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Reduction of chromate by bacteria isolated from the cooling water of an electricity generating station

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SUMMARY

Chromate-reducing bacteria were isolated from the cooling water of an electricity generating station where reduction of chromate had caused blockage of pipes by precipitation of chromium(III) oxide. Isolates identified included the genera *Alcaligenes*, *Vibrio*, *Bacillus*, *Micrococcus*, *Staphylococcus* and *Corynebacterium*. Isolate VMC-2 with the highest chromate-reducing activity was tentatively identified as *Comamonas testosteroni*. The concentration of added chromate (K_2CrO_4, 20 μM) decreased by 95% during 45 min incubation with whole cells of VMC-2. In comparison, two Fe(III)-reducing isolates, *Vibrio metschnikovii* and *Aeromonas hydrophila*, from lake sediments, showed similarly high chromate-reducing activities, and were able to reduce 99% of added chromate (20 μM) in 45 min. Moderate Cr(VI)-reducers included strains of *Bacillus*, *Vibrio* and *Corynebacterium*. *Micrococcus* and *Staphylococcus* did not reduce Cr(VI). Sulfate (0.5 and 1.0 mM) inhibited the reduction of chromate by VMC-2 suggesting competition between the two oxyanions. Chromate-reducing activity was located in the soluble fraction of this isolate. The intermediacy of Cr(V) in the reduction of chromate was confirmed by EPR spectroscopy. The bactericidal activity of hypochlorite towards isolate VMC-2 was determined.

INTRODUCTION

Chromium commonly exists in oxidation states (II), (III) and (VI) but is most toxic in the hexavalent form. Wastewaters containing Cr(VI) are generated in many industrial processes including recirculating water systems in which it is employed as an inhibitor of corrosion. Environmental concern lies in the fact that Cr(VI) is a strong oxidant, mutagen and carcinogen. It exists primarily in the two highly toxic oxyanionic forms, chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$), the latter being less toxic. Cr(III) is far less soluble than Cr(VI), inert to ligand substitution, and precipitates as a biologically less toxic form.

The chromate anion is transported across cell membranes via the sulfate pathway, reflecting the similar geometry and charge of the two ions. It may also inhibit the uptake of sulfate. Once in the cell, chromate exerts a variety of toxic effects [4,15]. Bacterial resistance to chromate is plasmid determined and has been described for several genera, notably *Pseudomonas* [3,14], *Alcaligenes* [13] and *Streptococcus* [5]. Resistance to chromate is associated with decreased uptake by resistant cells or outward translocation of chromate anions. Reduction of chromate is an independent process that is not plasmid determined but it may provide an additional mechanism of chromate resistance [4]. Indeed, both sensitive and resistant strains of *Ps. fluorescens* can reduce chromate [2,14].

Bacterially-mediated reduction of chromate has been demonstrated in bacteria including *Pseudomonas* [6,7,10], *Aeromonas* [9], *Enterobacter* [19], *Escherichia coli* [10] and sulfate-reducers [17]. Little is known of the enzymic pathways during reduction although chromate reductase activities have been associated with membrane [19] and soluble fractions [7]. An anaerobic respiratory chain has been implicated in chromate reductase activity in *Enterobacter cloacae* obtained from activated sludge of a wastewater treatment plant [19]. Commercial exploitation of this anaerobic activity is underway in pilot schemes for the removal of chromium from wastewaters.

In the present study, bacteria were isolated from the water of a subsidiary cooling system in a power station to which chromate had been added as an anticorrosive agent and to inhibit the growth of algae and slime. Subsequently, a problem arose due to the blockage of water pipes by the precipitation of Cr(III) oxide formed by reduction of Cr(VI). The objectives of this work were to isolate the bacteria responsible for the reduction of Cr(VI) and to study their chromate-reducing activities. In addition, the bactericidal action of sodium hypochlorite on these chromate-reducing bacteria was assessed.

