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Reduction of perchlorate by an anaerobic enrichment culture

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SUMMARY

A mixed bacterial culture capable of reducing perchlorate stoichiometrically to chloride under anaerobic conditions was enriched from municipal digester sludge. The reduction of 10 mM perchlorate resulted in oxidation of the medium and cessation of perchlorate reduction. The activity was recovered on addition of a reducing agent. Addition of air to the culture during perchlorate reduction immediately terminated the process and aeration for 12 h permanently destroyed the ability of the culture to reduce perchlorate. The culture also reduced nitrite, nitrate, chlorite, chlorate and sulfate. The presence of 10 mM nitrite or chlorite completely inhibited perchlorate reduction, whereas the same concentration of chlorate decreased the reduction rate. Nitrate or sulfate did not affect perchlorate reduction. Chlorate and chlorite, suspected intermediates in the reduction of perchlorate to chloride, were not detected in any cultures during reduction of perchlorate.

INTRODUCTION

Perchlorate salts are used extensively as oxidizers in the chemical, aerospace and defense industries. The most common applications are in propellants, explosives and pyrotechnic composites [19]. Ammonium perchlorate is used as an oxidizer in solid rocket propellants; composing up to 70% of the material by weight in some cases. Large amounts of aqueous ammonium perchlorate wastes are produced from the manufacture, refurbishment, and maintenance of large solid rocket motors and the perchlorate ion can be quite stable in these wastewaters. The fate of perchlorate in microbial systems is of interest from a biochemical point of view as well as aiding in the development of propellant waste treatment processes and bioremediation systems.

Vibrio dechloraticans Cuznesove B-1168 was reported to degrade perchlorate and chlorate to chloride when grown anaerobically on acetate or ethanol [14]. This organism when mixed with domestic wastewater reduced 3 mM concentrations of perchlorate at a maximum rate of 70 mg ClO_4^- h^{-1} per g dry weight [6]. A variety of heterotrophic bacteria containing nitrate reductase reduced perchlorate to chloride [1,2]. Nitrate inhibited perchlorate reduction in these cultures and repeated cultivation on perchlorate caused the loss of perchlorate- and nitrate-reducing ability. Reduction of perchlorate was linked to nitrate reductase activity [1,2,6,14].

Chlorate, an intermediate in perchlorate reduction [1,2] has been widely used as a suicide substrate in the study of nitrate reductase activity [11–13,17,18]. Enzymatic reduction of chlorate produces the toxic metabolites chlorite and hypochlorous acid. A wide range of microorganisms contain enzymes capable of reducing chlorate under non-growing conditions [8–10,15,16]. Recently, it has been demonstrated that chlorate can serve as the electron acceptor for anaerobic respiration by a mixed culture growing on acetate [7].

The aims of this study were to isolate a stable microbial culture capable of degrading higher perchlorate concentrations at rates superior to those previously reported and determine the optimum conditions for reduction to occur. We also investigated the effect of oxygen, oxidation-reduction potential, and other oxidized anions on the culture's perchlorate-reducing activity.

MATERIALS AND METHODS

Sample collection and enrichment

Anaerobic sludge was collected from a digester at the Denver municipal sewage plant, Denver, CO. In a series of enrichments, 10-ml samples of sludge were added to 120-ml sterile serum bottles containing 90 ml of the following sterile medium: K_2HPO_4, 5.0 g L^{-1}; NaH_2PO_4, 2.5 g L^{-1}; yeast extract (Difco Laboratories, Detroit, MI), 5.0 g L^{-1}; nutrient broth (Difco), 5.0 g L^{-1}; NH_4ClO_4, 0.5 g L^{-1} and resazurin, 0.001 g L^{-1} (final pH, 7.0). The serum bottles were crimp sealed with sterile butyl rubber stoppers and incubated at 30 °C. Ambient oxygen was not purged from the medium. The inoculum rapidly consumed the oxygen present as demonstrated by reduction of resazurin (E'_0, −51 mV [pH 7.00]) [3] from pink to colorless after incubation for 10–15 min. When perchlorate disappeared from the medium, 5 ml of the suspension was transferred to 100 ml of the

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above medium twice more. A fourth 5-ml transfer was made to 100 ml of medium containing an increased concentration of 1.17 g L^{-1} NH_4ClO_4. A stable perchlorate-reducing culture was obtained after three additional transfers.

