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### Title

Perchlorate Removal in groundwater by perchlorate reductases from the perchlorate respiring bacterium, perc1ace

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## ABSTRACT

Perchlorate ( $\text{ClO}_4^-$ ) is an important energetic component of solid rocket fuel. The major source of  $\text{ClO}_4^-$  pollution is the military, space program and supporting industries.  $\text{ClO}_4^-$  is recalcitrant in the environment and is potentially toxic. The California Department of Health Services adopted an action level of 4 ppb for perchlorate in potable water. Microorganisms that reduce  $\text{ClO}_4^-$  to chloride and molecular oxygen have been isolated. For designing an efficient biological-based ground water  $\text{ClO}_4^-$  remediation strategy, the biochemical and molecular data on the enzymatic reduction of  $\text{ClO}_4^-$  are needed. The  $\text{ClO}_4^-$  respiring organism, *perclace* when grown using either  $\text{ClO}_4^-$  or  $\text{NO}_3^-$  as a terminal electron acceptor produced  $\text{ClO}_4^-$  reductase to a significant extent. The  $\text{ClO}_4^-$  reductase activity appeared to be within the periplasmic space, with activities as high as  $14,000 \text{ nmol}^{-1} \text{ min}^{-1} \text{ mg protein}^{-1}$ , indicating that it is a soluble enzyme. A  $\text{ClO}_4^-$  reductase from cell-free extracts of *perclace* was purified 10-fold by ion-exchange and molecular exclusion fast protein liquid chromatography (FPLC). The  $\text{ClO}_4^-$  reductase catalyzed the reduction of  $\text{ClO}_4^-$  at a  $V_{\text{max}}$  and  $K_m$  of 4.8 Units  $\text{mg protein}^{-1}$  and 34.5  $\mu\text{M}$ , respectively. Maximal activity was recorded at 25-30°C and pH 7.5 – 8.0. *Perclace*  $\text{ClO}_4^-$  reductase is a dimer with molecular masses of 35.07 kDa and 75.1 kDa determined by SDS-PAGE. Matrix-Assisted Laser Desorption Ionization-Time of Flight/Mass Spectrometry (MALDI-TOF/MS) analysis of the 35 kDa protein revealed several tryptic peptides. To study the genetic determinants of  $\text{ClO}_4^-$  reductase, the amino terminal sequences of 22 tryptic peptides of the approximately 35 kDa  $\text{ClO}_4^-$  reductase subunit were obtained by electrospray mass spectrometry. GenBank Blast analysis of the amino acid sequences revealed similarity to reductases, dehydrogenases and heme proteins. In batch studies of *in vitro* reduction of perchlorate, *perclace*  $\text{ClO}_4^-$  reductase reduced perchlorate in water with either NADH or methyl viologen as an electron donor. Less enzyme activity was observed with methanol and ethanol. Experiments showed that  $\text{ClO}_4^-$  reductase immobilized to Ca alginate reduced  $\text{ClO}_4^-$ . Additional studies are focusing on optimization of reaction conditions for perchlorate reduction by immobilized perchlorate reductases, molecular characterization of the overall genetic determinants of  $\text{ClO}_4^-$  bioreduction by *perclace* by cloning the genes using degenerate primers designed from the amino acid sequences of  $\text{ClO}_4^-$  reductase tryptic peptides and over-expression of recombinant  $\text{ClO}_4^-$  reductase. Such a recombinant enzyme available in large quantities can be immobilized and safely used for the treatment of perchlorate contaminated ground water on site. Treatment systems designed to employ cell-free enzymes catalyze the  $\text{ClO}_4^-$  reduction reaction without the production of biomass wastes. Moreover, the spent enzymes can be regenerated and reused, substantially reducing cost.

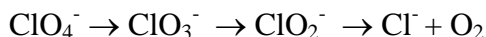
## INTRODUCTION AND PROBLEM STATEMENT

Increased usage of perchlorate ( $\text{ClO}_4^-$ ) for decades, has caused significant contamination of soils and ground water. Perchlorate is an important energetic component of solid rocket fuel, and chlorate is a potent herbicide as well as a defoliant. Wood bleaching in the manufacture of pulp and paper also leads to the generation chlorate. These oxyanions are recalcitrant in the environment and are potentially toxic to various forms of life, humans in particular (Lamm et al., 1999). The potential impact of perchlorate on human health has spurred regulatory agencies to regulate perchlorate concentration in drinking

water. In California, the Department of Health Services established an action level of 4 ppb for potable water.

Perchlorate contamination is a widespread problem. It has been detected in high concentrations in surface and ground waters in many states: Arizona, Arkansas, California, Indiana, Iowa, Louisiana, Maryland, Nevada, New York, Pennsylvania, Texas, Utah, Virginia and West Virginia. EPA expects  $\text{ClO}_4^-$  to be found in almost all of the lower 48 states except perhaps Maine, Vermont, Connecticut and Rhode Island. Perchlorate contamination of ground water has been estimated to potentially affect the drinking water supplies of at least 12 million people in the United States (Logan et al., 1999). A survey reported by the California Department of Health Services revealed that of 53 wells tested in Northern California, and 449 wells tested in Central and Southern California,  $\text{ClO}_4^-$  levels exceeded the previous  $18 \mu\text{g L}^{-1}$  action level in 8 and 25 wells, respectively. Testing of drinking water wells in Riverside and San Bernardino counties (California) revealed that some wells contain as much as  $216 \mu\text{g L}^{-1}$   $\text{ClO}_4^-$ , resulting in the closure of nine wells. Major surface water systems have also been shown to contain  $\text{ClO}_4^-$ , as indicated by levels as high as  $165 \mu\text{g L}^{-1}$  found in some areas of Lake Mead and  $8 \mu\text{g L}^{-1}$  in the Colorado River water south of the lake (Herman and Frankenberger, 1998).