MATERIALS AND METHODS

Isolation and identification of chromate-reducing bacteria

Samples (100 μl) of the cooling water were added to Luria Bertani (LB) medium comprising (g L^{-1}): tryptone (10), yeast extract (5) and NaCl (0.5) at pH 7.4. Plates were incubated in triplicate at a range of temperatures between 10 and 75 °C. Optimum growth was observed at 30 °C after 24 h. Morphologically-distinct colonies were picked from plates and repeat-

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edly subcultured for purification. Gram stains were performed for each isolate and first-stage diagnostic tables used for the identification of Gram-positive and Gram-negative bacteria. Primary tests for the identification of genera included acid-fastness, production of spores, motility, presence of catalase and oxidase, metabolism of glucose and the ability to grow under aerobic or anaerobic conditions.

Assaying for Cr(VI)

Cr(VI)-reducing activity was assayed from the decrease in [Cr(VI)] with time using the colorimetric reagent *s*-diphenylcarbazide. The reagent was stored in AnalaR acetone solution to minimize deterioration [18]. A solution of *s*-diphenylcarbazide (50 mM) in sulfuric acid (0.4 M), and chromate gave a maximum absorption at 535 nm with a molar absorptivity calculated as 31 300 M^{-1} cm^{-1}.

Screening of isolates for Cr(VI)-reducing activity

Each isolate was grown aerobically whilst shaking (200 r.p.m.) at 30 °C for 24 h in a LB broth starter culture (25 ml). LB broth (250 ml) was inoculated with 5% of its volume of the starter culture and grown under the same conditions. Cells were harvested by centrifugation at $16\,000 \times g$ for 15 min at 4 °C. The cell pellet was resuspended in buffer (104 ml) containing 10 mM Tris hydrochloride and 2 mM EDTA, pH 7.0 [7]. Cells and buffer were maintained in a flask at a constant temperature of 25 °C. Cells were not provided with an exogenous electron donor but presumably relied on an endogenous source for the reduction of chromate. At zero time, potassium chromate was added to the cell suspension to a final concentration of 20 μM. Immediately, 10 ml of suspension was removed and centrifuged at $24\,000 \times g$ for 10–15 min. A sample (5 ml) of the supernatant solution was added to 5 ml of reagent solution containing 50 mM *s*-diphenylcarbazide (0.5 ml), 0.4 mM sulfuric acid (3 ml) and deionized water (1.5 ml). Samples were then removed at 10–15 min intervals from the culture flask and similarly analysed for Cr(VI). Subsequently, isolate VMC-2 was used exclusively as this strain reduced chromate most rapidly.

Control assays were conducted with heat-killed cells and with all components minus whole cells. Two Fe(III)-reducing bacteria, *Vibrio metschnikovii* and *Aeromonas hydrophila*, previously isolated from the profundal sediments of tarns in the English Lake District, were assayed for Cr(VI)-reducing properties as a comparison, together with *Escherichia coli* RP1.

Effect of selenate and sulfate on the reduction of chromate by VMC-2

The inhibitory effect of the tetrahedral anions selenate (SeO_4^{2-}) and sulfate (SO_4^{2-}) on the uptake/reduction of chromate was examined. In *Pseudomonas fluorescens* direct competition between SO_4^{2-} and CrO_4^{2-} suggested the common use of the sulfate transport system [14]. However, a second transport system without the involvement of the sulfate carrier was responsible for partial uptake of radiolabelled chromate in this species.

Assays were performed at an initial chromate concentration of 20 μM with the addition of either sodium sulfate or sodium selenate solutions to give final concentrations of 0.1, 0.5 and 1.0 mM. Assays were also carried out with either selenate or sulfate in the absence of cells to confirm that the presence of these compounds in buffer did not contribute to the reduction of chromate (data not shown).

Effect of protonophore CCCP on the chromate-reducing activity of isolate VMC-2

Komori and coworkers showed that the uncoupling agent CCCP at 50 μM completely inhibited the reduction of chromate by *Enterobacter cloacae* H101 [8]. In this study, freshly prepared solutions of CCCP in AnalaR ethanol were added to whole cell chromate reduction assays to give final concentrations of 0.1 mM and 0.5 mM. It was confirmed that ethanol had no affect on absorbance values at 535 nm.

