RESPIRATORY PATHWAYS USED BY PERCHLORATE–RESPIRING
BACTERIA

A Thesis in
Environmental Engineering
by
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ABSTRACT

Systematic experiments were performed to examine the inhibitory effects of oxygen on perchlorate reduction and to identify the components and branches in the electron transport chains involved in perchlorate reduction and aerobic respiration by *Dechlorosoma* sp. KJ (ATCC strain BAA-592). To investigate the effect of dissolved oxygen (DO) on perchlorate reduction, anaerobically grown cultures of *Dechlorosoma* sp. KJ were exposed to near-saturated concentration of DO for various periods of time. It was determined that cells exposed to oxygen for more than 12 hours were incapable of reducing perchlorate. Cells exposed to oxygen for less than 12 hours, when re-introduced to anoxic conditions, quickly reduced the redox potential to highly negative values (-127 mV to -337 mV) and were able to reduce perchlorate or chlorite. This result suggested that aeration during backwashing of biofilm reactors, or exposure of perchlorate-degrading cell suspensions to oxygen for periods of less than 12 hours, will not be detrimental to the ability of perchlorate-degrading bacteria to use perchlorate as an electron acceptor.

Inhibitors were used to block specific respiratory enzymes, and thus to identify elements of the electron transport chain (ETC) involved in the reduction of molecular oxygen and perchlorate (Chapters 3 and 4). Inhibition of dissimilative perchlorate reduction by a low concentration of cyanide was due to oxygen build up, not inhibition of enzymes used for perchlorate reduction. The threshold DO concentration to inhibit perchlorate reduction was < 0.04 mg/L. The main ETC used by cells grown on high DO concentrations likely contains cytochrome *bc*₁, cytochrome *c* and cytochrome *aa*₃. When cells were grown on perchlorate, DO uptake occurred via a shorter ETC than that used by cells grown at higher DO concentrations. The ETC to oxygen at low DO concentrations is branched off the quinone to cytochrome *bd*, while the ETC to perchlorate is branched off the quinone to perchlorate reductase. These results should be useful in helping to improve the design and operation of biological perchlorate treatment systems.
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1. INTRODUCTION

1.1 Problem Statement and Research Objectives

Environmental contamination with compounds containing perchlorate and chlorate has been a constantly growing problem over the last fifty years due to the use of perchlorate as an oxidizer in solid rocket propellant and chlorate as a defoliant. Perchlorate, by interfering with hormone production in the thyroid gland, may endanger the drinking water supply of 15 million people [1]. Although there is no current drinking water limit for perchlorate, it has been included on the federal Contaminant Candidate List (CCL) [2]. Since perchlorate can be used as a terminal electron acceptor by many newly isolated strains of bacteria, several drinking water, wastewater, and in-situ treatment systems are being developed to biologically remove perchlorate. The three-step biochemical pathway for perchlorate reduction is proposed via $\text{ClO}_4^->$ $\text{ClO}_3^->$ $\text{ClO}_2^->$ $\text{Cl}^- + \text{O}_2$ [3]. In the last step, a chlorite dismutase produces molecular oxygen, which is a preferred electron acceptor [3, 4]. Under fully aerobic conditions, perchlorate reduction is inhibited by oxygen even for capable bacteria [5, 6]. Although the fact that microbes can respire using perchlorate, not much has been done to determine the relationship between perchlorate reduction and aerobic respiratory pathways, the knowledge of which will be important to the stability and long-term operation of such reactors.

The research discussed herein was initiated to analyze the electron transport chain (ETC) used by perchlorate-reducing bacteria. Aerobic respiration and anaerobic chlorate and perchlorate reduction were studied on pure, batch cultures of an isolate (Dechlorosoma sp. KJ), which is a gram-negative, heterotrophic, facultative anaerobe. The effect of molecular oxygen on perchlorate and chlorate ((per)chlorate) reductase was investigated on the batch culture of the same strain. Inhibitors were used to block specific respiratory enzymes in order to identify elements of the ETC involved in the reduction of molecular oxygen, chlorate and perchlorate. A chemostat system inoculated with KJ was established to explore the threshold of oxygen at which
(per)chlorate reduction is inhibited. The ultimate goal of this work was to characterize the branches of ETC for perchlorate-reducing bacteria and to understand the mechanism(s) in order to improve the operation of biological perchlorate treatment system.

1.2 Literature Review

1.2.1 Perchlorate respiring bacteria (PRB)

1.2.1.1 Ubiquity and diversity of perchlorate reducing bacteria

It has been known for several years that the ability to reduce perchlorate is not limited to a single bacterial species, although some of this earlier evidence of perchlorate degradation was inferred from information on biological chlorate degradation [7]. Bacteria capable of chlorate reduction were found to inhabit a variety of environments including rivers, sediments, soils, and wastewater treatment plants [8]. Utilizing media with chlorate as the only electron acceptor, Coates et al. [9] similarly found that the acetate-oxidizing chlorate reducing bacteria (CRB) represented a significant population whose abundance ranged from $2.31 \times 10^3$ to $2.4 \times 10^6$ cells per gram of samples obtained from a variety of sources, including pristine and hydrocarbon-contaminated soils, aquatic sediments, paper mill waste sludges, and farm animal waste lagoons. All thirteen isolates obtained in this study were also capable of growth on acetate using perchlorate, leading to early speculation, via the abbreviation of (per)chlorate, that all CRB were also capable of perchlorate reduction [9].

More recent studies have provided evidence that not all CRB are PRB. Wu et al. [10] used perchlorate or chlorate in anaerobic growth medium to compare the abundance of PRB and CRB in different environmental samples. They found that when perchlorate was the sole terminal electron acceptor in the medium, the number of PRB in a pristine soil was up to 1000-fold lower that found using chlorate in the medium to enumerate CRB for the same samples [10].
provided preliminary evidence that not all CRB were PRB, and that PRB were less abundant than CRB in these environments. Confirmation that not all CRB were PRB was provided later by three different studies. Only 8 of 10 CRB isolated from wastewater were found to be capable of respiring perchlorate [11]. In another study, it was similarly mentioned that a microorganism was isolated that was capable of chlorate, but not perchlorate, reduction [12]. Wolterink et al. [13] isolated a CRB strain *Pseudomonas chloritidismutans* AW-1 from an anaerobic bioreactor treating wastewater containing chlorate, and found that this strain did not reduce perchlorate. These studies indicate that while most CRB are able to degrade perchlorate, there is a subset of CRB that cannot use perchlorate as an electron acceptor for cell respiration.

Perchlorate-reducing isolates include *Vibrio dechloraticans* Cuznesove B-1168 [14], *Wolinella succinogenes* HAP-1 [15], *Dechloromonas agitata* CKB [16], *Dechloromonas* sp. SIUL, MissR, CL, NM and *Dechlorosoma* sp. SDGM, PS [9, 12], *Dechloromonas* sp. JM [17] and HZ [18], *Dechlorospirillum anomalous* WD [19], *Dechlorosoma* sp. KJ and PDX [11], GR-1 [3], perc1ace [20], *Citrobacter* sp. IsoCock1 [21], and *Dechloromonas* sp. JDS5 [22]. These PRB are all Gram-negative, facultative anaerobic rods. Analysis of the 16S rRNA sequences of tested strains indicated that all isolates were members of the class Proteobacteria. The majority of these PRB, including GR-1, perc1ace, KJ, PDX, HZ, JDS5, and 15 other strains [23], are located in the ß-subclass of Proteobacteria. Achenbach et al. [23] proposed two new genera, *Dechloromonas* and *Dechlorosoma* for these ß-subclass lineages, which represent the predominant PRB in the environment.

1.2.1.2 Electron donors and acceptors used by PRB for growth

All PRB are capable of dissimilatory reduction of chlorate to chloride for energy and growth, and many PRB can also reduce nitrate. For both perchlorate and chlorate, reduction does not occur in the presence of a high concentration of dissolved oxygen. Most isolates can use oxygen and many can respire using nitrate as a terminal electron acceptor. HAP-1 was initially
reported to be an obligate anaerobe [15], but in a later study it was reported that it was a microaerobic organism [24] although no further details on oxygen tolerance were provided. It seems reasonable that PRB should be able to use, or at least tolerate, dissolved oxygen as it is produced during the decomposition of chlorate and perchlorate. Although many PRB are capable of complete denitrification, CKB does not grow on nitrate [16], and Cuznesove B-1168 [14] and HAP-1 [15] reduce nitrate only to nitrite and do not produce ammonia or nitrogen gas.

