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Short Communication

A SURVEY OF EFFECTIVE ELECTRON DONORS FOR REDUCTION OF TOXIC HEXAVALENT CHROMIUM BY *ENTEROBACTER CLOACAE* (STRAIN HO1)

HISAO OHTAKE,* EIJI FUJII, AND KIYOSHI TODA

Institute of Applied Microbiology, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

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Hexavalent chromium (Cr(VI)) is very soluble in water, and forms divalent anion species: chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$). Cr(VI) is a strong oxidizing agent. As a result of chemical redox reactions, Cr(VI) is reduced to the trivalent form (Cr(III)), which readily forms insoluble chromium hydroxides at neutral pH (*2*). In biological systems, Cr(VI) passes easily through cellular membranes, and then is reduced to Cr(III) in the mitochondria and nuclei, as well as in the cytoplasm (*1*). Biological membranes are impermeable to Cr(III), but the Cr(III) generated inside the cell stably binds to protein and interacts with nucleic acids (*6*). This probably is why Cr(VI) is much more toxic than Cr(III).

Recently we isolated a CrO_4^{2-}-resistant bacterium, identified as *Enterobacter cloacae* (strain HO1), from an activated sludge sample (*12*). Interestingly, this bacterial strain reduced CrO_4^{2-} to the trivalent form anaerobically. In our previous work (*11*, *12*), we demonstrated that: (i) the reducing activity was sensitive to oxygen stress; (ii) the bacterial growth accompanied the reduction of CrO_4^{2-}; and (iii) the reductase activity was preferentially associated with the membrane fraction of the bacterial cells. This showed that this bacterium can use CrO_4^{2-} as a terminal electron acceptor.

In the present study, we conducted reduction experiments with growing and washed cells in a basal medium containing a variety of organic substances to survey appropriate electron donors for CrO_4^{2-} reduction in *E. cloacae* HO1.

E. cloacae HO1 was grown anaerobically at 30°C in KSC medium. The

* Address reprint requests to: Dr. Hisao Ohtake, Institute of Applied Microbiology, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan.

composition of KSC medium has been previously described (*11*). The anaerobic conditions were established by purging the cultures with purified nitrogen for 10 min before the start of incubation. Reduction experiments were conducted using a minimal salts solution supplemented with a defined organic substance as the basal medium. The minimal salts solution contained (grams per liter of distilled water): NH_4Cl (0.03); K_2HPO_4 (0.03); KH_2PO_4 (0.05); NaCl (0.01); $MgSO_4 \cdot 7H_2O$ (0.01). The minimal salts solution was adjusted to pH 7.0. Cells of *E. cloacae* HO1 grown overnight were harvested at 4°C by centrifugation (5,000 × *g*, 10 min), washed with the minimal salts solution, and resuspended in the same solution at a density of about 10^9 cells per ml. For experiments with growing cells, washed cells were inoculated into 25-ml screw-capped test tubes containing 20 ml of basal medium. The initial concentration of viable cells was about 3×10^6 cells per ml. Test tubes were incubated for 24 h at 30°C. For experiments with washed cell suspensions, washed cells were resuspended in the basal medium at a density of 10^8 cells per ml, and the cell suspensions were incubated at 30°C for 5 h. Chromate was determined spectrophotometrically using diphenylcarbazide (*9*). The concentration of viable cells was determined by plating 100 μl of appropriately diluted culture onto nutrient agar (Difco Laboratories, Detroit, U.S.A.) and incubating the plates at 30°C for 16 h. Growth in media containing no CrO_4^{2-} was also assayed by measuring turbidity with a spectrophotometer at 660 nm (model 101, Hitachi Co., Tokyo, Japan). Casamino acids, casitone, tryptone and yeast extract were purchased from Difco Laboratories.

E. cloacae HO1 grew anaerobically using a variety of organic substances as a sole carbon source (Table 1). The growth in Table 1 was assayed by measuring turbidity at 660 nm in media containing no CrO_4^{2-}. Acetate, aspartate, citrate, glutamate, lactate, malate, pyruvate, succinate and glycerol substantially supported the growth as sole carbon sources in the basal medium. There was no growth with formate, oxalate, propionate and tartrate. The growth was stimulated by adding organic nitrogenous compounds (casamino acids, casitone, tryptone and yeast extract). Oxalate, tartrate and glucose supported the growth when they were added along with 1 g·l^{-1} of casamino acids.