Physicochemical water treatment technologies are expensive and less attractive for perchlorate removal from ground water. Microbial reduction of perchlorate to environmentally-acceptable innocuous end products is currently an area of intense interest because the strategy is relatively cost-effective and environmentally compatible. The overall mechanism of microbial breakdown of perchlorate is not fully understood (Logan et al., 2000). However, current published information support the following pathway:



The first two reactions are catalyzed by (per)chlorate reductase. Chlorite dismutase catalyzes the disproportionation of chlorite into chloride and oxygen.

A number of studies have addressed bioremediation of  $\text{ClO}_4^-$  (Romenanko et al., 1976; Wallace et al. 1996; Logan, 1998; Herman and Frankenberger, 1999, 2000; Urbansky and Schock, 1999; Logan et al., 2000, Miller and Logan, 2000; Giblin et al., 2000ab, 2001, 2002, Frankenberger and Herman, 2000, Logan et al., 2001, Okeke et al. 2001, Losi et al. 2002). However, there is very little biochemical and molecular data on the enzymes involved in  $\text{ClO}_4^-$  reduction. A (per)chlorate reductase was purified from a chlorate-respiring strain GR-1 (Kengen *et al.* 1999). Chlorite dismutase was purified from a bacterial strain GR-1 (Ginkel *et al.* 1996), but only recently, was the chlorite dismutase gene of the  $\text{ClO}_4^-$  respiring bacterium *Dechloromonas agitata* cloned and sequenced (Bender et al. 2002). Such data would provide important information on the design and application of an efficient bacterial-based  $\text{ClO}_4^-$  remediation strategy and molecular data obtained can be applied in the detection of  $\text{ClO}_4^-$  reducing bacteria in ground water.

Nitrate is commonly found in ground water of agricultural regions in the United States. Nitrate reduction will occur under the same conditions as perchlorate reduction. Nitrate

reductase has been successfully immobilized onto solid support (Aylott et al. 1997). Enzymatic nitrate elimination using immobilized nitrate reductase has been demonstrated by other researchers (<http://es.epa.gov/ncer/progress/sbir/water/68d50127.html>). It is thus reasonable to explore the production and stabilization of recombinant perchlorate reductase for removal of perchlorate from water in containment systems. For environmental applications, high volume flow, single pass reactors discharging a clean effluent is preferred. This is best achieved with packed bed reactors (Rosevear et al., 1987). Enzyme immobilization simplifies the use of biological systems as well as providing more opportunities for novel reactor designs. Immobilized enzymes may be more effective than microbial cells at a lower  $\text{ClO}_4^-$  and  $\text{NO}_3^-$  concentrations. Perchlorate reductase act specifically on  $\text{ClO}_4^-$ , while microorganisms prefer electron acceptors higher up on the redox scale. Cell-free enzymes require no uptake mechanisms, whereas, diffusion and permeability problems may impair microbial uptake of  $\text{ClO}_4^-$ . Cell-free enzymes are not susceptible to microbial competition in which other organisms may eventually colonize a bioreactor. Moreover, the enzyme can be re-used for the same application, substantially reducing cost.

## OBJECTIVES

This study was designed to:

1. Identify the cellular location of perchlorate reductase.
2. Assess perchlorate reduction by cell-free perchlorate reductase from perchlorate.
3. Select potential electron donors for *in vitro* reduction of perchlorate by cell-free enzymes.
4. Immobilize perchlorate reductase and examine removal of perchlorate from water in a bioreactor system.
5. Purify and characterize the perchlorate reductase of perchlorate.
6. Obtain amino acid sequences of perchlorate reductase and clone relevant genes by reverse molecular genetics.

## PROCEDURE

### Localization of Perchlorate Reductase in Perch1ace Cells.

**Cultivation of perch1ace.** Strain perch1ace was isolated by Herman and Frankenberger (1999). Strain perch1ace was grown in flasks sealed with a teflon septum, on a mineral salts medium (FTW) at 29°C, as described in Herman and Frankenberger (1998). Acetate (16 mM) was the carbon source and the electron acceptor was either  $\text{NO}_3^-$  (8mM), perchlorate (5 mM) or a combination of both. After autoclaving, the medium was sparged with  $\text{N}_2$  gas to remove any residual oxygen. One liter of FTW medium containing acetate and  $\text{NO}_3^-$  or  $\text{ClO}_4^-$  or a combination of both (as described above) was inoculated with strain perch1ace and the culture was grown for one week at 29°C. Cells were removed by centrifugation at 11,000 x g and washed once with phosphate buffer (100 mM, pH 7.2, sparged with nitrogen gas following autoclaving). The wet weight of each cell pellet was recorded, and pellets were resuspended in 25 mL anoxic phosphate buffer (100 mM, pH 7.2). Cells were broken in a French press at 20,000 psi followed by fractionation via centrifugation at 110,000 x g for 2 h. The pellet (membrane fraction) was resuspended in 50 mM potassium phosphate buffer (pH 7.2) containing 100 mM

sucrose and 5 mM magnesium chloride. The supernatant (cytoplasm and periplasm) and the resuspended membranes were kept at 4°C.

**Spheroplast formation.** Cells were grown and harvested as described above. The whole cell pellet was resuspended in 100 mM PO<sub>4</sub> buffer (pH 7.2) (40 mL) containing 0.3 M sucrose, 5 mM NaEDTA and 100 mM lysozyme and incubated at 37°C for 1 h. Additional MgSO<sub>4</sub> and sucrose were then added to give final concentrations of 50 mM and 0.6 M, respectively. Spheroplasts were harvested by centrifugation at 11,000 x g (4°C) for 30 min. The supernatant was saved as the periplasmic fraction. The pellet was resuspended in buffer (30mM Tris-HCl, pH 7.2, 30% sucrose) and then broken by sonication (30 sec). Centrifugation at 11,000 x g for 30 min yielded a supernatant which contained the membrane and cytoplasmic portions of the cell. Cell manipulations were not performed under anoxic conditions.