In some cases PRB have been tested for their ability to use other electron acceptors such as metals and sulfate. GR-1 can utilize Mn (IV) as an electron acceptor [3]. Most PRB and CRB cannot reduce sulfate [3, 9, 13, 15, 16, 19, 20]. The only report of CRB (Acinetobacter spp.) capable of growth using sulfate was provided by Stepanyuk et al. [25], but the ability to respire perchlorate was not tested and these bacteria could not use nitrate. PRB have not found to be capable of using any other electron acceptors, including: Fe (?), selenate, malate, and fumarate [9].

Both heterotrophic and autotrophic PRB have been isolated. Acetate has been most frequently used as a single substrate for heterotrophic perchlorate reduction [9, 11, 14-16, 20], but hydrogen or formate was required as an electron donor for the growth of HAP-1 on acetate [15]. Perchlorate reduction by Citrobacter sp. IsoCock1 [21] was sustained on acetate, but yeast extract was found to improve growth. A wide variety of organic substrates, including alcohols and carboxylic acids, can be used as growth substrates by PRB although the use of these substrates is strain-dependent. Dechloromonas sp. JM [17], a strain isolated from the bacterial consortium in an autotrophic packed-bed biofilm reactor, reduced perchlorate using dissolved hydrogen, but could not grow using hydrogen as the sole electron donor. Two autotrophic isolates, Dechloromonas sp. HZ [18] and Dechloromonas sp. JDS5 [22], have recently been isolated that can grow in a minimal inorganic medium using perchlorate, hydrogen, CO₂ and nutrients.
1.2.1.3 Nutritional requirements for PRB

There is no detailed information on the best medium to use for PRB or what trace nutrients or metals are needed for growth. Several research groups have used a phosphate buffer system \cite{3, 5, 10, 11, 15, 20}, while others \cite{9, 16} have used a bicarbonate-buffered freshwater medium amended with a complex vitamin solution. There is no evidence that these additional vitamins are necessary for PRB growth, but in one study it was shown that several PRB strains could not grow without the trace metal solution \cite{19}. Iron, molybdenum and selenium appear to be important for PRB growth and perchlorate degradation. Perchlorate reductase purified from GR-1 was found to contain 11 mol of iron, 1 mol of molybdenum and 1 mol of selenium per mol of heterodimer \cite{6}. Wallace \textit{et al.} \cite{15} used yeast extract and peptone in their medium for HAP-1. Zhang \textit{et al.} \cite{18} found that yeast extract improved the growth of the autotrophic PRB strain HZ, but that yeast extract was not needed for growth.

It is likely that the different nutrients added for laboratory media are not needed for bioremediation of low perchlorate concentrations in natural systems. Several field studies have achieved perchlorate degradation only through the addition of an oxidizable substrate (acetate, ethanol, etc.) and nutrients (nitrogen and phosphorus) \cite{26, 27}.

1.2.2 Perchlorate degradation pathway

Much of what was originally known about bacterial degradation of perchlorate resulted from earlier studies on chlorate reduction. Quastel \textit{et al.} \cite{28} found that chlorite was produced from chlorate by one strain of \textit{E. coli} without further reduction of chlorite (ClO$_2^-$). The failure of chlorate respiration in this strain was likely due to the toxic effects of chlorite and an absence of the enzyme chlorite dismutase. Later, it was found that some bacteria could reduce chlorate to chloride \cite{29, 30}. The reduction of perchlorate to chloride by several species of heterotrophic bacteria was first demonstrated with the use of $^{36}$Cl-labeled perchlorate \cite{31}. Hackenthal \cite{32}
concluded that the chlorate was the product of the perchlorate reduction by cell-free extracts obtained from nitrate-adapted cells of *Bacillus cererus*. They also found that chlorate could be reduced by the same cell-free preparation and that it competitively inhibited perchlorate reduction. The first proposed perchlorate reduction pathway was $\text{ClO}_4^- \rightarrow \text{ClO}_3^- \rightarrow \text{ClO}_2^- \rightarrow \text{Cl}_2\text{O}^- \rightarrow \text{Cl}^- [31, 32]$. The reduction of perchlorate or chlorate to chloride by bacteria, but not this degradation pathway, was subsequently confirmed by other researchers [3, 9, 10, 14, 33].

Research on the perchlorate reduction pathway did not make further progress until a new enzyme, chlorite dismutase, was purified from the PRB strain GR-1 and found to produce oxygen from chlorite [3]. Rikken *et al.* [3] proposed a three-step mechanism of perchlorate reduction in which chlorate, chlorite, and dissolved oxygen were sequentially produced. This pathway, which is now widely accepted for bacterial respiration using perchlorate and chlorate, is: $\text{ClO}_4^- \rightarrow \text{ClO}_3^- \rightarrow \text{ClO}_2^- \rightarrow \text{O}_2 + \text{Cl}^-$. Chlorite dismutase has been isolated from two other bacteria, *Ideonella dechloratans* and CKB. Stenklo *et al.* [34] purified chlorite dismutase from *I. dechloratans* that had catalytic properties that were similar, but not identical, to those found for the chlorite dismutase enzyme obtained from GR-1. Stenklo *et al.* [34] determined the 22-residue N-terminal amino acid sequence for this enzyme and found no homologue in the protein sequence of the purified enzyme from *I. dechloratans*. Coats *et al.* [9] also purified a chlorite dismutase from the strain CKB, which is similar to that purified from the strain GR-1. The finding by Coats *et al.* [9] that all thirteen CRB isolates obtained in their laboratory could disproportionate chlorite into chloride and oxygen makes it likely that chlorite dismutase is the central enzyme for the dissimilatory reduction of perchlorate to chloride in PRB.

It is still not clear if only a single enzyme is used by PRB for chlorate and perchlorate reduction, or if there are separate enzymes used for perchlorate and chlorate reduction. Research by Kengen *et al.* [6] suggested only one enzyme is needed for chlorate and perchlorate respiration. They found that a single enzyme could catalyze both chlorate- and perchlorate-reduction, and that this oxygen-sensitive enzyme was located in the periplasm. However, the
maximum reaction rates (measured with methyl viologen) for perchlorate (3.8 U/mg) were actually less than those with either nitrate (6.2 U/mg) or chlorate (11.3 U/mg) [6]. Because it appears likely that separate enzymes are used for nitrate and perchlorate (as explained above), it may also be that there are separate chlorate and perchlorate enzymes. Additional evidence for separate enzymes is provided indirectly by the fact that not all CRB are capable of respiration with perchlorate, although this question will require further research to resolve.

1.2.3 ETC in PRB

Although little has been done about ETC in PRB, much is known about the enzymatic oxidase system and nitrate reductase system, which is instrumental to understand the perchlorate reduction ETC. The ETCs to oxygen in bacteria are branched, the branch point being at the quinone or cytochrome [35]. Many bacteria can alter their electron transport chains depending upon growth conditions (Fig. 1.1). Under aerobic conditions there are often two or three branches leading to different terminal oxidases. The ability to synthesize branched electron transport pathways to oxygen confers flexibility on the bacteria, because the branches may differ not only in the energy that can be generated (because they may differ in the number of coupling sites), but their terminal oxidases may also differ with respect to affinities for oxygen [35]. For example, in *Escherichia coli* cytochrome *o* has a low affinity for oxygen, whereas cytochrome *d* has a higher affinity for oxygen (Fig. 1.2). Switching to an oxidase with a higher affinity for oxygen allows the cells to continue to respire even when oxygen tensions fall to very low values. It has also been suggested that under microaerophilic conditions the use of an oxidase with a high affinity for oxygen would remove traces of oxygen that might damage oxygen-sensitive enzymes that are made under microaerophilic or anaerobic growth conditions [36]. This assumption leads to the belief that perchlorate-reducing bacteria could have branched terminal oxidase systems with enzymes having different affinities for oxygen.
The adaptability of the bacteria, with respect to their electron transport chains, can also be seen with many bacteria that can respire either aerobically or anaerobically. Under anaerobic conditions these bacteria do not make the oxidase complexes but instead synthesize reductases (Fig. 1.3). Electrons travel from quinones to reductases. For example, during anaerobic growth, *E. coli* synthesizes fumarate reductase, nitrate reductase, and tri-methylamine-N-oxide (TMAO) reductase. The different reductases enable the bacteria to utilize alternative electron acceptors under anaerobic conditions.

Little has been done to study branches in the ETCs of PRB. Oxygen is a preferential electron acceptor to perchlorate and high oxygen concentrations inhibit perchlorate reduction [6, 37]. Kengen *et al.* [6] found that perchlorate reductase activity was lost following exposure to oxygen. However, oxygen is evolved during chlorite disproportionation, and therefore perchlorate reduction can obviously occur in the presence of low concentrations of dissolved oxygen. It is unclear whether organisms utilize oxygen at different levels by a same ETC or a branched ETC, and where the branch point would be if a branched ETC was used. It is also not known what level of oxygen will destroy the perchlorate reductase activity and how long whole cells exposed to oxygen can maintain the capacity for perchlorate reduction.

