosa* HP014. The strain actively reduced 0.5 mM Cr(VI) to Cr(III) while it was growing under aerobic conditions. When 1:1 mixture of fresh medium and autoclaved cells were tested, no measurable reduction occurred over the same period of incubation time, indicating that there was no significant chemical Cr(VI) reduction or adsorption onto the cells.

Localization of Cr(VI) reduction activity

Upon the analyses of the cell extracts for Cr(VI) reduction activity, the supernatant fractions (S_{12} and S_{150}) reduced Cr(VI) readily under aerobic conditions with NADH as an electron donor, and showed similar levels of specific Cr(VI) reduction activity (Fig. 3). No activity was found in the membrane fraction. These data suggest that the enzyme responsible for the reduction of Cr(VI) located in soluble fraction of cytosol. To our knowledge, only *Enterobacter cloacae* was found to retain the activity within the membrane fraction (25). Wang *et al.* (24) showed that an *E. cloacae* strain was resistant to chromate under both aerobic and anaerobic conditions, although, only the anaerobic culture showed chromate reduction, suggesting that the reduction of Cr(VI) by this bacterium is tightly coupled to the energy production in which Cr(VI) is utilized as a terminal electron acceptor. Unlike *E. cloacae*, however, our strain utilize oxygen as a terminal electron acceptor and soluble fraction has the Cr(VI) reduction activity. This finding is not contradictory to other reports performed with different species of *Pseudomonas* such as *P. ambigua* G-1 and *P. putida* PRS 2000 (11, 23).

To observe any substantial Cr(VI) reduction activity in our assay system, an addition of reduced pyridine nucleotide was necessary. This finding suggests that the activity of Cr(VI) reduction catalyzed by endogenous reserves as electron donor was negligible. However, the activity was enhanced by addition of external electron donor, either NADH or

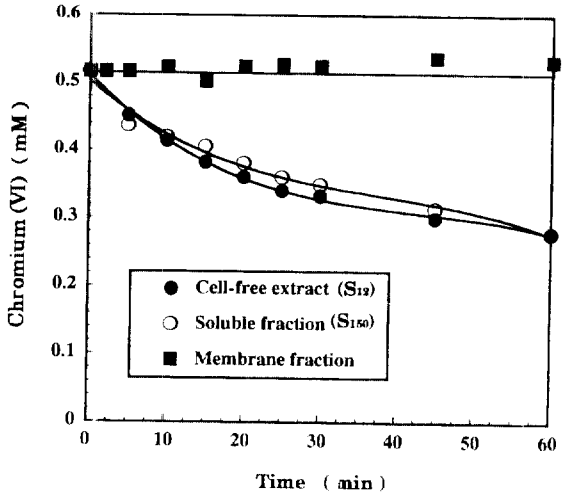

Fig. 3. Localization of Cr(VI) reduction activity. Upon the analyses of the cell extracts for Cr(VI) reduction activity, the supernatant fractions (S_{12} and S_{150}) reduced Cr(VI) readily under aerobic conditions with NADH as an electron donor, and showed similar levels of specific Cr(VI) reduction activity. No activity was found in the membrane fraction.

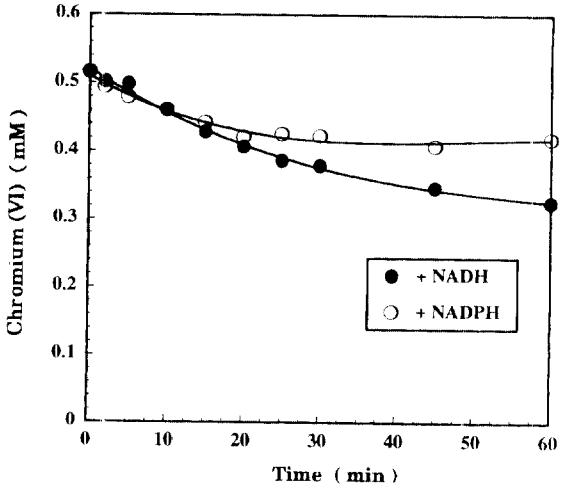

Fig. 4. Pyridine nucleotide specificity for Cr(VI) reduction. The cell extracts required reduced pyridines for activity and had a preference for NADH over NADPH when compared at the same concentration of 0.4 mM.