**Enzyme activity and protein determination.** Activity of perchlorate reductase (PR) and nitrate reductase (NR) were measured in cell extracts by monitoring the oxidation of reduced methyl viologen at 578 nm using 10 mM NO<sub>3</sub><sup>-</sup> or 10 mM ClO<sub>4</sub><sup>-</sup> as the substrate, as described by Kengen et al. (1999). The assays were performed at pH 8.0 for PR and 8.5 for NR, which was determined to be near the optimal pH for each enzyme by measuring activity at various pH levels.

**Analytical Techniques.** The ClO<sub>4</sub><sup>-</sup> concentration was determined using ion chromatography (Dionex, Sunnyvale, CA) as described by Wirt et al. (1998). Briefly, an AS-11 column was used with 100mM NaOH as the eluent and a 0.740 mL sampling loop. The suppressor was operated at 300mA with external water regeneration. Nitrate was also determined by ion chromatography using the AS-11 column with 10mM NaOH as the eluent.

### **Molecular Analysis of Perclace Perchlorate Reductase**

**Microorganisms and culture conditions.** The bacterium, perclace (ATCC 202172), isolated from biosolids from a water quality control plant, Riverside, CA, USA by Herman and Frankenberger (1999) was used. Perclace was pre-grown in 100 ml FTW mineral elements medium using 125 ml Erlenmeyer flasks. FTW mineral salts medium comprised of the following (in g/L): K<sub>2</sub>HPO<sub>4</sub>, 0.225; KH<sub>2</sub>PO<sub>4</sub>, 0.225; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.225; MgSO<sub>4</sub> x 7H<sub>2</sub>O, 0.05; CaCO<sub>3</sub>, 0.005; FeCl<sub>2</sub>.4H<sub>2</sub>O, 0.005, NaC<sub>2</sub>H<sub>3</sub>O<sub>4</sub>, 1.39 and 1 ml of trace elements solution (Focht, 1994). The medium was supplemented with 0.1g/L tryptone and 1g/L ClO<sub>4</sub><sup>-</sup>. Headspaces were flushed with nitrogen and incubated at 30°C for five days. The 100 ml culture was used to inoculate sterile 900 ml FTW mineral medium (Herman and Frankenberger, 1999) and further incubated at 30°C for one week. Aliquots of cells from a total of 10-liter culture were centrifuged (4,500 x g, 15 min, 4°C) and washed using nitrogen saturated buffer (100 mM Tris-chloride buffer pH 7.2, 2 mM DTT).

**Extraction of Enzymes.** Perclace cells were re-suspended in fresh 100 mM Tris-chloride buffer pH 7.2, 2 mM DTT, were lysed in a pre-chilled French pressure cell, at 20,000 psi using a French press. The extraction procedure was repeated. Cellular debris

were separated by centrifugation (4,800 x g, 15 min, 4°C) and reddish supernatant fraction was passed through 0.22 µ filter and the filtrate served as the cell-free enzyme (CFE) for further studies.

**Enzyme Assays.** Perchlorate reductase was assayed in capped cuvetts by monitoring the oxidation of reduced methyl viologen at 578 nm as described by Kengen et al. (1999). The 4 mL assay mixture comprised of 100 mM Tris-chloride buffer pH 7.2, 0.5 mM methyl viologen, 1 µM sodium dithionate, 10 mM ClO<sub>4</sub><sup>-</sup> and enzyme. For the surrogate assay mixture, the enzyme solution was replaced with the Tris-chloride buffer. The enzyme assay mixture was incubated at 30°C for 10 min. All solutions were purged with pure nitrogen for 5 min to remove O<sub>2</sub>. For the calculation of specific activities, protein concentration was quantified by the standard Bradford method (Bradford, 1976) using Coomassie<sup>R</sup> protein assay reagent (Pierce, Rockford, IL).

**Enzyme purification.** Cell-free enzyme extract of perchance was concentrated by passing it through a 10 kDa molecular weight cutoff using a Macrosep<sup>TM</sup> centrifugal membrane concentrator (Pall Filtron Corporation, Northborough MA, USA) to approximately 0.5 ml. A Pharmacia model LCC-500-PLUS fast protein liquid chromatography (FPLC) system (Amersham Bioscience Corp., NJ, USA) was used for ion-exchange and gel filtration chromatography. The concentrate was applied to HiTrap Q Sepharose high performance anion-exchange column (1.6 x 2.5 cm), previously equilibrated with 20 mM Tris-chloride buffer pH 8.2 to which 2 mM DTT (buffer A) was added. The column was washed with 50 ml of buffer A to elute unbound proteins. Bound proteins were then eluted using a linear gradient of 0-1 M NaCl in buffer A, at a flow rate of 4ml/min. Thirty 1 ml fractions were collected at 4°C and assessed for perchlorate reductase activity. Active fractions were pooled and subjected to gel filtration chromatography as follows. Pooled fractions were concentrated to about 0.5 ml using the 10 kDa centrifugal concentrator. The concentrate was applied to Superose 12 HR 10/30 column (1 x 30 cm) previously equilibrated with elution buffer (150 mM KCl in 20 mM potassium phosphate buffer, pH 7.5). Protein elution was with the same buffer at a flow rate of 0.36 ml/min. Fractions (1ml) were collected at 4°C and subjected to ClO<sub>4</sub><sup>-</sup> reductase assay. Approximate relative molecular mass ( $M_r$ ) of the native enzyme was estimated from the gel-filtration column according to the method of Andrews (1964).