### 1.3 Organization of this Dissertation

This dissertation is organized as separate and complete chapters and presented in a manuscript style based on the work performed for my Ph.D. The literature review section in Chapter 1 is part of a manuscript that has been published in *Environmental Engineering Science*, entitled “Microbial Degradation of Perchlorate: Principles and Applications” by J. Xu, Y. Song, B. Min, L. Steinberg and B. Logan. The part included in chapter 1 was written by me and edited by Logan.
Chapter 2 is a manuscript that has been published in *Water Research*, entitled “Effect of O₂ Exposure on Perchlorate Reduction by *Dechlorosoma* sp. KJ” by Y. Song and B. Logan. One of the goals for my Ph.D. research was to explore oxygen inhibition of perchlorate reduction. Although during perchlorate reduction molecular oxygen is produced by a chlorite dismutase, perchlorate reduction is inhibited by oxygen even for perchlorate-degradation-capable bacteria under fully aerobic conditions. I approached this study from an engineering viewpoint after I had worked on a pilot-scale perchlorate bioreactor in Redlands, California for three weeks. During that time, the influent pump of the reactor was accidentally shut down due to a power surge. The perchlorate-reducing culture completely lost the capability to reduce perchlorate after being exposed to oxygen for two days. Thereafter, I designed the experiments in conjunction with Logan to explore the inhibitory effect of oxygen on perchlorate reduction by determining the recovery time of perchlorate degradation following different time periods of oxygen exposure. I performed all experiments, analyzed the data, and wrote the paper that was then edited by Logan.

One other concept that had been explored with respect to perchlorate-reducing pathways was the idea of using specific inhibitors to block specific respiratory enzymes in order to identify elements of the electron transport chain involved in the reduction of molecular oxygen and perchlorate. During my early Ph.D. studies, it was not known whether the PRB used aerobic respiratory enzymes, whether some of the denitrifying enzymes were necessary, or whether new respiratory enzymes were used for electron transport. I first used cyanide to explore if cytochrome oxidases were involved in the electron transport chain of perchlorate reduction since cyanide is known to inhibit heme-based cytochrome oxidases. One potential problem in using cyanide to inhibit cytochrome oxidase was that chlorite dismutase (a non-respiratory enzyme) is inhibited by cyanide [4]. I designed and performed the experiments for the manuscript given in Chapter 3, “Cyanide Inhibition of Enzyme Activities Involved in Perchlorate Reduction”. I wrote the manuscript and Logan edited it.
It was my hypothesis that PRB contain a complete high-oxygen aerobic respiratory chain including at least cytochrome c oxidase [9], but that a low-oxygen enzyme is involved in oxygen and perchlorate reduction. This hypothesis was based on my work with strain KJ and my observations in preliminary experiments regarding the physiological roles of oxygen. All PRB are facultative anaerobes (except for HAP-1, which is microaerobic.) [9, 24]. Oxygen never accumulates to detectable level during perchlorate reduction indicating that it is rapidly scavenged. At the same time, high oxygen concentrations completely inhibit perchlorate reduction. Thus, I believe two membrane-bound oxidases are produced by the bacteria. This work is described in Chapter 4, “Different Electron Transport Chains to Molecular O$_2$ in Perchlorate-Respiring Bacteria”, which has not yet been submitted for publication. I planned and executed all experiments. I wrote the manuscript with suggestions and editing by Logan.
1.4 References


Figure 1.1 General electron transport pathways for aerobic respiration found in bacteria [35]. A dehydrogenase complex removes electrons from an electron donor and transfers these to a quinone. The electrons are transferred to an oxidase complex via a branched pathway. Depending upon the bacterium, the pathway may branch at the quinone or at the cytochrome. Many bacteria have cytochrome complex $bc_1$, cytochrome $c$, and cytochrome $aa_3$ in one of the branches, and in this way resemble mitochondria. Other bacteria do not have a cytochrome $bc_1$ complex, and may or may not have cytochrome $aa_3$. 
Figure 1.2 Aerobic respiratory chain in *E. coli* [35]. The chain branches at the level of ubiquinone (UQ) to two alternate quinol oxidases, cytochrome *bo* and *bd*. 
Figure 1.3 General anaerobic electron transport pathways found in bacteria [35]. Under anaerobic conditions the electrons are transferred to reductase complexes, which are synthesized anaerobically. Y represents either an inorganic electron acceptor other than oxygen, *e.g.*, nitrate, or an organic electron acceptor, *e.g.*, fumarate. More than one reductase can simultaneously exist in a bacterium.
2. EFFECT OF O$_2$ EXPOSURE ON PERCHLORATE REDUCTION BY

*Dechlorosoma* sp. KJ

**Abstract**

Anaerobic bioreactors have been developed to remove perchlorate from water, but backwashing and operational interruptions can expose biofilms to oxygen. While it is well known that oxygen is a preferential electron acceptor to perchlorate for perchlorate-respiring bacteria, little is known about the effect of oxygen exposure or redox potentials on perchlorate reduction. Four different dissolved oxygen scavengers were tested for their ability to quickly restore anaerobic conditions and allow perchlorate reduction by a facultative, perchlorate respiring bacterium *Dechlorosoma* sp. KJ. Of the four different oxygen scavengers tested (Oxyrase™, L-Cysteine, Na$_2$S and FeS), only Oxyrase™ was able to rapidly (<30 minutes) scavenge dissolved oxygen and allow cell growth. There was no cell growth after addition of Na$_2$S and FeS, and L-Cysteine produced a long lag in cell growth. To investigate the effect of dissolved oxygen on perchlorate reduction, anaerobically grown cultures *Dechlorosoma* sp. KJ, were exposed to dissolved oxygen for various periods ranging from 1 to 32 hours. Perchlorate reduction and redox potential were then measured for cells returned to an anaerobic environment containing an oxygen scavenger. It was determined that cells exposed to dissolved oxygen for more than 12 hours were incapable of reducing perchlorate. Cells exposed to dissolved oxygen for less than 12 hours quickly reduced the redox potential to negative value (-337 mV, -333 mV, -315 mV and –127 mV) and were able to reduce perchlorate or chlorite. Our results suggest that aeration during backwashing of biofilm reactors, or exposure of perchlorate-degrading cell suspensions to dissolve oxygen for less than 12 hours, will not be detrimental to the ability of perchlorate-degrading bacteria to use perchlorate as an electron acceptor.

*Keywords:* perchlorate reduction; bioremediation; oxygen scavengers; redox potential.
2.1 Introduction

Perchlorate has been found in ground waters in the United States, at typical concentrations of 50 to 200 µg/L, primarily as a result of its production and use in solid rocket propellant [1]. The U.S. EPA has identified perchlorate users and manufacturers in 44 states, and perchlorate releases in at least 20 states [2]. Perchlorate contamination poses a significant health threat to humans because even at low concentrations perchlorate is known to affect the function of the thyroid gland by inhibiting the uptake of iodide and affecting hormone production [2-4]. Perchlorate releases are estimated to have affected the drinking water of 15 million people that reside mostly in the states of California, Nevada, Utah, and Arizona. The U.S. EPA proposed a draft reference dose of 0.03 µg per kg of body weight per day, which could lead to a drinking water standard of 1 µg/L to protect human health [2]. Based on this information, the California Department of Health Service in California decreased the action level of perchlorate in drinking water in California from 18 µg/L to the detection limit of ion chromatograph, 4 µg/L [5].