In media containing 2 mM CrO_4^{2-}, CrO_4^{2-} reduction occurred in the presence of casamino acids, casitone, tryptone or yeast extract. Aspartate, glutamate, isocitrate, malate, oxalate, pyruvate and tartrate substantially enhanced CrO_4^{2-} reduction when they were added along with casamino acids. Control culture which was grown solely with 1 g·l^{-1} of casamino acids reduced only about 30% of the initial amount of CrO_4^{2-}. Glucose and citrate depressed CrO_4^{2-} reduction, but they supported the growth in the presence of casamino acids.

Reduction experiments were also conducted with washed cell suspensions (Fig. 1). In these experiments, washed cells were resuspended in the basal medium at a relatively high density of 10^8 cells per ml. Changes in cell viability were not monitored after the addition of CrO_4^{2-}. Washed cells showed some CrO_4^{2-} reduction even in the absence of added electron donors. However, the reduction virtually ceased

Table 1. Anaerobic growth of CrO_4^{2-} and its reduction by *E. cloacae* HO1 in a minimal salts solution supplemented with a variety of organic substances.[a]

Organic substance[d]	Growth (A_{660})[b]		Reduction (%)[c]	
	Casamino acids		Casamino acids	
	−	+[e]	−	+
Acetate[f]	0.047	0.087	n.d.[g]	55
Aspartate	0.052	0.087	25	100
Citrate	0.048	0.165	n.d.	18
Formate	0.014	0.044	n.d.	24
Glutamate	0.052	0.100	12	89
Isocitrate	0.032	0.083	n.d.	88
Lactate	0.063	0.130	n.d.	30
Malate	0.062	0.139	5	100
Oxalate	0.019	0.108	n.d.	89
Propionate	0.015	0.046	n.d.	10
Pyruvate	0.072	0.137	5	85
Succinate	0.048	0.065	7	39
Tartrate	0.019	0.108	n.d.	87
Glucose	0.031	0.133	n.d.	20
Glycerol	0.055	0.102	n.d.	55
Casamino acids	0.112		100	
Casitone	0.088		100	
Tryptone	0.085		100	
Yeast extract	0.178		100	
Control	0.015	0.067	n.d.	32

[a] Values are the means from three independent experiments.
[b] Turbidity measured at 660 nm after 24-h incubation. The growth was assayed in the media without CrO_4^{2-}.
[c] Percentage of CrO_4^{2-} reduced during 24-h incubation. The initial concentration of CrO_4^{2-} was 2 mM.
[d] Initial concentrations were $2\,g \cdot l^{-1}$.
[e] Initial concentration was $1\,g \cdot l^{-1}$.
[f] For organic acids, the sodium salts were tested.
[g] Not detected.

about 1 h after the start of incubation. Casamino acids, casitone, tryptone and yeast extract greatly increased CrO_4^{2-} reduction by washed cells. Interestingly, a relatively high rate of CrO_4^{2-} reduction was observed with glucose which did not support CrO_4^{2-} reduction by growing cultures of *E. cloacae* HO1. Though the mechanism is unclear, it appears that induced cells can use glucose as an electron donor. Except for acetate, glutamate and pyruvate, the tested organic acids only slightly stimulated the rates of CrO_4^{2-} reduction by washed cell suspensions.

To determine whether amino acids are also effectively utilized as electron donors for CrO_4^{2-} reduction, another reduction experiment was conducted with washed

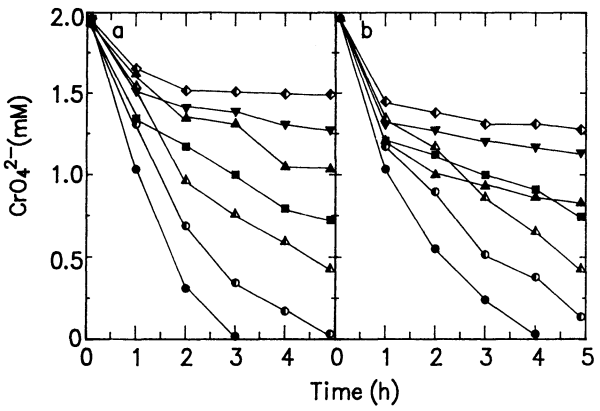

Fig. 1. CrO_4^{2-} reduction by washed cells in the basal medium supplemented with a variety of organic substances ($2\,g \cdot l^{-1}$). Two separate experiments (a and b) were conducted under the same conditions except for the organic substances added. In experiment (a), the basal medium was supplemented with: yeast extract (●), tryptone (◐), glutamate (◭), glucose (■), malate (▲), formate (▼), or none (◇). In experiment (b), the basal medium was supplemented with casamino acids (●), casitone (◐), pyruvate (◭), glycerol (▲), tartrate (▼), acetate (■) or none (◇).