**Determination of molecular mass.** The purity and molecular mass of enzyme fractions were examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using 7.5 % polyacrylamide. Broad range molecular mass markers (BIO-RAD, Hercules, CA) ranging from 6,400 to 198,000 Da were used. Gels were stained with Coomassie brilliant blue G-250. Molecular mass was then calculated from a regression equation [ $Y = -1.1105X + 2.3757$ ,  $R^2 = 0.98$ ; where  $Y = \text{Log}_{10}$  Molecular mass and  $X = \text{Relative mobility (R}_f\text{)}$ ].

**In-gel digestion of perchlorate reductase.** In-gel digestion was carried out essentially according to the method of Clauser *et al.* (1999). After PAGE analysis, gel slices were excised from the 7.5% polyacrylamide gel and trimmed. The gel slice was diced into small pieces and transferred into 650 µl tubes and sufficient 50 % 25 mM NH<sub>4</sub>HCO<sub>3</sub> in acetonitrile was added to cover the gel pieces and vortexed for 10 minutes to remove

Coomassie blue stain. This extraction procedure was repeated until Coomassie was completely removed. Destained pieces were dried using a speed vac. The dried gel was covered with 3 volume sequencing grade modified trypsin solution (Promega Corporation, Madison, WI), vortexed for 10 min and incubated at 4°C for 30 min. Excess trypsin was removed and  $\text{NH}_4\text{HCO}_3$  (25 mM) was added to cover the gel pieces. The tubes were covered with parafilm and incubated at 37°C overnight. The trypsin solution was thereafter removed. The digest was extracted twice with 50  $\mu\text{l}$  of 1% formic acid in 50% acetonitrile by soaking for 30 min. The extract was combined with the trypsin solution recovered from the gel and dried to about 10  $\mu\text{l}$ . The sample was then purified and concentrated using ZipTip $\text{C}_{18}$  (ZTC18S024 Millipore cooperation, Bedford, MA) according to the manufacturer's instructions.

**Matrix-Assisted Laser Desorption Ionization-Time of Flight/Mass Spectrometry (MALDI-TOF/MS) Analysis.** Tryptic peptides were subjected to MALDI-TOF/MS analysis of molecular mass. Exactly 0.5  $\mu\text{l}$  of  $\alpha$ -cyano-4-hydroxy-cinnamic acid matrix solution (5 mg in solution containing 500  $\mu\text{l}$  acetonitrile, 500  $\mu\text{l}$  water and 1  $\mu\text{l}$  formic acid) and 0.5  $\mu\text{l}$  of the protein sample were mixed on sample plate, air-dried for 5 min and subjected to MALDI-TOF analysis. Monoisotopic masses of all peptides were measured using a Voyager DE-STR BioSpectrometry Workstation (PerSeptive Biosystems Inc., Applied Biosystem, Foster City, USA) according to the manufacturer's instructions, with delayed extraction operated in the reflection mode.

**Peptide sequencing by electrospray mass spectrometry (MS).** MS/MS spectra of peptides were obtained on a QTOF-Ultima-Global mass spectrometer (Micromass, Manchester, UK) with an external nanoelectrospray ion source. Mass resolution was routinely obtained in the neighborhood of 1000 (for both conventional MS and MS/MS modes of operation), and a mass accuracy of at least 0.02 dalton with external calibration was achieved. Approximately 5  $\mu\text{l}$  of sample was loaded into the nanoelectrospray tip. Conventional mass spectra were first obtained to measure mass values (parent ions) of trypsin digested peptides and to assign their charge states from observation of stable isotope spacing. The parent ions of interest were selected for sequence analysis by tandem mass spectrometry. The Biolynx peptide sequence program in the Masslynx software package (Micromass, Manchester, UK) was used to predict the peptide sequence based on its MS/MS spectrum.

**N-terminal sequencing.** This was carried out essentially as described by Okeke *et al.* (2001). After SDS-PAGE of protein sample, the protein was electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane using a Bio-Rad electroblotting apparatus (Bio-Rad, Hercules, CA, U.S.A.). The amino terminal sequence was analyzed by automated Edman degradation performed using an Applied Biosystems 491 protein sequencer (Perkin Elmer Applied Biosystem, Foster city, USA); equipped with a PROCISE<sup>TM</sup> 1.1a data analysis software.

**Determination of enzyme properties.** Active gel filtration fractions were pooled for the determination of enzyme properties. Assays were then performed as described under the enzyme assay procedure described above. Temperature activity profile was determined by incubating at 20, 25, 30, 35 and 40°C for min. Temperature stability was examined by incubating enzyme at each temperature for 1 h and residual activity was thereafter determined



at 30°C for 10 min. For pH activity profile, this was examined using 100 mM Tris buffer adjusted to pH 5.0 - 9.0 with HCl. The pH stability was determined by incubating the enzyme at each pH value for 1 h, at room temperature, using the same buffers, after which remaining activity was measured. The effects of metal ions ( $\text{Ag}^{3+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Na}^{2+}$ ,  $\text{Mn}^{2+}$  and  $\text{Zn}^{2+}$ ), were examined at a final concentration of 1 mM, using the enzyme assay procedure. Effect of substrate concentration was determined using different concentrations of perchlorate (0.01 to 4 mM). Kinetic parameters ( $K_m$  and  $V_{\max}$ ) were determined using the hyperbolic equation described for Michaelis-Menten ( $v_o = V_{\max}[\text{S}]/[\text{S}]_{1/2} + [\text{S}]$ ; where  $[\text{S}]_{1/2} = K_m$ ) (Farrel and Ranallo, 2000). The kinetic parameters were obtained after fitting the results to the equations using the software, Sigma Plot 2000 (Jandel Scientific Software, San Rafael, CA).