Recent studies have demonstrated the ubiquity and diversity of perchlorate-respiring bacteria (PRB) that can couple growth to the reduction of perchlorate under anaerobic conditions [6-13]. Analysis of the 16S rRNA sequences of these strains has shown that all were members of the Proteobacteria and that a majority of these PRB were located in the β-subclass [14]. Perchlorate is degraded via a three-step process of: ClO$^{4−}$ → ClO$^{3−}$ → ClO$^{2−}$ → O$_2$ + Cl$^{−}$, and the molecular oxygen evolved is immediately used for biomass generation [13]. Although a single enzyme (perchlorate reductase) has been found that can catalyze both chlorate- and perchlorate-reduction [15], not all chlorate-respiring bacterial are capable of respiration with perchlorate [7]. Thus, it may be that another enzyme is involved in perchlorate reduction in a single organism or that different forms of the enzyme are inactive with some bacteria. The last step of chlorite disproportionation does not yield any energy for the cell and is catalyzed by an enzyme (chlorite dismutase) only recently isolated [16, 17].
Bioreactors have been developed to remove perchlorate from drinking water sources and wastewaters [18-20]. PRB can be exposed to dissolved oxygen in different ways in these reactors, but the effects of oxygen exposure on perchlorate reduction are not well understood. It is well known that oxygen is a preferential electron acceptor to perchlorate by PRB, and that high dissolved oxygen concentrations are known to completely inhibit perchlorate reduction [15, 21]. However, while oxygen is released during perchlorate degradation it is rapidly used by PRB and does not accumulate to high concentrations in solution during perchlorate degradation. Operation of fixed bed reactors can result in instances where PRB are exposed to high concentrations of dissolved oxygen [22]. For example, periodic backwashing of fixed bed systems must be performed to limit the growth of PRB bacteria and prevent clogging. Air sparging enhances the efficiency of backwashing, but exposure of PRB to oxygen for long periods could inhibit perchlorate reduction when the system is returned to service. Alternatively, temporary reactor shut down for servicing could also expose the biofilm to high concentrations of dissolved oxygen.

The effects on perchlorate reduction of long-term exposure of PRB to high concentrations of dissolved oxygen has only been indirectly studied. Attaway et al. [21] found that addition of air to the microbial community during perchlorate reduction immediately terminated the process and aeration for 12 hours permanently destroyed the ability of the culture to reduce perchlorate. However, the applicability of this finding to other PRB is not known as most PRB are facultative anaerobes, but the PRB used in their study (Wolinella succinogenes HAP-1) [8] is only aero-tolerant and not facultative. The effect of dissolved oxygen on two key enzymes involved in perchlorate reduction is known, but cannot explain Attaway et al’s findings. Kengen et al. [15] reported that the half time of inactivity of perchlorate reductase following exposure to oxygen was about 2-3 days, not hours, as found for the PRB HAP1. Chlorite dismutase activity is unaffected by dissolved oxygen [13].

In order to better understand the effect of dissolved oxygen on perchlorate reduction, we examined the effect on perchlorate reduction of exposure of perchlorate-degrading cultures of the
PRB *Dechlorosoma* sp. KJ to dissolved oxygen for periods ranging from 1 to 32 hours. While the redox potentials needed for denitrifying and methanogenic cultures are well known, they have not been previously reported for PRB. We therefore tested four different reducing agents for their ability to scavenge oxygen and provide a low redox potential for cell growth. Using one of these oxygen scavengers, we then monitored the redox potential and perchlorate reduction of anaerobic cell suspensions exposed to oxygen for various time periods that were returned to anaerobic conditions.

### 2.2 Material and methods

#### 2.2.1 Bacterium and media

*Dechlorosoma* sp. KJ, originally isolated from a perchlorate-degrading packed bed bioreactor [7], was used in all experiments unless stated otherwise. Media were prepared using ultrapure water (Milli-Q system; Millipore Corp., New Bedford, Mass.) and research-grade chemicals in the amounts (per liter) indicated below. The basal medium contained: 1.55 g K$_2$HPO$_4$$\cdot$3H$_2$O, 0.85 g NaH$_2$PO$_4$$\cdot$H$_2$O, 0.5 g NH$_4$H$_2$PO$_4$, 50 mg MgSO$_4$$\cdot$7H$_2$O, 3 mg EDTA, 2 mg ZnSO$_4$$\cdot$7H$_2$O, 1 mg CaCl$_2$$\cdot$2H$_2$O, 4 mg FeSO$_4$$\cdot$7H$_2$O, 0.4 mg Na$_2$MoO$_4$$\cdot$2H$_2$O, 0.2 mg CuSO$_4$$\cdot$5H$_2$O, 0.4 mg CoCl$_2$$\cdot$6H$_2$O, 1 mg MnCl$_2$$\cdot$4H$_2$O, 0.1 mg NiCl$_2$$\cdot$6H$_2$O, 0.15 mg Na$_2$SeO$_3$, and 0.6 mg H$_3$BO$_3$ [7, 13]. Unless stated otherwise, acetate (C$_2$H$_3$O$_2^-$, 2 g/L) and perchlorate (ClO$_4^-$, 0.5 g/L) salts were added as the electron donor and acceptor, respectively. The pH of the media was adjusted to 7.0 using NaOH. Anaerobic medium was sterilized by autoclaving and degassed in an anaerobic glove box (Coy Scientific Products, Grass Lake, Mich.).
2.2.2 Preparation of cell suspensions

Anaerobically grown cell suspensions of strain KJ were used in all oxygen scavengers and oxygen exposure experiments unless stated otherwise. Cells were harvested during late-log-phase growth (optical density at 600 nm (OD\textsubscript{600}) of 0.3 to 0.4), washed once at 5,000 ×g for 10 min, and resuspended in medium to the same OD\textsubscript{600} in the glove box.

2.2.3 Oxygen scavengers

Four different reducing agents were tested for their ability to scavenge dissolved oxygen from the media while allowing bacterial growth: Oxyrase\textsuperscript{TM}, L-Cysteine•HCl [23], Na\textsubscript{2}S [24] and FeS [24]. Chemicals were added from sterile anoxic aqueous stock solutions to the medium at concentrations typically used or recommended for these compounds: Oxyrase\textsuperscript{TM}, 0.02 ml/ml; L-Cysteine•HCl, 0.5 mg/ml; Na\textsubscript{2}S, 20 mg/L; and FeS, 0.07 ml-precipitant/ml (added in excess).

2.2.4 Dissolved oxygen probe measurements of oxygen removal rates

Oxygen removal rates of four different oxygen scavengers were examined in abiotic growth medium by using an oxygen probe (YSI Model 5331, YSI Incorporated, Yellow Springs, Ohio) and monitor (YSI Model 5300). The probe was calibrated with an air-saturated water at 25°C before each experiment. Each oxygen scavenger was added at the final concentration as above to a 20-ml chamber containing 3 ml of air-saturated abiotic growth medium, and constantly stirred. A redox potential indicator, resazurin (E\textsubscript{o}° = -51 mV [pH 7.00]) [25], was added at a final concentration of 0.5 mg/L. Oxygen removal rates were calculated from the slope of the dissolved oxygen concentration change over time.
2.2.5 Effect of different oxygen scavengers on cell growth

Each oxygen scavenger was added at the final concentration as above to 130 ml serum bottles prepared under anaerobic conditions containing 95 ml of medium and 5 ml of cell suspension (1.67x10⁹ cells/ml). The serum bottles were fitted with butyl rubber stoppers and aluminum crimp seals and incubated at 25°C. The effect of the oxygen scavengers was monitored on cell growth and perchlorate reduction. Resazurin was added at a final concentration of 0.5 mg/L.

2.2.6 O₂ exposure procedures

Cell suspension (75 ml) was transferred to 2000-ml flask prepared under anaerobic conditions containing 1425 ml of medium. After incubation 20 hours at 25°C in the anaerobic glove box, the cell suspension (150 ml) was split and placed into ten separate flasks (200 ml each). One flask served as an anaerobic control (ANC) and was kept in the anaerobic glove box for the duration of the experiment. Eight flasks were taken out of the glove box, aerated for a different period of time (1, 2, 4, 8, 12, 22, 28 and 32 hours) and then returned back to the glove box. Oxyrase™ (0.02 ml/ml) was added to each flask when it was returned to the glove box in order to scavenge any remaining dissolved oxygen. One flask, (aerobic control, AC) was aerated continuously for the duration of the experiment. The effect of aeration was monitored on cell growth and perchlorate reduction. Following the return of samples to the glove box redox potentials (E₀, potential relative to the normal hydrogen electrode) were measured with two platinum redox electrodes (96-78-BN; ThermoOrion, Beverly, Mass.) filled with 4M KCl saturated with Ag/AgCl.
2.2.7 Analytical techniques

Cell suspensions were monitored by optical density at 600 nm (OD<sub>600</sub>). Concentrations of perchlorate, acetate, and chloride anions were determined with an ion chromatograph (DX500; Dionex, Sunnyvale, Calif.) equipped with an AS11 column and guard column, a self-regenerating suppressor, and an autosampler. Perchlorate ion was measured using 100 mM NaOH eluent. For acetate and chloride measurements, a 10 mM NaOH eluent was used. The minimum detection limit for perchlorate as 4 µg/L. Dissolved oxygen concentrations were measured using a YSI Model 5331 Standard Oxygen probe with a YSI Model 5300 biological oxygen monitor (YSI Incorporated, Yellow Springs, Ohio).