NADPH, as demonstrated in Fig. 4. Recently it was proposed that Cr(VI) can be reduced nonenzymatically by some bioreductants, such as NAD(P)H, ascorbic acid, glutathione, and reducing sugars (1, 6, 22). In our reaction assay system NAD(P)H was included but the extent of nonenzymatic reduction of Cr(VI) was carefully subtracted from the total Cr(VI) reduction activities. Therefore we are confident that the observed activities are not due to the nonenzymatic reduction. This was also confirmed by following data from kinetic approaches on the reduction of Cr(VI).

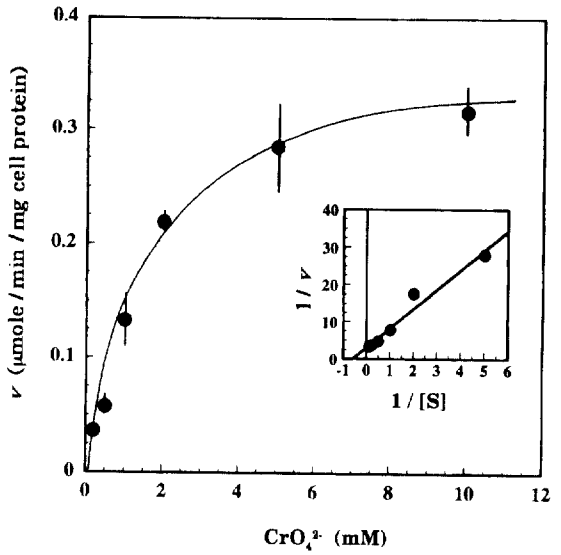

Fig. 5. Kinetic parameters of Cr(VI) reduction activity. An apparent Michaelis-Menten constant (K_m) of 1.73 mM chromate and a maximum specific activity of 0.33 μmol min^{-1} mg cell $protein^{-1}$ were estimated from the Lineweaver-Burk plot (inset). The reaction was performed at the pH of 7.0 and 37°C.

Table 1. Comparison of Cr(VI) reduction specific activity of *P. aeruginosa* HP014 with those of previously described Cr(VI) reducers. The Cr(VI) reductase activity of strain HP014 appears to be the highest among known activities, suggesting that the strain can serve as an useful model system for studying the biochemistry of Cr(VI) reductase and its potential use on the treatment of Cr(VI)-contaminated wastewater might be advantageous

Microbial strain	V_{max} (units[a])	K_m	Ref.
P. aeruginosa HP014	0.33	1.73	this study
P. ambigua G-1	0.027	0.0013	(10)
P. utida PRS 2000	0.006	0.04	(11)
D. vulgaris ATCC 29579	0.05	ND[b]	(16)
E. cloacae HO1	0.105	ND	(24)
E. coli ATCC 33456	0.001	ND	(20)

[a] Units area defined as μmol Cr(VI) reduced min^{-1} mg cell $protein^{-1}$
[b] ND: not determined.

Cr(VI) reduction activity

The Cr(VI) reduction activity by *P. aeruginosa* HP014 was shown to be catalyzed by an enzymatic reaction, specifically a reductase reaction, judging from the saturation kinetics of the activity (Fig. 5). It showed somewhat lower affinity (K_m of 1.73 mM) to Cr(VI) compared to other strains of *Pseudomonas* (10, 11). The fact that we can observe a K_m value, however, confirms that the activity we observed was purely biological not originated from any physicochemical change of Cr(VI) valency to Cr (III). When compared with those of previously described Cr(VI) reducers, the Cr(VI) reductase activity of *P. aeruginosa* HP014 appears to be the highest among known activities, suggesting that the strain HP014 can serve as an useful model system for studying the biochemistry of Cr(VI) reductase and its potential use on the treatment of Cr (VI)-contaminated wastewater or soils might be advantageous (Table 1).

In summary, this study showed that Cr(VI) was quantitatively transformed by an environmental isolate to Cr(III), which precipitates at neutral pH and therefore decreases its toxic effects on biota. The fact that the isolate, *P. aeruginosa* strain HP 014, was found to be the most effective Cr(VI) reducer among the strains isolated so far, promises possible use of the isolated strain for the remediation of Cr(VI)-contaminated sites.

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