## **Immobilization of Cell-free Perchlorate Reductase and Bioreactor Experiments**

**Immobilization of Cell-free perchlorate reductase.** Celite and Ca-alginate were used as supports. For Ca-alginate, sodium alginate (2%) was added to 50 mL of the cell-free extract. Cell-free  $\text{ClO}_4^-$  reductase were immobilized onto alginate by dripping into a cross-linking solution from an 18 gauge needle attached to a 60 mL syringe. Ca-alginate beads of immobilized intact cells and CFE ranging from 0.5-2 mm were achieved by this procedure. After about 1.5 h, the hardened beads were washed with a fresh calcium crosslinking solution and packed into the column. To immobilize perchlorate reductase, cell-free extract (25 mL) was introduced into the influent sample port of the bioreactor packed with Celite at a flow rate of 0.33 mL min<sup>-1</sup> and the eluent was recycled.

**Bioreactor.** The reactor was designed to operate in an upward flow mode. The reactor columns consisted of 60 mL sterile polypropylene syringes (2.5 cm, internal diameter x 13.5 cm). Columns were packed in duplicate with 45 mL of sterile Celite R-635 (Celite Corporation Lompoc, CA) and Ca-Alginate beads prepared from alginic acid (Acros Organics, NJ, U.S.A.). Celite pellets were crushed to obtain particles between 0.3 to 0.9 mm in diameter. Celite particles were washed in water, autoclaved twice and oven dried. The reactor was set up at ambient room temperature (22°C). A Master Flex 4S (Cole Parmer, Venon Hills, IL) multi-channel peristaltic pump was used to deliver the influent feed to the reactor units.

**Reactor startup and operating conditions.** The flow rate of the reactor was calibrated for 20 mL h<sup>-1</sup> corresponding to retention times of 0.9 h and 1.2h respectively for Ca-alginate and Celite columns, respectively. The column were washed with sterile Tris-HCl buffer, pH, 7.5 and thereafter fed with 10 mg/L perchlorate in the same buffer. For controls, columns were packed with the supports without the biocatalyst. The influent tank was purged with N<sub>2</sub> gas to minimize dissolved O<sub>2</sub> before the electron donor (5mM NADH or methanol) was added to the influent tank. Thereafter the headspaces were kept under N<sub>2</sub> atmosphere.

## **PCR Amplification of Putative Regions of $\text{ClO}_4^-$ Reductase**

**Preparation of genomic DNA.** Genomic DNA template was prepared as follows. Perclace was grown in FTW mineral salts medium (Herman and Frankenberger, 1999) containing perchlorate (100 mg L<sup>-1</sup>), acetate (1000 mg L<sup>-1</sup>) and yeast extract (300 mg L<sup>-1</sup>)

at 25°C for 5 days. Bacterial cells were recovered by centrifugation and suspended in sterile nuclease-free water. DNA was extracted from the cell suspension according to the method described by Ausubel et al. (1997). Degenerate primers (Table 1) were designed from the amino acid sequences of tryptic peptides and synthesized (SigmaGenosys, TX). The following primer combinations were used for PCR: A (grF and phosR), B (grF and oxiredR), C (phosF and oxiredR), D (cytF and phosR), E (cytF and oxiredR), F (oxiredF and oxiredR) and G (oxiredF and phosR). PCR master mix (Promega, Madison, WI) was used according to the manufacturer's instructions. Genomic DNA (1 µl) was the template. DNA was amplified using a 35-cycle PCR (initial denaturation, 95°C for 3 min; subsequent denaturation, 95°C for 1 min; annealing temperature, 55°C for 1 min; extension temperature was 72°C for 1 min and final extension was 72°C for 5 min).

Table 1. Primers used in this study.

Primer code	Sequence <sup>a</sup>	T <sub>m</sub> (°C)	Source <sup>b</sup>
grF 1999	CCNCGNCGNCARCTNAACNTAYGT (ANVMKAP <b>RRQLTYV</b> TDXN)	60.9	Kengen et al.,
phosF	GGNCTNACNGGNGGNTTYGGNGT (KSYV <b>GLTNFGTV</b> LGGR)	64.9	This study
phosR	ACNCCRAANCCNCCNGTNAGNCC (KSYV <b>GLTNFGTV</b> LGGR)	60.4	This study
oxiredR	GCNSHNSHNSHNACNGCNGCNCC (KVNASY <b>GAAVSSSA</b> AGG)	63.8	This study
cytF	GCNCCNACNCCNAARCCNHSNGG (KSGSL <b>APTPKPSG</b> PLNK)	62.9	This study
oxiredF	GGNGCNGCNGTNHSNHSNHSNGC (KVNASY <b>GAAVSSSA</b> AGG)	67.2	This study

<sup>a</sup>Primers were designed from the amino acid sequences (in bold) of the tryptic peptides sequences in parenthesis. <sup>b</sup>Source of amino acid sequences used for the construction of primers. R=A+G, Y=C+K, S=G+C, H=A+T and N=A+C+G+T.

**Purification and cloning of PCR products.** The PCR product was analyzed on 2% agarose gel and purified using a Qiaex II gel kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Briefly, 20 µl ligation mixture comprised 4 µl of PCR product, 1 µl of pGEM vector, 10 µl of 2X rapid ligation buffer and 5 µl of water, at 4°C overnight. The ligation mixture was transformed in *E. coli* by adding 10 µl of the ligation to High Efficiency JM109 competent cells ((Promega) according to the manufacturer's instructions. Clones were selected by blue and white screening on Luria-Bertani, Miller agar plates containing 100 µg/ml ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranose (X-Gal). Plasmid DNA was isolated from transformant cells with inserts (white) using Wizard Plus Minipreps DNA purification system (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions.

**DNA sequencing.** DNA sequencing was performed by cycle sequencing using the ABI Prism BigDye terminator kit (Perkin Elmer Applied Biosystem, Foster city, USA) and an Applied Biosystem ABI 3100 genetic analyzer.