2.3 Results

2.3.1 Oxygen scavengers

All four oxygen scavengers tested removed over 50% of the dissolved oxygen in 30 minutes (Fig. 2.1). FeS removed dissolved oxygen the most rapidly, while Oxyrase<sup>TM</sup> reduced the dissolved oxygen the most slowly. In tests with bacteria, however, only Oxyrase<sup>TM</sup> did not adversely affect cell growth on perchlorate. The rate of perchlorate reduction and cell growth was more rapid with Oxyrase<sup>TM</sup> than with FeS, Na<sub>2</sub>S or L-Cysteine. The growth yield using Oxyrase<sup>TM</sup> was larger than the control (Fig. 2.2), suggesting that some of the proprietary ingredients in Oxyrase<sup>TM</sup> were used as a substrate for cell growth. Cells grown with L-Cysteine exhibited a long lag phase (25 hours) prior to cell growth. Although FeS and Na<sub>2</sub>S removed the dissolved oxygen in the abiotic medium more rapidly than the other two scavengers (Fig. 2.1), there was no appreciable cell growth or perchlorate reduction in the presence of these two chemicals for sixty hours (Fig. 2.2). Cells grown with FeS and Na<sub>2</sub>S eventually (>60 h) removed all perchlorate in
2.3.2 Redox potential

The redox potential in abiotic tests (Fig. 2.1) was below −110 mV for all oxygen scavengers except Oxyrase™ as indicated by the color change of resazurin from pink to clear. In the absence of bacteria, Oxyrase™ reduced the redox potential to only 277 mV. In tests where bacteria respired perchlorate (Oxyrase™, L-Cysteine, and the control; Fig. 2.2), the redox potential remained above −110 mV as the color of the tubes containing resazurin remained pink. A positive redox potential was confirmed by the presence of ca. 1.6 mg/L of dissolved oxygen in all actively growing samples. Changes in redox potential during cell growth on perchlorate were further examined in tests described below.

2.3.3 Effect of oxygen exposure on redox potential change

Anaerobically growing cells exposed to oxygen were capable of rapidly reducing the redox potential to a low level (≤127 mV) after being exposed to oxygen for 8 hours (T8, Figure 2.3). The redox potential of this suspension exposed to dissolved oxygen for 8 hours was carefully monitored following exposure to aerobic conditions. There was a rapid drop in redox potential observed just prior to rapid perchlorate reduction, followed by a gradual increase in redox potential due to oxygen evolution after the culture began rapidly reducing perchlorate (Fig. 2.3).

The rapid drop in redox potential was always observed in other experiments when cells were returned to anaerobic conditions following exposure to dissolved oxygen for up to 12 hours (Fig. 2.4). During aeration, dissolved oxygen concentrations were about 6-7 mg/L. The final
dissolved oxygen concentration of most samples (except the aeration control, AC) containing Oxyrase™ was below 0.03 mg/L. The initial redox potential ($E_0$) of the cell suspension growing anaerobically in degassed medium in the glove box was –327 mV. This redox potential increased to 420 mV when samples were removed from the glove box and aerated (Fig. 2.4). Cells returned to the glove box after exposure to dissolved oxygen for less than 12 hours were able to rapidly decrease redox potential after being returned to the glove box. For example, when cells were exposed to dissolved oxygen for one hour (T1) and then were returned to the anaerobic glove box and amended with Oxyrase™, the redox potential rapidly (<30 min) decreased to -337 mV. Similarly, other samples returned to the glove box within 12 hours (T2, T4 and T8), exhibited a rapid decrease in redox potential as well. The most negative redox values achieved by these samples seemed to decrease (-333 mV, -315 mV and –127 mV for T2, T4 and T8 respectively) with the extent of exposure to dissolved oxygen (Fig. 2.4). For the sample exposed to oxygen for 12 hours (T12), a negative redox potential was never reached, and after 60 minutes the redox potential began to increase. Samples exposed to oxygen for periods longer than 12 hours (T22, T28 and T32), had redox potentials (281 mV, 295 mV and 282 mV; Fig. 2.4) similar to that produced solely by the Oxyrase™ (277 mV).

2.3.4 Effect of oxygen exposure on perchlorate reduction

Growing cells exposed to dissolved oxygen for up to 8 hours retained the ability to rapidly reduce perchlorate (Fig. 2.5). This exposure time of 8 hours was shown above to be critical for cells to resume a negative redox potential (T8, Figure 2.4). The rate at which perchlorate could be degraded was inversely proportional to time of exposure to dissolved oxygen up to 12 hours. Aeration for 12 or more hours destroyed the ability of the culture to completely reduce perchlorate even though a low redox potential was rapidly restored by the addition of Oxyrase™ (Fig. 2.4). With aeration for more than 22 hours, cultures completely lost the capability
to reduce perchlorate for at least 10 days (data not shown). Washed cells of the culture exposed to oxygen for 12 hours or more eventually grew using perchlorate, but only after 3-7 days of incubation. In general, the incubation time needed to remove perchlorate increased with the time that the sample had been aerated.

2.4 Discussion

Oxygen scavengers such as L-Cysteine are routinely used to maintain strict anaerobic conditions for methanogenic cultures under laboratory conditions, but they have not been previously tested or reported for use in maintaining anaerobic conditions for perchlorate reduction. Of the four oxygen scavengers tested, Oxyrase™ appeared to be the most useful oxygen scavenger for growing PRB. L-Cysteine produced long lags in growth, and PRB did not grow in cultures containing FeS and Na$_2$S.

Exposure of cultures growing anaerobically, and using perchlorate, to dissolved oxygen (DO) for 12 hours or more destroyed the capability of these bacteria to reduce perchlorate even when they were returned to an anaerobic environment and the remaining DO was scavenged using Oxyrase™ (Fig. 2.5). This exposure time is consistent with that found by others. Attaway et al. [21] found that exposure of perchlorate respiring culture containing primarily the aero-tolerant bacterium W. succinogenes HAP1 [8] to DO for 12 hours terminated the ability of the cell to reduce perchlorate. However, the short time needed to inhibit perchlorate reduction is inconsistent with an explanation that oxygen affected key enzymes known to use for perchlorate reduction. Perchlorate reductase activity is oxygen sensitive, but it has a half time of inactivation of 2 to 3 days [15], which is much larger than the 12 hours observed here. Chlorite dismutase activity is not affected by dissolved oxygen [9, 16, 17], consistent with other tests in our laboratory showing that cells retained chlorite dismutase activity when cells were exposed to dissolved oxygen for 12 hours (data not shown). Thus, the lack of perchlorate reduction by cells
exposed to high dissolved oxygen concentrations for 12 or more hours does not appear to be due to the effect of oxygen on either perchlorate reductase or chlorite dismutase.

Cells were only capable of resuming perchlorate reduction after exposure to dissolved oxygen when they were able to rapidly reduce the redox potential to negative values. Thus, there must be a link between the inability of cells to reduce perchlorate and the ability to create this negative redox potential. When anaerobically growing cells were exposed to dissolved oxygen, perchlorate reduction resumed only when cells reduced the redox potential to less than \(-127\) mV in the presence of Oxyrase™ (Fig. 2.4). This large initial decrease in redox potential for cells able to degrade perchlorate can not be attributed solely to Oxyrase™, as this chemical reduced the redox potential to only 277 mV in an abiotic medium. The ability to produce a redox potential of less than \(-127\) mV must therefore due to microbial activity.

The lack of an effect of dissolved oxygen on the two enzymes known to be involved with perchlorate reduction (perchlorate reductase and chlorite dismutase), suggest that there must be at least one oxygen-sensitive enzyme involved in perchlorate respiration reduction. The activity of this enzyme would function to sharply reduce the redox potential of the medium when cells were first returned to anaerobic conditions. A logical candidate for this unknown oxygen-sensitive enzyme would be a high-affinity terminal oxidase [26, 27]. Other bacteria, such as \textit{E. coli}, have branched electron transport pathways that lead to different terminal oxidases. In \textit{E. coli}, cytochrome \textit{bd} complex has a high affinity for oxygen and is the dominant oxidase under low oxygen tension [28, 29], while the cytochrome \textit{bo} complex has a low affinity for oxygen and is predominant under high tension. Thus, it is possible that there are two different terminal oxidases expressed by PRB under different oxygen levels, and that perchlorate reduction is coupled with the activity of a low oxygen tension cytochrome. The ability of cells to reduce the redox potential to negative values demonstrated the presence and activity of a low oxygen tension enzyme.
When perchlorate reduction was lost (between 8 and 12 hours), this high-affinity terminal oxidase was either blocked or became damaged by exposure to high oxygen concentrations (Fig. 2.4).