Growth and testing medium

The culture medium contained per liter: K_2HPO_4, 6.0 g; Na_2HPO_4, 2.0 g; NH_4ClO_4, 1.17 g; yeast extract (Difco), 10.0 g; peptone (Difco), 10.0 g; and resazurin 1 mg. The pH was adjusted to 7.5 with HCl. The medium was added to appropriate size serum bottles, crimp sealed with butyl rubber stoppers and sterilized by autoclaving them. Ambient oxygen was not purged from the bottles but was removed by the microbial inoculum as demonstrated by the rapid reduction of resazurin. Cultures were initiated with a 1% inoculum and incubated at 32 °C. All sampling and culture manipulations were done with nitrogen-flushed sterile syringes. $NaClO_2$, $NaClO_3$, $NaClO_4$, $NaNO_2$, $NaNO_3$, or Na_2SO_4 were dissolved in water to the appropriate concentrations and filter sterilized before addition to the medium. Growth was monitored spectrophotometrically at 600 nm. Dry weight of biomass was determined by filtration of 5-ml aliquots of growth medium through dried and preweighed 0.2-μm pore size filters (Gelman). The filters were then dried overnight at 105 °C. The differences between the preweighed filters and the dried samples were used to determine dry weights.

Analytical methods

Perchlorate was measured with a perchlorate ion-selective electrode (pHoenix Electrode Co., Houston, TX) and model 901 pH/ion meter (Orion Research, Boston, MA). Chlorite, chlorate, nitrite, nitrate and sulfate were measured with an ion chromatograph (Model 4500i, Dionex Corp., Sunnyvale, CA) equipped with a conductivity detector, AG5A-5u anion precolumn and AG5A-5u column (Dionex Corp.). The mobile phase was sodium hydroxide at a flow rate of 1 ml min^{-1}. The mobile phase gradient was 5 mM NaOH for 4 min followed by a linear gradient from 5 mM NaOH to 50 mM NaOH for 12 min.

RESULTS

The initial culture inoculated with active sludge contained 4.25 mM perchlorate which disappeared after a lag of 12 days. The first transfer produced a lag of only 3 days. Subsequent transfers to medium containing 10 mM perchlorate demonstrated a reproducible 15–25 h lag time. The microbial community was composed of a diverse range of Gram-positive and negative rods as well as Gram-positive cocci. Initial attempts to isolate the organism(s) responsible for perchlorate reduction from the mixture were unsuccessful.

The enrichment culture stoichiometrically reduced perchlorate to chloride. In a typical experiment 9.2 ± 0.2 mM perchlorate yielded 9.3 ± 0.3 mM chloride at a rate of 107 mg ClO_4^- h^{-1} per g dry weight biomass. Another experiment demonstrated initial growth of batch cultures was rapid and extensive before perchlorate was reduced (Fig. 1). Secondary growth observed at about hour 30 during perchlorate reduction was not seen in cultures grown without perchlorate and implies perchlorate reduction is associated with biomass production. No chlorate or chlorite was detected during the incubation period. Microscopic examinations during the growth and reduction phase did not indicate noticeable morphological changes in the cells in the microbial population.

The pH range for perchlorate reduction was 6.6–7.5, with an optimum of 7.1, although luxurious growth occurred below 6.0 and above 8.5.

The temperature range for perchlorate reduction was 25–42 °C with an optimum of 42 °C. The culture grew at temperatures as low as 15 °C and as high as 45 °C, but no perchlorate loss was detected at these temperatures.

The nutritional requirements for perchlorate reduction were met by rich protein-based carbon sources. Simple sugars, organic acids, and alcohols alone provided good overall culture growth but did not support perchlorate reduction (Table 1).

Perchlorate reduction was inhibited at concentrations of 77.5 mM or higher (Table 2). Complete reduction of residual perchlorate concentrations up to 29.1 mM occurred with the addition of 25 g L^{-1} of both yeast extract and peptone.

The reduction of perchlorate depended on the oxidation-reduction potential of the medium. No activity was detected above the color change of resazurin (−110 mV [3]). In 10–15 min after the medium was inoculated with the mixed culture, resazurin was reduced from pink to colorless as residual oxygen in the medium was consumed. Perchlorate reduction began after 15–24 h. During perchlorate reduction resazurin in the medium was reoxidized and perchlorate reduction ceased. Perchlorate reduction resumed upon addition of a reducing agent. Fig. 2 shows the results of an experiment in which cysteine hydrochloride (in N_2-flushed, boiled water) was added to a culture when the medium was oxidized from colorless to a faint pink and perchlorate reduction had ceased (hour 46). The addition of cysteine hydrochloride to both cultures A1 and B1 resulted in color loss within 5 min and resumption of perchlorate reduction.