**Database analysis for similarities.** Determined amino acid sequences were analyzed for relevant similarities by GenBank Protein BLAST (search for short nearly exact matches).

## RESULTS

**Cellular location of perchlorate and nitrate reductases.** Regardless of growth substrate, perchlorate reductase (PR) and nitrate reductase (NR) activities were present in crude extracts of strain per1ace cells. For example, cells grown only in the presence of  $\text{NO}_3^-$  possessed PR activity and those grown only in the presence of  $\text{ClO}_4^-$  possessed NR activity (Table 2). PR and NR activities were found in both the soluble (cytoplasmic/periplasmic) fraction and the membrane fraction. The level of PR activity was very similar in both fractions, whereas the majority of NR activity was associated with the membrane fraction. Further fractionation of cells was necessary to determine the cellular locations of the enzymes. Treatment of cells with lysozyme to separate the periplasmic contents from the cytoplasmic and membrane fractions revealed that the NR was present in lysed spheroplasts (membrane and cytoplasm) but not in the periplasmic fraction. The exception was in  $\text{ClO}_4^-$  grown cells, in which the periplasmic fraction showed slight NR activity. The PR activity was found in the periplasm but not associated with the membrane/cytoplasmic fraction, except in  $\text{NO}_3^-$  grown cells where the membrane/cytoplasmic fraction displayed significant PR activity.

Table 2. Perchlorate (PR) and nitrate reductase (NR) activity in fractionated cells grown with perchlorate or nitrate or perchlorate plus nitrate.

Extract	Average activity ( $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ )					
	$\text{ClO}_4^-$ grown		$\text{NO}_3^-$ grown		$\text{ClO}_4^- + \text{NO}_3^-$ grown	
	PR	NR	PR	NR	PR	NR
Crude cell extract	2.02	1.31	4.24	6.61	1.29	1.13
Fractionated cells						
supernatant	1.71	.015	1.85	0.07	1.34	0.03
membrane	1.41	1.7	2.28	1.88	0.88	0.51
Spheroplasts						
periplasm	14	1.0	0.77	<0.01	8.17	<0.01
sonicated spheroplasts	<0.01	2.9	3.08	4.33	<0.01	4.45

**Extraction and purification of per1ace perchlorate reductase.**  $\text{ClO}_4^-$  reductase from cell-free extracts of  $\text{ClO}_4^-$  respiring-bacterium, per1ace, was purified by ion-exchange and molecular exclusion fast protein liquid chromatography (FPLC).  $\text{ClO}_4^-$  reductase activity was detected in the ion-exchange fractions 7-16. A second  $\text{ClO}_4^-$  reductase activity was detected in the ion-exchange fractions 20-23 but low compared to fractions 7-16. The purification scheme (Table 3) resulted in an approximately 10-fold purification of  $\text{ClO}_4^-$  reductase after superose 12HR molecular exclusion chromatography, with

approximately 22% yield of  $\text{ClO}_4^-$  reductase activity. The specific activity of the  $\text{ClO}_4^-$  reductase increased from  $0.48 \text{ U mg}^{-1}$  to  $4.74 \text{ U mg}^{-1}$  protein. In the molecular exclusion FPLC, fractions 10-12 (data not shown) displayed  $\text{ClO}_4^-$  reductase activity.

Table 3. Summary of purification of  $\text{ClO}_4^-$  reductase

Step	Total Activity (U)	Yield (%)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)
Cell-free extract	10.70	100	22.10	0.48	1.00
HiTrap Q Sepharose	6.71	62.7	7.60	0.88	1.82
Superose 12 HR	2.56	22.0	0.54	4.74	9.81

**Enzyme properties.** SDS-PAGE analysis of perclace  $\text{ClO}_4^-$  reductase revealed that the enzyme is a heterodimer with a relative molecular mass of approximately 35 kDa and 75 kDa. Approximate  $M_r$  of the native enzyme was estimated to be 384 kDa by molecular exclusion chromatography. Perclace  $\text{ClO}_4^-$  reductase catalyzed  $\text{ClO}_4^-$  reduction in the temperature range of 20 to 40°C and optimally at 25°C to 30°C. The enzyme was most stable at 20°C and relatively stable at 25 to 35°C for 1h. The enzyme was optimally active and stable at initial pH 7.5-8.0. Enzyme activity was not dependent on metal ions such as ( $\text{Ag}^{3+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Na}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ ) but activity was slightly stimulated by  $\text{Fe}^{2+}$ . The  $\text{ClO}_4^-$  reductase catalyzed the reduction of  $\text{ClO}_4^-$  at a  $V_{\text{max}}$  of  $4.79 \text{ U mg}^{-1}$  and  $K_m$  of  $34.5 \mu\text{M}$ .

**MALDI-TOF MS analysis and amino acid sequencing.** MALDI-TOF MS analysis of trypsin digested band of the small subunit revealed several tryptic peptides (Fig. 1). The amino acid sequence of 22 tryptic peptides were obtained by electrospray mass spectroscopy. The amino acid sequence of the tryptic peptides were different. The total mass of the 22 peptides was calculated to be approximately 30.49 kDa representing 86.9% of the parent 35.1 kDa subunit. The molecular mass of the tryptic peptides ranged from approximately 1000 Da to 2700. A typical electrospray MS spectra of a tryptic peptide showing sequencing pattern is presented in Fig. 2. GenBank BLAST analysis of the amino acid sequences obtained from the tryptic peptides showed relevant similarities to reductases, dehydrogenases and heme proteins (Table 4).