These results suggest that the ability of bacteria to reduce perchlorate, which produces dissolved oxygen from chlorite, can occur as long as low oxygen tensions are maintained. However, exposure of cells to high DO concentrations for 12 or more hours will inhibit perchlorate reduction likely due to the loss of some key respiratory enzyme that has not yet been isolated from PRB. The identification and isolation of this low-oxygen tension respiratory enzyme are interesting areas for future research, and may lead to new clues for understanding the ability of bacteria to respire using perchlorate.

2.5 Conclusions

The following conclusions can be made regarding the effect of oxygen exposure on perchlorate reduction by Dechlorosoma sp. KJ:

a. Dissolved oxygen can be reduced to low levels using Oxyrase™, but other oxygen scavengers tested were not suitable for use with perchlorate respiring bacteria. Even with Oxyrase™, low levels of dissolved oxygen persisted (~1.6 mg/L) in abiotic cultures.

b. At the onset of perchlorate reduction by Dechlorosoma sp. KJ, the redox potential was initially reduced to a negative value (-127 mV to –333 mV), but it increased as DO was evolved during perchlorate reduction.

c. PRB growing anaerobically that were exposed to high DO levels retained the ability to rapidly reduce perchlorate for up to 8 hours, but could not reduce perchlorate after 12 hours.
2.6 Acknowledgement

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2.7 References


http://www.dhs.ca.gov/ps/ddwem/chemicals/perchl/actionlevel.htm


Figure 2.1 Oxygen removal of different oxygen scavengers in abiotic growth media.
Figure 2.2 Effect of different oxygen scavengers on *Dechlorosoma* sp. KJ growth with acetate as the electron donor and perchlorate as the sole electron acceptor. (A) Effect on perchlorate reduction. \( C/C_0 \): Concentration of perchlorate left in the solution / initial perchlorate concentration. (B) Effect on cell growth by optical density at 600 nm. (Note that absorbance data could not be obtained in the presence of FeS due to the formation of a black precipitate). The experiment with L-Cysteine was performed separately from the one with the other three
chemicals. The results of perchlorate reduction and cell growth with L-Cysteine were adjusted to compare with those of other oxygen scavengers by zero the length of lag-phase growth by 15 hours.
Figure 2.3 Perchlorate reduction and redox potential change of culture with 8 hours (T8) of oxygen exposure. $E_0$: Redox potential readings relative to the normal hydrogen electrode.
Figure 2.4 Redox potential change during perchlorate reduction of culture with different time period of oxygen exposure. $E_0$: Redox potential readings relative to the normal hydrogen electrode. ANC: anaerobic control without oxygen exposure; T1: anaerobic culture with 1 hour of aeration; T32: anaerobic culture with 32 hours of aeration; AC: aerobic control with anaerobic overnight incubation and aeration afterwards.
Figure 2.5 Perchlorate reduction of culture with different time period of oxygen exposure (Same legend as Figure 2.4). The experiment for perchlorate reduction was performed separately from the one for redox potential change with different time periods of oxygen exposure.
3. CYANIDE INHIBITION OF ENZYME ACTIVITIES INVOLVED IN PERCHLORATE REDUCTION

Abstract

Although microbes can respire using perchlorate, little is known about how these bacteria regulate enzymes used for aerobic and perchlorate respiration. It has previously been shown that the activity of chlorite dismutase can be inhibited using high concentration of cyanide (520 mg/L). It was assumed that this inhibition would result in cyanide inhibition of perchlorate reduction. However, cyanide may indirectly affect perchlorate reduction through its effect on other enzymes. The effect of a low concentration of cyanide on dissimilative perchlorate and chlorate reduction, and aerobic respiration, was studied using pure cultures of *Dechlorosoma* sp. KJ (ATCC strain BAA-592). Cyanide (1 mg/L) inhibited cell growth on perchlorate, chlorate and molecular oxygen. Cyanide did not inhibit chlorite dismutation by the cell suspension. When oxygen accumulation was prevented by adding an oxygen scavenger (Oxyrase™ or L-cysteine), however, cells completely reduced perchlorate in the presence of cyanide. It was concluded that the inhibition of dissimilative perchlorate reduction by cyanide at this concentration was a consequence of oxygen accumulation, not inhibition of the enzymes used for perchlorate reduction. This finding on the effect of cyanide on respiratory enzymes provides a new method to control and study respiratory enzymes used for perchlorate reduction. By using a low concentration of cyanide (1 mg/L) it is possible to inhibit aerobic, but not anaerobic cell respiration. The finding that inhibition of oxygen utilization stops perchlorate reduction reinforces the critical role that oxygen plays in regulating perchlorate reduction by perchlorate-respiring bacteria such as strain KJ.
3.1 Introduction

Perchlorate has been detected in impacted ground waters generally at levels of 50-200 µg/L primarily at sites near its production and use as a solid propellant for rockets, missiles, and fireworks [1]. Due to health concerns related to its effect on hormone production by the thyroid gland, the U.S. EPA proposed a draft reference dose of 0.03 µg per kg of body weight per day, which could produce a drinking water equivalent level of 1 µg/L [2]. The National Academy of Science is currently reviewing the risk assessment study on the reference dose study.

Rikken et al. first demonstrated that perchlorate reduction required an enzyme, chlorite dismutase, that produced oxygen from chlorite [3, 4]. They proposed a three-step mechanism of perchlorate reduction in which chlorate, chlorite, and dissolved oxygen were sequentially produced. This pathway, which is now widely accepted for bacterial respiration using perchlorate and chlorate, is: ClO$_4^-$ → ClO$_3^-$ → ClO$_2^-$ → O$_2$ + Cl$^-$ [4-8]. Although oxygen is evolved during chlorite disproportionation, high concentrations of oxygen are known to inhibit perchlorate reduction [9]. Therefore, perchlorate-respiring bacteria must maintain an aerobic respiration pathway during perchlorate reduction in order to maintain a low dissolved oxygen level [10].

It is important to understand how dissolved oxygen affects perchlorate degradation. Perchlorate reductase activity has a half time of inactivation by oxygen of 2 to 3 days [9], but high concentrations of oxygen quickly halt perchlorate respiration [11]. Cyanide, a potent inhibitor of heme-protein-catalyzed reactions, has been used to study aerobic respiratory chains in order to determine when heme-based enzymes are expressed. While cyanide is known to inhibit cytochrome oxidase, for example, it has also been shown to inhibit chlorite dismutase [4]. The activity of chlorite dismutase was lost completely and immediately by the addition of 520 mg/L cyanide [4]. The inhibition of cytochromes used for removal of dissolved oxygen (DO) means that DO will accumulate during perchlorate reduction. Thus, it is possible that perchlorate reduction is affected through inhibition of cytochromes, and through inhibition of chlorite
dismutase which could allow dissolved oxygen or chlorite to accumulate in solution. Given the importance of the role of DO on perchlorate inhibition, additional information was needed on the effect of cyanide on these enzymes needed for aerobic and anaerobic respiration.

In this paper I examined the effect of cyanide on bacterial growth by cells using perchlorate, chlorate, or molecular oxygen as a terminal electron acceptor, and the effect of cyanide on chlorite dismutase activity. It is shown that by using a low concentration of cyanide it is possible to inhibit aerobic respiration, but not perchlorate and chlorate reduction. However, an oxygen scavenger must be added in order to prevent oxygen accumulation from inhibiting perchlorate reduction.

3.2 Material and methods

3.2.1 Bacterium and media

*Dechlorosoma* sp. KJ (ATCC BAA-592), originally isolated from a perchlorate-degrading packed bed bioreactor [12], was used in all experiments. The basal medium was prepared as described by Song and Logan [11] using ultrapure water (Milli-Q system; Millipore Corp., New Bedford, Mass.) and research-grade chemicals. Sodium acetate (C$_2$H$_3$O$_2^-$, 2 g/L) was added as the electron donor in all experiments. Sodium perchlorate (ClO$_4^-$, 0.5 g/L) or chlorate (ClO$_3^-$, 0.5 g/L) was added as the terminal electron acceptor in anaerobic experiments. The pH of the medium was adjusted to 7.0 using NaOH. The anaerobic medium was sterilized by autoclaving, and degassed in an anaerobic glove box (Coy Scientific Products, Grass Lake, Mich.). The aerobic medium was prepared without the addition of chlorate or perchlorate, and saturated with dissolved oxygen using air.

Cells were harvested during late-log-phase growth [optical density (OD) at 600 nm of 0.3 to 0.4, 1.74 to 2.32 ± 0.04×10$^9$ cells/ml], washed once by centrifuging at 5,000 xg at 4ºC for 10 min and resuspending in fresh medium to the same OD either in a glove box (anaerobic...
culture) or in a laminar flow hood (Model 36212; Labconco Corporation, Kansas City, MO.) (aerobic culture).