Reduction of chromate by subcellular fractions of VMC-2

Bacteria were grown aerobically whilst shaking (200 r.p.m.) at 30 °C for 24 h in a LB broth pre-starter culture (25 ml). A starter culture of LB broth (500 ml) was inoculated with 5% of its volume of the pre-starter culture and grown in the same conditions. LB broth (10 L) in a 12-L Braun Fermenter (B. Braun Biotech, Aylesbury, UK) was subsequently inoculated with the starter culture (500 ml) and 1 ml antifoam. Cells were grown overnight at 30 °C and harvested by centrifugation in an Alfa Laval continuous centrifuge (Alfa Laval, Camberley, Surrey, UK). Cells were washed twice in disruption buffer (as described earlier) and resuspended in approximately 140 ml buffer plus a few grains of DNase. Disruption of the cell suspension was carried out by three passages through a French pressure cell (SLM-Aminco, DG Electronics, Farnborough, UK) at 100–150 MPa and centrifuged at $12\,000 \times g$ for 20 min to remove cell debris. A few ml of this supernatant fluid was removed and kept aside as the 'cell-free' fraction and stored at −70 °C until use. The remaining supernatant fluid was centrifuged at $225\,000 \times g$ for 90 min to yield a supernatant fraction, known as the 'soluble' fraction, to be stored until further use, and a pellet. Cell pellets were then resuspended in a small volume of disruption buffer using an homogenizer and the suspensions subjected to a further high speed spin. The resulting supernatant fluid was retained and added to the first supernatant fraction to give a combined 'soluble' fraction. Pellets were resuspended in a small volume of buffer and known subsequently as the 'membrane' fraction. The protein content of each fraction was determined by a modification of the Lowry procedure [12].

Portions of subcellular fractions (0.05 ml) were dispensed into 1·5-ml microcentrifuge tubes (Scotlab, Coatbridge, Strathclyde, Scotland) and the reaction started by addition of potassium chromate solution to give a total volume of 0.5 ml and a final chromate concentration of 0.2 mM. In experiments with membrane fractions, NADH (10 μl from a 100-mM stock solution) was added as the electron donor during incubation with chromate. Reactions were stopped at appropriate time intervals by the addition of the analytical reagent for chromate. This resulted in the precipitation of protein which was removed by centrifugation at $11\,600 \times g$ for 5 min in a

microcentrifuge. The residual Cr(VI) in the supernatant solution was then determined spectrophotometrically.

Bactericidal activity of sodium hypochlorite

In these experiments sodium hypochlorite was chosen as the bactericidal agent. Because of its fast disinfecting property, relative low toxicity, broad antimicrobial activity and its cheapness, hypochlorite is widely used by the electricity-generating industry. Cells were grown aerobically whilst shaking (200 r.p.m.) for 24 h at 30 °C in LB medium to an OD_{600} of 4.1–4.4, determined after dilution. This complex medium was considered appropriate for growth, as the bacteria were isolated from cooling water which contained waste organic matter.

Cultures were harvested and washed twice in chilled 0.1 M buffer comprising ($g\,L^{-1}$), K_2HPO_4 (17.41) and KH_2PO_4 (13.60) and adjusted to the required pH. Experiments were usually conducted at pH 7.0 except when a concentration of 0.282 mM NaOCl was used. In this case, experiments were also performed at pH 6.0 and 8.0 using mixed phosphate buffer. Bacteria were suspended in approximately 50 ml buffer and held in a flask in a water bath at 25 °C. At time zero, the sodium hypochlorite solution (to give final concentrations ranging from 0.028 mM to 1.08 mM) was added to cells in buffer and swirled. At various time intervals after the addition of NaOCl, 100-μl samples were removed and viable counts were undertaken. The concentration of any residual NaOCl was presumed to be diluted to a value below the minimum inhibitory concentration.

RESULTS

Seven isolates were purified from the cooling water. Isolates included representatives of the genera: *Alcaligenes*, *Bacillus*, *Vibrio*, *Corynebacterium*, *Micrococcus* and *Staphylococcus*. The isolate referred to as VMC-2 has been tentatively identified as *Comamonas testosteroni*.