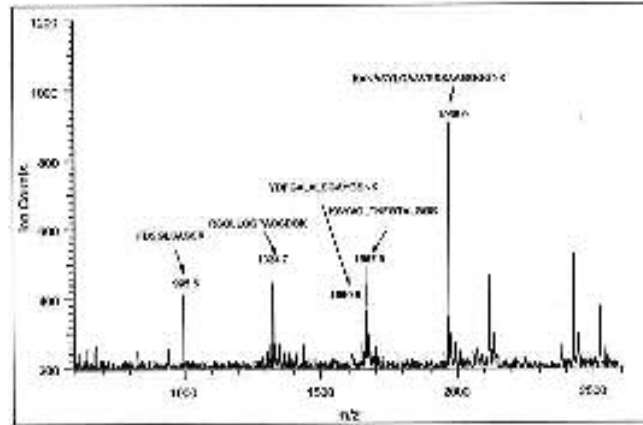


Fig. 1. MALDI-TOF spectrum of a 35.1 kDa subunit of perchlorate reductase digested with trypsin.

KVNASYLGAAVSSAAGGGNK MII<sup>+</sup>:1966.0

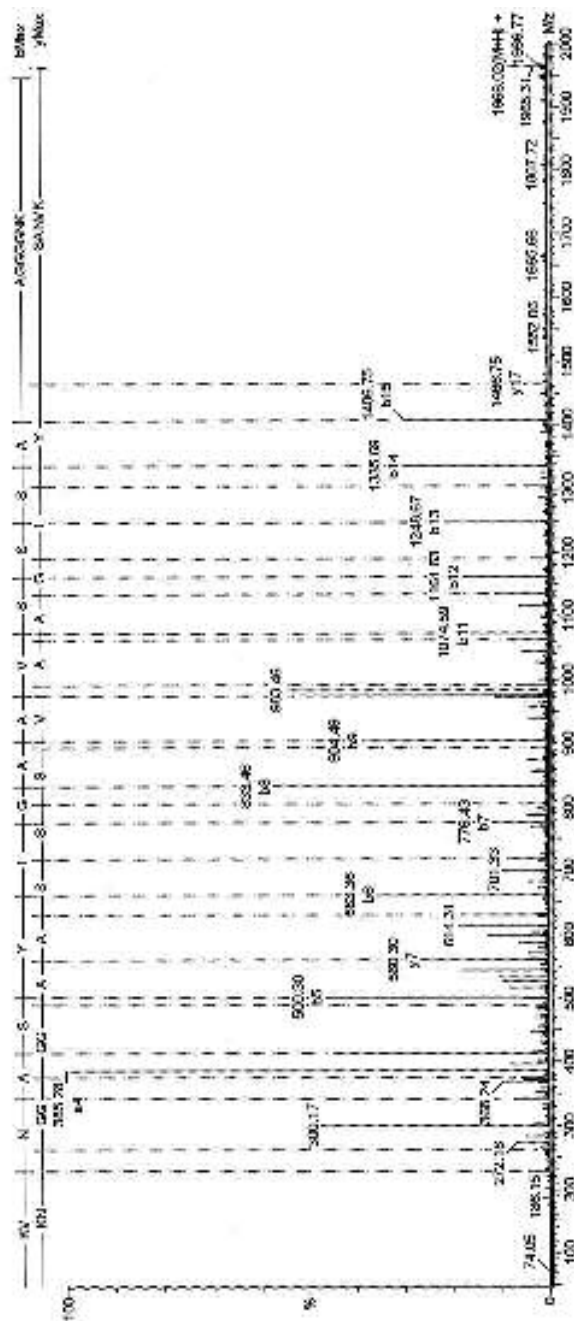


Fig. 2. Electrospray MS spectra of a tryptic peptide showing sequencing fragmentation pattern.

Table 4. Amino acid sequences of tryptic peptides of ClO<sub>4</sub><sup>-</sup> reductase (number of residues sequenced for each peptide are in parenthesis).

Predicted amino acid sequence	Monoisotopic mass (M+H <sup>+</sup> )	<sup>a</sup> Relevant short sequence similarities (GenBank accession number)
KVNASYLGAAVSSSAAGGGGNK (22)	1966.13	Reductases (XP145980.1, CAB72693.1, AAF21802.1, Dehydrogenase (CAD24097.1, Q52459, AAM94037.1, AAF10524.1, AAK44392.1, AAD12612.1, AAK23921.1)
KSGSLAPTPKPSGPLNK (17)	1677.94	Cytochrome C oxidase III (AF095622)
KSYVGLTNFGTVLGGGR (16)	1667.89	Cytochrome P450 (P21505), NADH dehydrogenase (CAD48168.1), <i>Rhodobacter sphaeroides</i> protein (ZP 00007460.1)
NQGAGMPGGGACGGGSR (17)	1432.59	Cytochrome C4 (AAF42142.1)
KNSLGGVFETTR (12)	1307.68	Oxidoreductase (CAB67715.1), Alanine dehydrogenase (NP646469.1)
RSGLLGPPAGSDGK (14)	1324.75	Dehydrogenase (P42515, AAC31183.1), <i>R. sphaeroides</i> protein (ZP 00005270.1, )
FDSGLSAGSR (10)	995.47	Oxidoreductase (NP 437896.1)
YLAAYLALSALGHNK (16)	1660.89	Dehydrogenase (NP 420615.1, NP 295218.1, NP 704747), <i>R. sphaeroides</i> protein (ZP 00006390.1)
RSGLLGPPAGLATR (14)	1324.76	Thioredoxin reductase (NP636459.1, P56431, Q9ZL18), Oxidoreductase (NP 519688.1, NP 015001.1, P42515, QO8822), Nitrate reductase (NP335644.1).
VEANGNDADKVR (12)	1286.62	Chlorophyllide reductase (Q02432), Ribonucleotide reductase (P48591) Gamma-glutamyl phosphate reductase (Q9RDK1)
RTAAAGGAPGGGAAATL (17)	1368.71	Hypothetical oxidoreductase (P71825) Nitrous-oxide reductase (Q59746, Q59105) Beta-ketoacyl reductase (P19097) Maleylacetate reductase II (P94135)
KGDVAHSGSGGAGPL (16)	1365.66	Dehydrogenase (NP 280387.1, BAA13047.1, P23312, 1808317A)
FDSGLSAGSR (10)	995.47	Oxidoreductase (NP 437896.1), Dehydrogenase (NP 014779.1, AAC69609.1)
YDFGVLALSAYGSNK (16)	1660.81	Heme/hemopexin-binding protein (P44602, P45355), Oxidoreductase (NP 280033.1)
VYSGLASPGPA (11)	1017.51	Nitrate reductase (AAL177669.1, BAA 13047.1), Reductase (NP714572.1)
KNSLGGVFETTR (12)	1307.68	Oxidoreductase (CAB67715.1), Dehydrogenase (BAB13146.1)
KLDSGPELPYL (11)	1230.65	Precorrin-6X reductase (Q10680)
KCSPGPGASAGP (16)	1027.48	Ribonucleotide reductase (P08543), Heme enzyme (NP 520477.1), Dehydrogenase (NP 636859.1)
KSGSLAPTPKPSGPLNK (17)	1677.94	Cytochrome c oxidase polypeptide (Q9ZZ61, P24989, O47693, O47693, P48892)
RSPSPSSGLM (10)	1017.49	Heme protein (NP520477.1)
KTASPVGGPTGGAGPL (16)	1365.73	Ketoreductase (AAD04717.1), Oxidoreductase (P80265), Dehydrogenase (B49732).
RGDPQLALAIHVQWGR (16)	1815.98	Dehydrogenase (AAL66805.1)