3.2.2 Culture growth in the presence of CN⁻

The concentration of CN⁻ needed to inhibit aerobic cell respiration was determined by growing cells on perchlorate and acetate in the presence of CN⁻. Washed cell suspensions (0.50 ml each, triplicate samples) were transferred in an anaerobic glove box into 23-ml test tubes (final volume 10 ml) containing basal medium, acetate, and perchlorate or chlorate. CN⁻ was added at the final concentration of 1 mg/L except in one experiment where several concentrations were tested (1, 10, 100, 200, 500, and 800 mg/L of CN⁻). Test tubes were fitted with butyl rubber stoppers and aluminum crimp seals and incubated at 25°C. The inhibition of CN⁻ on cell growth was monitored based on cell density (OD₆₀₀), and by loss of the electron acceptor (perchlorate or chlorate) and the electron donor (acetate). Cell dry weights were taken at the beginning and the end of each experiment. The concentrations of chlorite and chloride were monitored during cell growth on chlorate. DO concentrations were measured at the beginning and the end of experiments using an oxygen probe (YSI Model 5331, YSI Incorporated, Yellow Springs, Ohio) and monitor (YSI Model 5300). The probe was calibrated with an air-saturated water at 25°C before each experiment.

In aerobic growth experiments, CN⁻ was added at a final concentration of 1 mg/L (treatment) or 0 mg/L (biotic control). The test tubes were aerated during experiment by shaking tubes on their sides at 150 rpm (Model 3250; Lab-line Instrument Inc., Melrose Park, Ill.). The inhibition of CN⁻ on cell growth was monitored based on cell density (OD₆₀₀) and loss of the electron donor (acetate). DO concentrations were measured at the beginning and the end of each experiment.
3.2.3 Accumulation of DO during anaerobic cell respiration

In separate experiments, DO levels were measured during anaerobic cell respiration of perchlorate to explore CN⁻ inhibition of oxygen utilization. Cell suspensions (5 ml) were inoculated in the 130 ml serum bottles prepared under anaerobic conditions, containing 95 ml of medium with perchlorate and acetate. The serum bottles were fitted with butyl rubber stoppers and aluminum crimp seals and incubated at 25°C. Cyanide was added during the early-log growth phase at a concentration of 1 mg/L. Samples were taken and analyzed for OD\textsubscript{600}, and concentrations of DO, perchlorate, and acetate.

3.2.4 Perchlorate culture growth with CN⁻ and chemical oxygen scavengers

To scavenge oxygen produced during perchlorate degradation, two reducing agents, Oxyrase\textsuperscript{TM} and L-cysteine•HCl [11, 13], were used. The effect of cyanide on the effectiveness of the two chemical oxygen scavengers was also measured. Reducing agents were added from sterile anoxic aqueous stock solutions to the medium at a concentration either used by others (L-cysteine•HCl, 0.5 mg/ml ) [13] or at a concentration recommended by the manufacturer (Oxyrase\textsuperscript{TM}, 0.02 ml/ml). Oxygen removal rates were examined at final CN⁻ concentrations of 0 mg/L (control), 1 mg/L, 5 mg/L, 10 mg/L, 20 mg/L, and 50 mg/L. Each oxygen scavenger was added to a 20-ml chamber containing 3 ml of an air-saturated abiotic growth medium, and constantly stirred. CN⁻ was then added 2.5 minutes after each experiment started. Oxygen removal rates were calculated from the slope of the dissolved oxygen concentration change over time. Free cyanide concentration in medium containing L-cysteine was measured by precipitating cyanide with 10 mg/L AgNO\textsubscript{3} [14].

Oxyrase\textsuperscript{TM} and L-cysteine•HCl were used to remove dissolved oxygen produced during anaerobic cell growth in the presence of CN⁻. Oxyrase\textsuperscript{TM} or L-cysteine•HCl were added at the same concentrations as described above. When L-cysteine was used, cells were pre-acclimated to
this chemical by growth in the presence of this oxygen scavenger. The test tubes lacking CN\(^{-}\) or a reducing agent served as control for cell growth. The test tubes containing one of the reducing agents and no CN\(^{-}\) served as controls on the effect of the reducing agents (Oxyrase\textsuperscript{TM} control: O\(_C\); L-cysteine control: L\(_C\)) on cell growth. The inhibition of CN\(^{-}\) on cell growth in the presence of oxygen scavenger was determined by monitoring cell density (OD\(_{600}\)) and loss of the electron donor (acetate).

3.2.5 Chlorite dismutation in the presence of CN\(^{-}\)

Cyanide inhibition of chlorite dismutase activity was determined by measuring the rate of chlorite disproportion in the presence CN\(^{-}\) using anaerobic culture. Cell suspensions (0.3 ml) were added to a 20-ml YSI DO chamber containing 2.7 ml of phosphate buffer and CN\(^{-}\) (1 mg/L final concentration), and constantly stirred. Chlorite (30 \(\mu\)L; 675 mg/L, final concentration) was added 1.5 minutes after the experiment started. Dissolved oxygen measurements were recorded every 0.5 minute for 7 minutes.

3.2.6 Analytical Techniques

Cell suspensions were routinely monitored by optical density (OD) at 600 nm. Concentrations of perchlorate, chlorate, and acetate were determined with an ion chromatograph (DX500; Dionex, Sunnyvale, Calif.) equipped with an AS11 column and guard column, a self-regenerating suppressor, and an autosampler. Perchlorate ion was measured using a 100 mM NaOH eluent. For chlorate, chlorite, acetate and chloride measurements, a 10 mM NaOH eluent was used. The minimum detection limit for perchlorate was 4 \(\mu\)g/L. Cell dry weight (DW) was determined using membrane filters (25 mm, 0.2-\(\mu\)m pore diameter; Osmonics Corp.,
Minnetomka, Minn.) and a microbalance (±0.1 µg; duplicate samples; Mettler Toledo UMT2, Greifensee, Switzerland).

3.3 Results

3.3.1 CN\textsuperscript{-} inhibition of cell growth on ClO\textsubscript{4}\textsuperscript{-}

Cyanide at concentrations of 1 mg/L or more inhibited growth of *Dechlorosoma* sp. KJ on perchlorate (Fig. 3.1). In the absence of cyanide (positive control), cells grew from an initial optical density of $\text{OD}_{600} = 0.02$ ($1.2 \pm 0.4 \times 10^8$ cells/ml) to a final optical density of $\text{OD}_{600} = 0.37$ ($2.15 \pm 0.04 \times 10^9$ cells/ml). With 1 mg/L cyanide, the cells reached a final optical density of $\text{OD}_{600} = 0.06$ ($3.5 \pm 0.4 \times 10^8$ cells/ml). Perchlorate was completely removed in the control lacking cyanide, while only 2.5% of perchlorate was degraded by cells containing 1 mg/L of cyanide. Less than 1% of perchlorate was degraded at cyanide concentrations of 10 – 200 mg/L, and no perchlorate was removed at a cyanide concentration of 500 mg/L or more. Cell dry weight of the control after incubation for 70 hours (0.222 mg cell/ml culture) was five times the initial cell dry weight (0.041 mg cell/ml culture). Dry weights of samples containing cyanide were the same as that of control at cyanide concentrations of 1 – 100 mg/L. Cell dry weights were lower (0.025 and 0.017 mg cell/ml, respectively) when 500 mg/L or 800 mg/L of cyanide were used.

The final DO concentration of the control was < 0.10 mg/L (initial DO 0.81 mg/L). Dissolved concentrations of all samples containing cyanide, however, were similar to that of the initial sample (0.79 - 0.84 mg/L), suggesting a lack of cell respiration using oxygen in the presence of 1 mg/L or more of cyanide.
3.3.2 Culture growth on ClO$_3^-$ or O$_2$ in the presence of 1 mg/L of CN$^-$

Experiments conducted with cells grown on chlorate or oxygen also demonstrated that 1 mg/L of cyanide inhibited *Dechlorosoma* sp. KJ growth on chlorate or molecular oxygen (Fig. 3.2 A and 3.2 B). The optical density of cells grown on chlorate in the presence of CN$^-$ increased only slightly (from 0.01 to 0.02), while that of control increased to OD$_{600} =$ 0.12 (Fig. 3.2 A). The concentration of aerobically grown cells also increased slightly (from OD$_{600} =$ 0.02 to 0.05), while that of control increased to OD$_{600} =$ 0.37 (Fig. 3.2 B). Chlorate was completely degraded by the control. However, only 3.2 ± 0.3 % (22 ± 2 mg/L) of chlorate was degraded in the culture containing cyanide, while 15 ± 3 mg/L of acetate was consumed and 11± 2 mg/L of chloride was generated. The stoichiometric ratio of chlorate to chloride was 0.85:1, similar to that of the control (0.89:1). No chlorite was detected in any samples. The final DO concentration of the chlorate control was much lower than that of initial sample as observed for perchlorate. The DO level of the sample with cyanide was the same as that of initial sample. For the aerobic culture, all the DO was depleted, while no DO was used by bacteria in the presence of cyanide.