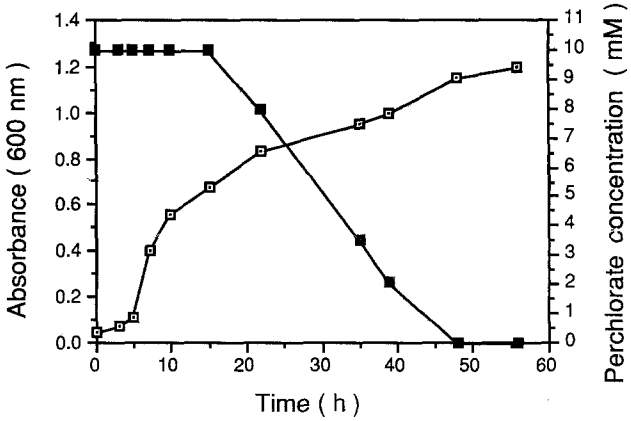

Fig. 1. Culture growth during perchlorate reduction. Symbols as follows: ⊡, absorbance; ■, perchlorate.

TABLE 1

Growth substrates tested for perchlorate reduction

Substrate[a]	Growth[b]	Perchlorate reduction
Acetate	+	−
Butyrate	+	−
Citrate	+	−
Lactate	+	−
Propionate	+	−
Pyruvate	+	−
Succinate	+	−
Glucose	+	−
Fructose	+	−
Lactose	+	−
Sucrose	+	−
Ethanol	+	−
Methanol	+	−
Nutrient broth	+	+
Peptone	+	+
Yeast extract	+	+
Casamino acids	+	+

[a] Each substrate was tested at a concentration of 5 g L^{-1} in the following buffer: (grams per liter) K_2HPO_4, 6.0; Na_2HPO_4, 2.0; NH_4ClO_4, 1.17; MgCl, 0.1; $MnSO_4$, 0.01; $FeSO_4$, 0.01; CaCl, 0.01; resazurin, 0.001; pH adjusted to 7.5

[b] Growth was defined as an increase in culture absorbance (600 nm) above 0.1 after 24 h.

TABLE 2

Concentration range of perchlorate degradation

Perchlorate concentration (mM)		Reduction (mmol)
Day 0	Day 9	
10.3 ± 0	0.1 ± 0.4	10.2 ± 0.4
22.5 ± 0	8.3 ± 0.8	14.2 ± 0.8
42.6 ± 1.5	29.1 ± 1.1	13.5 ± 0.4
60.5 ± 0	48.0 ± 4.9	12.5 ± 4.9
77.5 ± 3.5	75.0 ± 3.5	2.5 ± 3.5
103.8 ± 6.1	103.8 ± 6.1	0

Values represent triplicate samples ± the standard deviation.

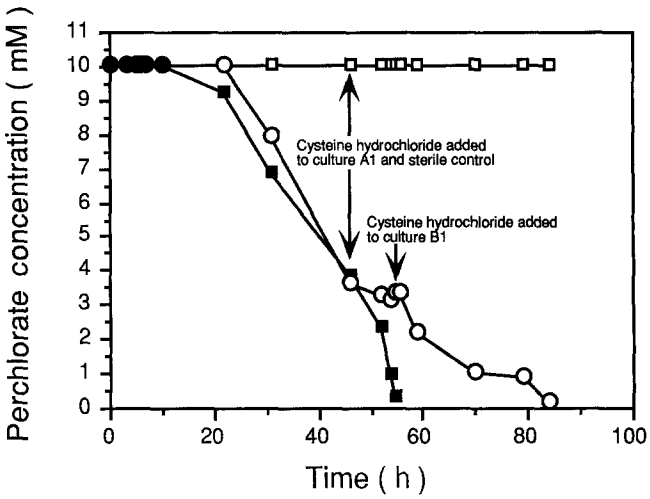

Fig. 2. Effect of 0.02% cysteine hydrochloride amendment on perchlorate reduction. The pH of cultures following amendment was 6.9. Symbols as follows: □, sterile control; ■, A1; ○, B1.