The fastest rate of reduction of Cr(VI) of the isolates obtained from the cooling water was shown by isolate VMC-2 (Fig. 1(A)). In 45 min, 95% of the added chromate had been reduced from an initial concentration of 20 μM. This result compares favourably with that of Ishibashi and coworkers [7] who demonstrated that the reduction of chromate (initial concentration of 20 μM as used in this study) at 45 min for *Pseudomonas fluorescens* was 79% and for *Pseudomonas putida* was 15% (interpolated from their graphical data). The lake isolates, *V. metschnikovii* and *A. hydrophila* reduced chromate at a similarly fast rate. At 45 min, 99% of the chromate had been reduced by the *Vibrio*. The corresponding value for *E. coli* RP1 was 40%. In the control experiments, minus bacteria or with heat-killed bacteria, chromate was not reduced.

The Gram-positive strains, *Micrococcus* and *Staphylococcus*, were unable to reduce chromate (Fig. 1(B)). The rate of reduction of chromate by the *Alcaligenes* isolate was lower than isolate VMC-2 (Fig. 1(B)). An even slower rate of reduction of chromate was observed for the strains of *Bacillus*, *Corynebacterium* and *Vibrio* (Fig. 1(C)).

Reduction of chromate by isolate VMC-2 was further

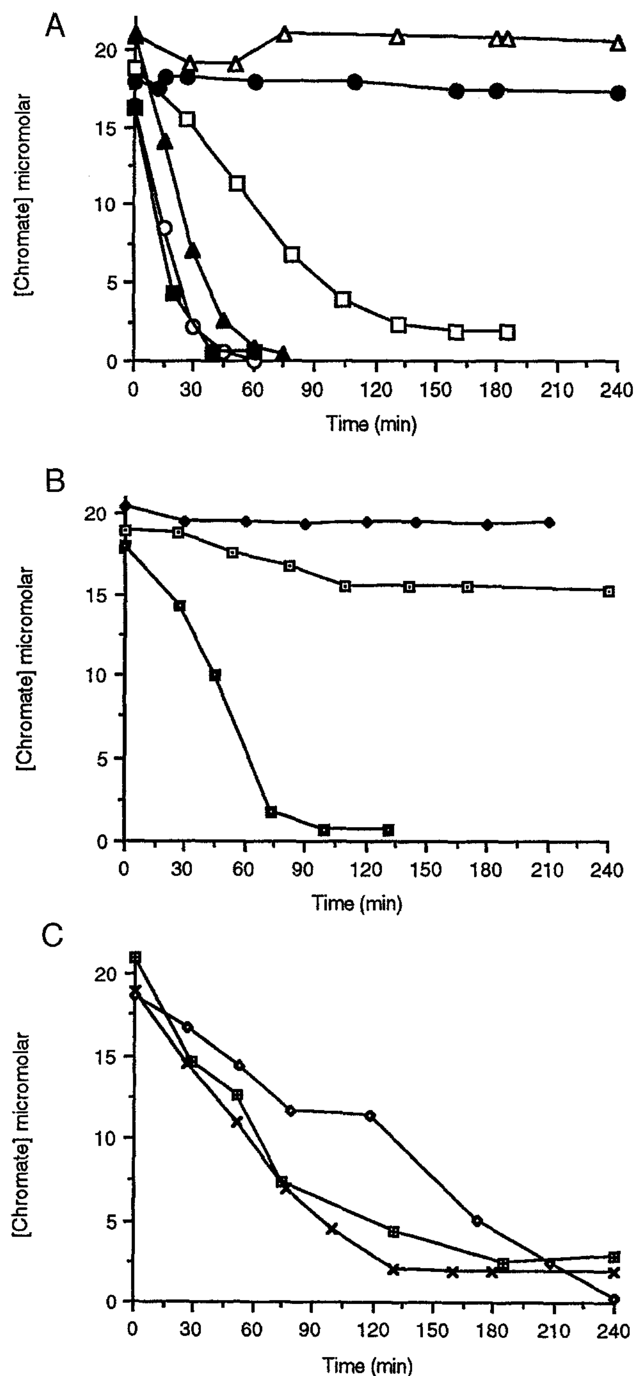

Fig. 1. Reduction of chromate by bacteria. Cells were harvested after 24 h at the following culture densities (OD_{600}) and suspended at 2.4 × these concentrations in buffer, to which was added 20 μM chromate. Samples were withdrawn at intervals and Cr(VI) assayed as described in the text. In (A) *Vibrio metschnikovii* (3.1,–○–); *Aeromonas hydrophila* (6.7,–■–); isolate VMC-2 (4.2,–▲–); *E. coli* RP1 (3.2,–□–); no bacteria (–●–); and heat-killed bacteria (–△–). In (B) *Alcaligenes* (4.0,–◘–); *Micrococcus* (4.8,–◆–) and *Staphylococcus* (4.2,–⊡–). In (C) *Corynebacterium* (3.4,–⊞–); *Bacillus* (4.0,–×–) and *Vibrio* (3.4,–◇–).