<sup>a</sup>Proteins related to reductases.

**Perchlorate removal by immobilized cell-free perchlorate reductase.** Cell-free perchlorate reductase immobilized on both Ca-alginate and Celite displayed perchlorate reductase activity. Perchlorate removal was highest in 1h and thereafter the rate decreased. The cell-free perchlorate reductase immobilized to Ca-alginate caused 26.8±2.9% and 32.2±4.9% removal of perchlorate in 1h. The rate of removal, however, decreased over time in both Ca-alginate and Celite columns.

**PCR amplification of putative regions of  $\text{ClO}_4^-$  gene.** Four of the seven PCR primer combinations successfully amplified putative regions of perchlorate reductase gene. An approximately 0.35 kb and 0.3 kb; 0.35 kb and 0.25 kb; 0.25 kb; and 0.35 kb were amplified with primer combinations A, C, D and G respectively. No amplification was achieved with primer combinations B and F.

**Cloning and sequencing of PCR products.** PCR products were cloned and sequenced. Total nucleotides for the PCR products were determined to be 359 bp (A1), 297 bp (A2), 351 bp (C1) and 262 bp (C2). Homology searches by GenBank blastx of PCR products A1, A2, C1 and C2 revealed significant homologies (accession numbers are given in parenthesis) to other *proteobacteria*. The sequence of PCR product clone A1 showed strong similarity with hypothetical proteins of *proteobacteria* such as *Ralstonia solanacearum* (NP-518586.1), *R. metallidurans* (ZP-00024614.1) and *Burkholderia fungorum* (ZP 00034261.1). The sequence of A1 was also slightly similar to electron transfer protoheme ferro-lyase (007401), a flavoprotein and a dehydrogenase (Q06401). PCR product clone A2 showed substantial similarity to dehydrogenases (AF273256.1, P93257) and a nitrate reductase (P16081). PCR product clone C1 displayed strong similarity to an oxidoreductase protein (NP 519448.1) of *Ralstonia solanacearum* and several dehydrogenases (AAF21129.1, NP252425.1, NP 743628.1, NP519448.1, NP 532274.1, NP385828.1) and a hypothetical protein of *R. metallidurans* (ZP 00024057.1, *B. fungorum* (ZP 00028531.1) and *Rhodobacter sphaeroides* (AF108766), a chlorate and nitrate reducing bacterium. The translated protein sequences of PCR product clone C2 showed substantial similarity to a hypothetical protein of *B. fungorum* (ZP 00030623.1), *R. metallidurans* (ZP 00024715.1) and *R. sphaeroides* (ZP 00006875.1); and anthranilate phosphoribosyltransferase protein of *R. solanacearum* (NP 521005.1) and *R. metallidurans* (ZP 00024715.1).

## CONCLUSIONS

- 1.) Perclace produces a periplasmic perchlorate reductase that is active *in vitro* on both nitrate and perchlorate. This study suggests that separate terminal reductases are present in strain perclace to reduce  $\text{ClO}_4^-$  and  $\text{NO}_3^-$ .
- 2.) Biochemical properties of perclace  $\text{ClO}_4^-$  reductase were characterized, providing relevant information for optimization of reaction conditions.
- 3.) Perchlorate reductase of perclace immobilized to Ca-alginate reduced perchlorate. However, the rate of reduction decreased over time.
- 4.) Amino terminal sequences of 22 tryptic peptides of  $\text{ClO}_4^-$  reductase  $\beta$  subunit were obtained by electrospray mass spectrometry. GenBank protein Blast analysis of the amino acid sequences revealed similarities to relevant proteins such as reductases, dehydrogenases and heme proteins.



- 5.) Degenerate primers constructed from selected tryptic peptides amplified relevant bands from perclace genomic DNA. Data obtained are useful towards the identification of the overall genetic determinants of  $\text{ClO}_4^-$  reduction, specific *in situ* detection of  $\text{ClO}_4^-$  reducing bacteria and prediction of  $\text{ClO}_4^-$  reduction in ground water.

## LIST OF PUBLICATIONS

Okeke, B.C., Giblin, T. and Frankenberger Jr. W.T., Reduction of perchlorate and nitrate by salt tolerant bacteria. *Environmental Pollution*, 118, 357-363, 2002.

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