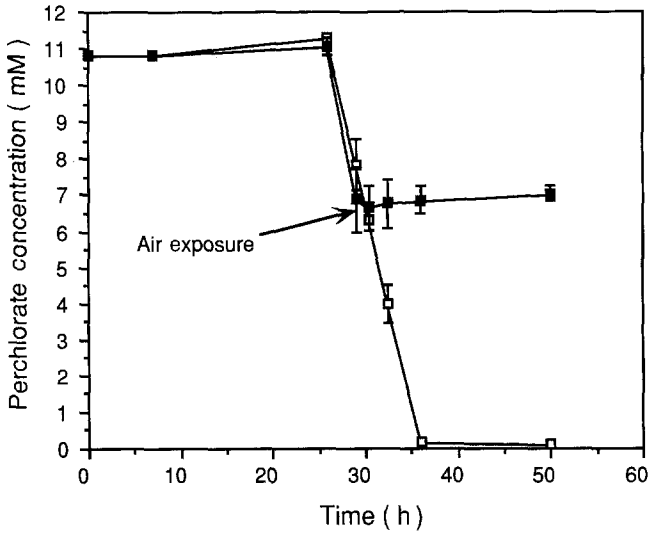

Fig. 3. Effect of air on perchlorate reduction. Two series of sealed cultures were shaken at 37 °C at 250 rpm. At hour 29 series A culture seals were replaced with sterile aluminum foil allowing air introduction. Series B remained sealed. Error bars represent the standard deviation of duplicate samples. Symbols as follows: ■, series A2; □, series B2.

The addition of cysteine hydrochloride to the sterile control did not cause chemical reduction of perchlorate. The addition of sodium dithionite, peptone or yeast extract also re-established perchlorate reduction under the above conditions (data not shown). A concentration of 5 g L^{-1} of both peptone and yeast extract supported the reduction of approximately 6–7 mM of perchlorate before the culture redox potential rose to an inhibitory level.

Perchlorate reduction was inhibited by oxygen. A culture series aerated during perchlorate reduction immediately ceased activity whereas a sealed anaerobic series continued to reduce perchlorate (Fig. 3). Transfers from the aerobic series to sealed anaerobic media after 12 or 24 h of aeration produced heavy growth, but did not reduce perchlorate and the activity could not be recovered. Transfers from anaerobic mixtures at 12 and 24 h readily reduced perchlorate. These results indicate that the organism(s) responsible for perchlorate reduction are strictly anaerobic. The culture contains a variety of undefined facultative anaerobes and these appear to provide a protective low-oxygen environment during normal culturing and transfer.

Cultures containing perchlorate alone catalyzed typical reduction with concurrent production of chloride (Fig. 4(A)). Addition of nitrate did not inhibit perchlorate reduction (Fig. 4(A,B)). Addition of nitrite produced both a lag in

perchlorate reduction until nitrite was removed and a slower rate of reduction (Fig. 4(C)). These results suggest that perchlorate reduction is inhibited by nitrite but not nitrate.

In the enrichment culture chlorate was reducd preferentially over perchlorate (Fig. 4(D)). The production of chloride was stoichiometric. The addition of an equimolar concentration of chlorate decreased perchlorate reduction rates by approximately one half (Fig. 4(A,D)).

Ten millimolar chlorite inhibited both perchlorate reduction (Fig. 4(E)) and culture growth. Increased chloride was presumably formed by reduction of chlorite. In a separate experiment a lower concentration of chlorite (4 mM) was completely reduced to chloride without perchlorate present within 48 h.

The effect of sulfate was investigated on the premise that perchlorate reduction may be metabolically related to sulfate reduction. Some sulfate disappeared, but perchlorate metabolism was unaffected by its presence (Fig. 4(A,F)). None of the compounds tested in Fig. 4 were reduced in sterile controls.

DISCUSSION

The reduction in lag times for perchlorate loss in enrichment transfers and the high rates of removal suggest that the reduction of perchlorate to chloride confers a selective advantage on a component of the microbial community. The increase in culture biomass during perchlorate reduction supports the previous suggestion that perchlorate is used as an electron acceptor for anaerobic respiration [14]. The degradation rate of 107 mg ClO_4^- h^{-1} per gram dry biomass is higher than the 70 mg ClO_4^- h^{-1} per gram dry biomass reported for *Vibrio dechloraticans Cuznesove* B-1168 [6] and 10–50 fold higher than rates reported for other bacterial isolates [1,2]. The rate is also comparable to that reported for chlorate in a chemostat system [7]. The upper degradable perchlorate concentration (60 mM) for the culture is twenty-fold higher than previously reported [6] and represents a significant improvement for the treatment of concentrated wastewaters.