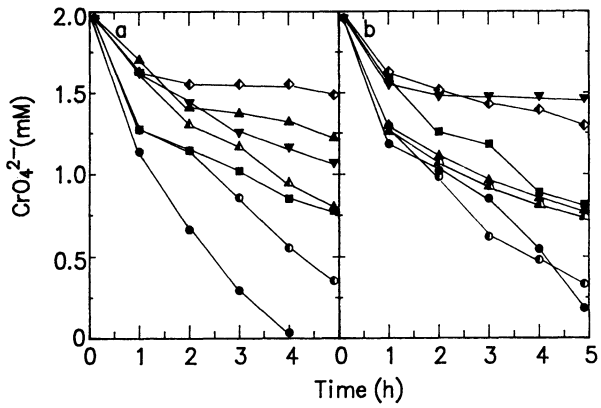

Fig. 2. CrO_4^{2-} reduction by washed cells in the basal medium supplemented with a variety of amino acids ($1\,g \cdot l^{-1}$). Two separate experiments (a and b) were conducted under the same conditions except for the amino acids added. In experiment (a), the basal medium was supplemented with threonine (◐), alanine (■), methionine (◭), proline (▲), leucine (▼), casamino acids (●) or none (◇). In experiment (b), the basal medium was supplemented with glycine (◐), glutamine (●), isoleucine (■), valine (▼), serine (▲), arginine (◭) or none (◇).

cells in the basal medium supplemented with a variety of amino acids (Fig. 2). Clearly, alanine, glutamine, glycine, methionine and threonine stimulated the rate of CrO_4^{2-} reduction by washed cells. CrO_4^{2-} reduction occurred to a lesser extent with isoleucine, leucine, proline and valine.

Very few papers have reported microbial reduction of toxic CrO_4^{2-} under anaerobic conditions (*3*, *4*, *8*). This is the first report of a survey on effective electron donors for CrO_4^{2-} reduction by bacterial cells. Organic nitrogenous compounds (casamino acids, casitone, tryptone and yeast extract) had a substantial effect on CrO_4^{2-} reduction by *E. cloacae* HO1. Though CrO_4^{2-} is known as a strong oxidizing agent, this can not be attributed to chemical redox reactions between the organic nitrogenous compounds and CrO_4^{2-}. CrO_4^{2-} reduction never occurred unless metabolically active *E. cloacae* cells existed. It is highly possible that these compounds, i.e. mixtures of amino acids, were utilized as electron donors for CrO_4^{2-} reduction. This was supported by the results of reduction experiments with washed cells in the basal medium supplemented with a variety of amino acids. Amino acids including threonine, glycine, glutamine, alanine and methionine were effectively utilized as electron donors for CrO_4^{2-} reduction by this organism.

Except for acetate, glutamate and pyruvate, the organic acids tested only slightly increased the rate of CrO_4^{2-} reduction by washed cells. However, aspartate, isocitrate, malate, oxalate and tartrate stimulated CrO_4^{2-} reduction by growing cultures when they were added to the basal medium along with casamino acids. CrO_4^{2-} is toxic and mutagenic in a number of bacterial systems (*7*, *10*). Prior to CrO_4^{2-} reduction, bacteria must tolerate its toxicity without their viability being affected. Bacteria generally tolerate higher concentrations of CrO_4^{2-} in complex media than in minimal defined media (*5*). The increase in the level of resistance may allow the cells to utilize effectively the organic acids as carbon sources, and this may stimulate CrO_4^{2-} reduction. The relation between CrO_4^{2-} resistance and reduction is now being investigated.

The microbial reduction of CrO_4^{2-} is of interest because of the potential applications of this activity in biological removal of toxic CrO_4^{2-} from wastewaters. By the appropriate choice of electron donors, it is conceivable that a new microbial method may be developed for detoxification of hexavalent chromium in contaminated wastewaters.

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