investigated by increasing by ten-fold the initial chromate concentration employed in the assay, to 200 μM. Within 188 min, 137 μM Cr(VI) had been reduced. The reaction was followed for a further 52 min but no further reduction was evident probably due to the depletion of endogenous electron donors (data not shown).

Reduction of Cr(VI) (20 μM) by whole cells of isolate VMC-2 was inhibited by 0.5 mM, and to a greater extent by 1.0 mM, sulfate (Fig. 2(A)). At 40 min, 70% of the original chromate had been reduced by the cells in the presence of 0.5 mM sulfate compared to 50% in the presence of 1.0 mM sulfate. The corresponding value in the absence of added sulfate was 85%. This suggests that sulfate is a competitive inhibitor of chromate reduction. No inhibition was observed with 0.1 mM sulfate; indeed there was a slight stimulatory effect.

In the presence of 1 mM selenate, reduction of chromate was slightly inhibited (Fig. 2(B)). At 40 min, 75% of the original chromate had been reduced. The two lower concentrations of selenate, 0.1 mM and 0.5 mM, stimulated reduction of chromate by isolate VMC-2. Therefore, there appears to be no direct competition between selenate and chromate for uptake sites, via the sulfate transport pathway or alternative pathways.

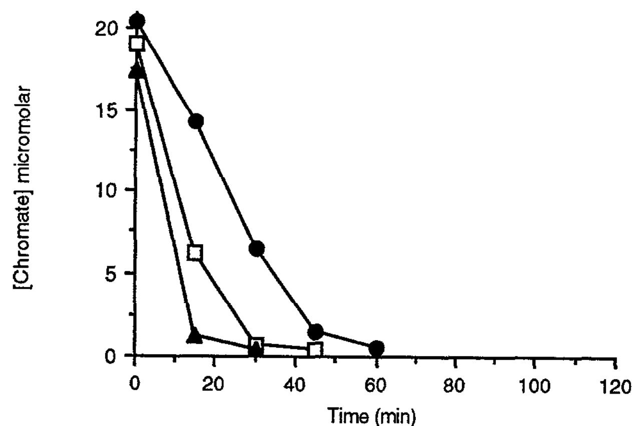

Fig. 3. Enhanced reduction of chromate by VMC-2 in the presence of CCCP. Cells were suspended in buffer to which was added CCCP in ethanol. Samples were withdrawn at intervals and assayed for Cr(VI) as described in the text. The initial concentration of chromate was 20 μM. Final concentrations of CCCP in cell suspensions were, 0.1 mM (–□–); 0.5 mM (–▲–) and zero (–●–). Each plot is representative of two experiments.