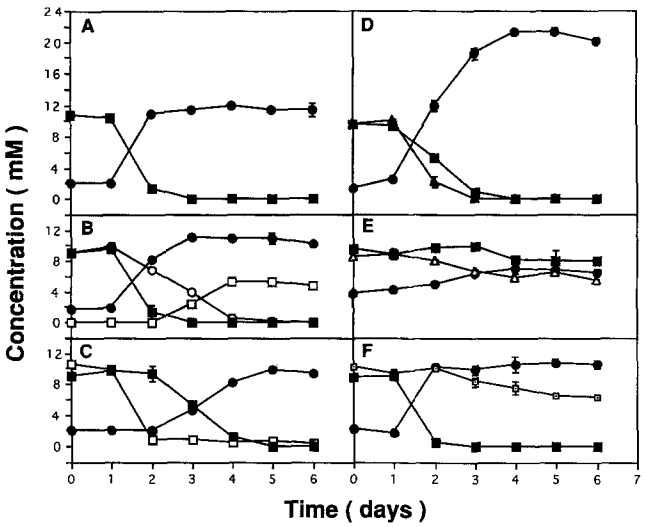

Fig. 4. Effect of alternate anions on perchlorate reduction and their fate during culture growth. **A**, perchlorate only; **B**, perchlorate plus nitrate; **C**, perchlorate plus nitrite; **D**, perchlorate plus chlorate; **E**, perchlorate plus chlorite; and **F**, perchlorate plus sulfate. Symbols as follows: ■, perchlorate; ●, chloride; ○, nitrate; □, nitrite; ▲, chlorate; △, chlorite; ⊡, sulfate.

Our results imply that the organism(s) responsible for perchlorate reduction within the mixed culture have narrower environmental and nutritional requirements than the culture as a whole. These include strict anaerobiosis, a more confined pH and temperature range and a requirement for yeast extract, casamino acids or peptone amendments. The requirement for protein-based amendments in the medium differs from previous reports for both perchlorate [14] and chlorate [7] where the metabolism of acetate served as the sole carbon and reductant source.

Perchlorate-degrading organism(s) within the mixed culture seem to require a highly reduced environment provided by the initial growth of facultative organisms. It may also be that the initial fermentative growth of the mixed culture provides nutrients used by the perchlorate-reducing population.

Because resazurin in the medium is oxidized during perchlorate reduction and remains oxidized until sufficient reductant is added, it suggests that a product of perchlorate metabolism is responsible for its oxidation. The metabolite is probably not oxygen because it is readily removed by facultative organisms. The oxidation of medium containing resazurin by nitrogen oxide intermediates during denitrification has been well documented [4,5]. In these reports the metabolites nitrous oxide and nitric oxide were shown to be responsible for the oxidation of resazurin in oxygen-free media. Analogous chloride oxide metabolites may be responsible for the oxidation in our cultures. Chlorite or hypochlorite oxidized sterile oxygen-free media while perchlorate and chlorate did not. It is possible that these anions were produced at concentrations below our levels of detection during the bioreduction and are responsible for the oxidation.

Previous reports have implied that nitrate reductase was responsible for perchlorate reduction [1,2,6,14]. Nitrate inhibited the reduction of perchlorate [1,2]. In contrast, in our mixed cultures nitrate did not inhibit perchlorate reduction. Had nitrate reductase activity been responsible for perchlorate removal, preferential use of nitrate would have been expected.

Previous work with *Bacillus cereus* extracts showed nitrite to be a non-competitive inhibitor of perchlorate reduction [1]. However, it is not clear in our culture whether nitrite is an inhibitor of the enzymatic process or simply toxic to the organism(s) carrying out the reduction.

It has been suggested that chlorate and chlorite are stepwise intermediates in the reduction of perchlorate [1,2]. This was supported by observations that chlorate was indeed formed during perchlorate reduction by *Bacillus cereus* extracts [2] and chlorite was a product of the reduction of chlorate under anaerobic conditions [13,16]. In our results the decrease of perchlorate reduction in the presence of

chlorate suggests that both compounds may be reduced by the same enzyme system and that chlorate is the preferred substrate. The preferential reduction of chlorate would prevent its accumulation.

Since sulfate was not a competitive inhibitor of reduction, it implies that sulfate reduction is not involved with the perchlorate process.

Because it is a highly oxidized compound, perchlorate represents a potential electron acceptor in anaerobic systems. The use of anaerobic bioreduction for the elimination of this compound in concentrated wastestreams is quite promising. We have obtained higher reduction rates with greater perchlorate concentrations than previously reported and are attempting to increase the culture activity in both regards. Our work describes new information about perchlorate reduction including: (1) A redox potential requirement below −110 mV; (2) oxygen toxicity to the organism(s) carrying out the reduction; and (3) inhibition by nitrite and chlorite, both of which are reduced by the culture. We are currently trying to determine what organisms within the culture are responsible for the reduction, what their carbon and energy requirements are, and how these organisms reduce perchlorate or chlorate without poisoning themselves with toxic intermediates. It will be of interest to see how similar this activity is to that observed by others [7]. This information will allow for the future development of potential biotreatment processes for dealing with perchlorate-laden wastewaters and contaminated sites.

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