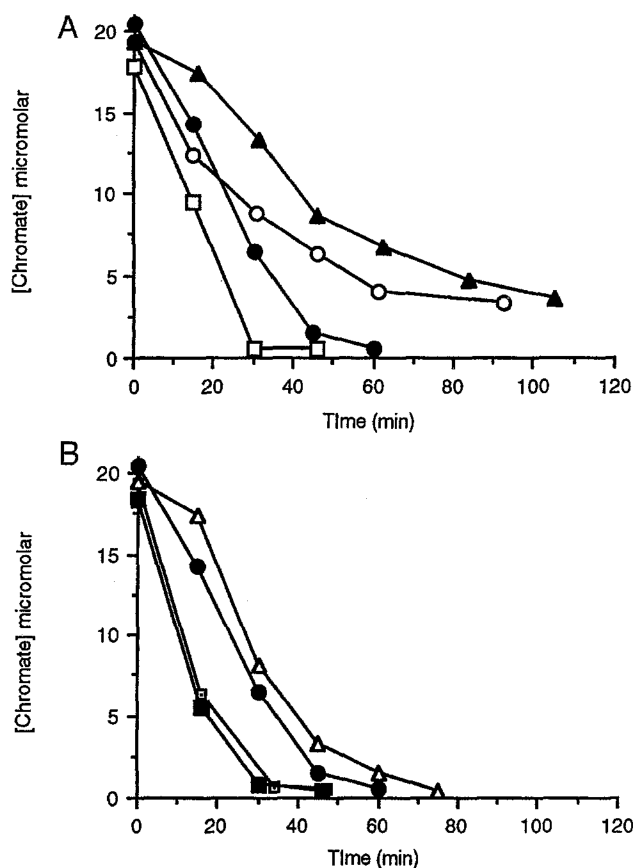

Fig. 2. Reduction of chromate by VMC-2 in the presence of sulfate or selenate. Cells were suspended in buffer to which was added various concentrations of sodium sulfate or sodium selenate. Samples were withdrawn at intervals and assayed for Cr(VI) as described in the text. The initial concentration of chromate was 20 μM. In (A) final concentrations of sulfate were, 0.1 mM (–□–); 0.5 mM (–○–); 1 mM (–▲–) or zero (–●–). In (B) final concentrations of selenate were, 0.1 mM (–⊡–), 0.5 mM (–■–), 1 mM (–Δ–) or zero (–●–). Each plot is representative of the results from two similar experiments.

The rate of reduction of chromate increased with increasing concentration of CCCP (Fig. 3). The simplest explanation of this result is that the reduction of chromate is linked to a proton-translocating respiratory chain.

Reduction of chromate by subcellular fractions

In 60 min, 90% chromate (from an initial concentration of 0.2 mM) had been reduced by the cell-free extract (Fig. 4). Most of this activity was located in the soluble fraction which was able to reduce 83% chromate in the same time period. No chromate-reducing activity was apparent in the membrane

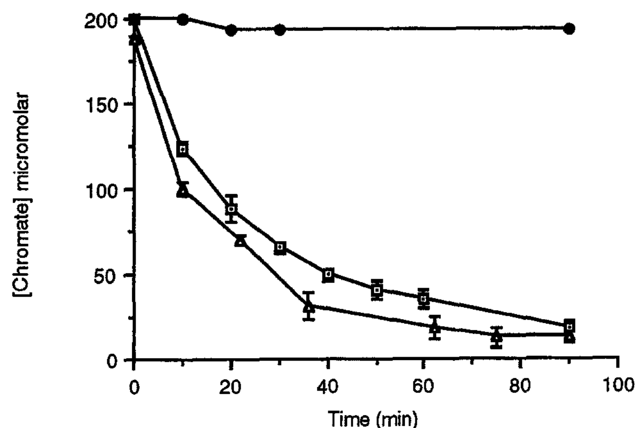

Fig. 4. Reduction of chromate by subcellular fractions of VMC-2. Fermenter-grown cells were disrupted by a French Pressure cell and fractionated. Cell fractions were incubated with 200 μM chromate. Samples were diluted before measurement and Cr(VI) assayed as described in the text. Protein concentrations in mg per 0.5 ml incubation mixture were: cell-free extract, 6.43, (–Δ–); soluble fraction, 4.35, (–⊡–); and membrane fraction 4.75, (–●–). Plots represent the mean of three replicates, ± standard deviation.

fraction with or without the addition of NADH. Reduction of chromate was not observed in the absence of cell material (data not shown).

This result compares favourably with a cell-free extract prepared from *Ps. ambigua*, with a protein content of 100 mg per 2 ml reaction mixture, which was able to reduce 40% chromate from an initial concentration of 0.2 mM chromate in 30 min. In the present study, the cell-free extract with a protein content of 6.43 mg per 0.5 ml reduced 78% chromate from an initial concentration of 0.2 mM in 30 min. Additionally, cell-free extracts or soluble fractions from this study did not require any exogenous electron donor for reduction of Cr(VI) unlike the extract prepared from *Ps. ambigua* G-1 and *Ps. putida* which both required NADH [6].

Studies on the bactericidal activity of sodium hypochlorite

Various concentrations of NaOCl between 0.028 mM and 0.226 mM were added to suspensions of cells and buffer at pH 7.0 without any noticeable decrease in viability over a 90 min period. A total kill of 9.0×10^9 cells ml^{-1} was only observed using 1.08 mM NaOCl, while approximately 75% kill was achieved at 0.282 mM NaOCl. The bactericidal action of NaOCl occurred within 1 min and even when viable counts were extended over 24 h a gradual rate of kill was not evident. Since an appreciable kill was observed at 0.282 mM, this concentration was recommended for use. Additional experiments were conducted at this concentration at pH 6.0 and 8.0 to assess the effect of varying the concentration of HOCl on the viability of bacterial cells. The fraction of total hypochlorite present as HOCl at these pH values was calculated using the pK_a for HOCl of 7.55. Results are shown in Table 1.

Increased kill was achieved at the lower pH values, suggesting the active species is HOCl, although there is not an exact correlation between the fraction of total hypochlorite present as HOCl and the percentage kill. Possibly the hypochlorite anion, OCl^-, is also active. Relatively poor disinfection was obtained with NaOCl, which in part may be due to the high density of cells used. It was assumed that the concentration of free residual NaOCl decreased during the course of these experiments, as would be found for the industrial situation owing to reaction with organic compounds present as contaminants in the cooling water. It should be noted that in industrial applications the effective bactericidal activity of NaOCl is diminished as it reacts with various organic compounds in the water to give chlorosubstituted products [11].

TABLE 1

Variation of the fraction of hypochlorite present as HOCl with pH and its relation to percentage kill

pH	% HOCl (Calculated)	Percentage kill ± SD of 9.0×10^9 cells ml^{-1} at 10-min exposure to NaOCl (0.282 mM)
6.0	96.6	76.5 ± 9.3
7.0	73.8	68.8 ± 14.8
8.0	21.9	55.5 ± 11.0

DISCUSSION

The results in this paper demonstrate that the isolate VMC-2 contains a chromate-reductase activity which is soluble after cell disruption. However, the observation that the protonophore CCCP stimulates this activity suggests that it might be membrane-associated in vivo.

Sulfate at 0.5 mM and 1.0 mM showed an inhibitory effect on the reduction of chromate. At low concentrations of sulfate (0.1 mM), chromate effectively competed with sulfate for transport. A possible explanation for this is that chromate and sulfate may use the same transport pathway. Selenate, on the other hand, did not inhibit the reduction of chromate even though selenate is frequently a substrate for sulfate transport systems.

Analysis of the product of chromate reduction in the cooling water system confirmed it to be Cr(III). The reduction of Cr(VI) to Cr(III) involves transfer of three electrons. The chemical reduction of oxyanions, such as chromate, usually involves the transfer of oxo groups with a change in the oxidation state of two. Such oxo group transfers are also known for redox reactions involving molybdoenzymes and nitrate/nitrite where both the molybdenum and nitrogen centres undergo changes of two in oxidation state. The reduction of Cr(VI) could involve a combination of 2-electron and/or a 1-electron transfer. However, it is noteworthy in the present study that Fe(III)-reducing isolates reduced Cr(VI) at a comparable rate to that achieved by VMC-2. This suggests that Cr(VI) is also reduced in 1-electron steps, as it seems unlikely that an Fe(III)-reducing isolate would catalyse 2-electron reductions efficiently.

The pathways for reduction of Cr(VI) by isolate VMC-2 have been investigated by electron spin resonance spectroscopy, which has confirmed the intermediacy of Cr(V) in the reduction of chromate by whole cells and soluble extracts (Cammack, R., V.M. Cooke, M.N. Hughes and R.K. Poole, in preparation). Such Cr(V) intermediates have previously been identified during the reduction of chromate and are responsible for certain toxic effects of chromium [1,16,20].

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