3.3.3 DO accumulation in anaerobic cultures treated with CN$^-$

Dissolved oxygen accumulation during anaerobic growth of cultures treated with cyanide was higher than that of the control lacking cyanide. The DO concentration reached 0.05 mg/L immediately after the addition of 1 mg/L of cyanide during early-log growth phase, resulting in the loss of cell growth. However, the DO concentration of the control lacking cyanide was below the detection limit of the DO probe (0.04 mg O$_2$/L) during the early-log growth phase. It is known that high concentrations of DO inhibit perchlorate respiration, but this demonstrated that very low DO concentration (< 0.04 mg/L) also inhibited cell growth. Thus in the presence of cyanide, oxygen cannot be used by anaerobically grown cells.
3.3.4 Perchlorate culture growth in the presence of CN⁻ and oxygen scavengers

To demonstrate that a lack of cell growth on perchlorate was due to inhibition caused by the accumulation of DO, reducing agents were added to scavenge oxygen. *Dechlorosoma* sp. KJ grew on perchlorate in the presence of a low concentration of cyanide when Oxyrase™ or L-cysteine was added to scavenge oxygen (Fig. 3.3 A and 3.3 B). With Oxyrase™ and 1 mg/L of cyanide, culture growth rate was similar to that of the control. Higher concentrations of cyanide inhibited both aerobic respiration and perchlorate reduction. With Oxyrase™ and 5 mg/L of cyanide, culture growth was much slower than that of the control (Fig. 3.3 A). With cyanide at 10 mg/L or more there was no culture growth on perchlorate even with Oxyrase™ after 80 hours.

Cell yields using Oxyrase™ but no cyanide (O_C) (0.279 mg cell/ml) were larger than the control (0.216 mg cell/ml) (Fig. 3.3 A), suggesting that some of the proprietary ingredients in Oxyrase™ were used as a substrate for cell growth. Although growth was slower in the presence of a chemical oxygen scavenger, perchlorate was still completely degraded by samples with 0, 1 and 5 mg/L of cyanide. There was no perchlorate removal at cyanide concentrations > 10 mg/L.

When L-cysteine was used to scavenge oxygen, growth was slower in the presence of 1 mg/L of cyanide than that of the control (Fig. 3.3 B). Perchlorate was completely degraded by samples with 0 and 1 mg/L of cyanide. With cyanide at 5 mg/L or more there was no culture growth and no perchlorate degradation for over 90 hours.

In order to show that a low concentration of cyanide did not completely inhibit the effectiveness of the reducing agents in scavenging oxygen, Oxyrase™ and L-cysteine were used to remove dissolved oxygen in abiotic medium in the presence of cyanide. Oxygen removal rates with Oxyrase™ were reduced by 45% with cyanide at 1 mg/L and by 91% with cyanide at 5 mg/L (Fig. 3.4). Cyanide at 10 mg/L completely inhibited oxygen removal by Oxyrase™ (data not shown). Cyanide at 1 mg/L and 5 mg/L decreased oxygen removal rates in the presence of L-cysteine by 41% and 80% (Fig. 3.4), respectively, while cyanide at 10 - 50 mg/L decreased
oxygen removal rates by 93% (data not shown). AgNO₃ precipitation results indicated that 95% of the cyanide was present as a free anion in an abiotic solution containing L-cysteine. Because the activity of the reducing agents is inhibited at higher concentration of cyanide, it is not possible to examine inhibition of perchlorate respiration separately from aerobic pathways at higher cyanide concentrations via cell growth or oxygen utilization.

3.3.5 Chlorite dismutation with different concentrations of CN⁻

In order to examine cyanide inhibition of chlorite dismutase activity, chlorite disproportion was measured in the presence of CN⁻ using perchlorate-grown cells. A low concentration (1 mg/L) of cyanide had no inhibition effect on chlorite dismutation (Fig. 3.5). Oxygen generation due to the addition of chlorite (675 mg/L) was similar to that of the control. A concentration of > 10 mg/L of cyanide, however, partially inhibited chlorite dismutation by the culture. Cyanide at a concentration of 100 mg/L or more completely inhibited chlorite dismutation (Fig. 3.5).

3.4 Discussion

Cyanide at a concentration of 1 mg/L inhibited Dechlorosoma sp. KJ growth on perchlorate or chlorate (Fig. 3.1 and 3.2) due to the accumulation of dissolved oxygen, not the loss of perchlorate reductase or chlorite dismutase activities. Aerobic cell growth was completely inhibited using 1 mg/L of cyanide (Fig. 3.2B). However, scavenging the oxygen produced during anaerobic growth, using chemical reducing agents, allowed cells to grow anaerobically in the presence of 1 mg/L of cyanide (Fig. 3.3A and 3.3B). The enzymes affected by cyanide are heme-based enzymes, such as chlorite dismutase and cytochrome oxidase, both of which are needed for perchlorate reduction [4]. It was found that a cyanide concentration of 100 mg/L or more was
needed to inhibit chlorite disproportionation by *Dechlorosoma* sp. KJ. This is consistent with a previous report that a concentration of 520 mg/L of cyanide inhibited the activity of chlorite dismutase isolated from strain GR-1 [4]. Thus, the lack of anaerobic cell growth on perchlorate in the presence of cyanide at a concentration of 1 mg/L was not due to inhibition of chlorite dismutase activity.

Maintaining a low dissolved oxygen concentration is critical for perchlorate reduction. By using a chemical oxygen scavenger, it was shown that perchlorate respiration will continue in the presence of 1 mg/L of cyanide while aerobic respiration will not. This shows that it is oxygen accumulation, not inhibition of enzymes (perchlorate reductase and chlorite dismutase) that results in the lack of perchlorate reduction at low DO concentrations. A DO concentration as low as 0.05 mg/L was sufficient to inhibit perchlorate reduction.

The above results provide direct evidence of a branched respiration pathway for perchlorate reduction. It was shown using 1 mg/L of cyanide that the aerobic pathway could be inhibited, but not the respiration enzymes needed for perchlorate respiration. Thus there must be a branch point to perchlorate reductase in the electron transport chain used by these bacteria under anaerobic conditions. These findings of the cyanide effect on the different enzymes used for perchlorate and aerobic degradation will allow further examination of the respiratory pathways used by perchlorate respiring bacteria.

3.5 Acknowledgement

This research was supported by a National Science Foundation Grant (BES-0001900).
3.6 References


Figure 3.1 Inhibition of CN⁻ at different concentrations on *Dechlorosoma* sp. KJ growth on ClO₄⁻.

1 mg/L: Culture growth with 1 mg/L of CN⁻; 800 mg/L: Culture growth with 800 mg/L of CN⁻.
Figure 3.2 Inhibition of CN\(^-\) at 1 mg/L on *Dechlorosoma* sp. KJ growth on ClO\(_3^-\) or molecular oxygen. (A) Culture growth on ClO\(_3^-\) with 1 mg/L of cyanide; (B) Culture growth on molecular oxygen with 1 mg/L of cyanide.
Figure 3.3 Inhibition of CN$^-$ at different concentrations on *Dechlorosoma* sp. KJ growth on ClO$_4^-$ in the presence of Oxyrase$^\text{TM}$ or L-cysteine. (A) Inhibition of CN$^-$ on culture growth in the presence of Oxyrase$^\text{TM}$. Control: Culture growth in the absence of Oxyrase$^\text{TM}$ and CN$^-; O\_C$: Culture growth in the presence of Oxyrase$^\text{TM}$ and absence of CN$^-; O\_1\text{mg/L}: Culture growth in the presence of Oxyrase$^\text{TM}$ and 1 mg/L of CN$^-$. (B) Inhibition of CN$^-$ on culture growth in the presence of L-cysteine. Same legends as (A) except “L” standing for L-cysteine.
Figure 3.4 Oxygen removal rates of Oxyrase™ and L-cysteine in abiotic growth medium in the presence of CN⁻ at different concentrations. O_C: Oxygen removal rate by Oxyrase™; O_1mg/L: Oxygen removal rate by Oxyrase™ in the presence of 1 mg/L of CN⁻; L_C: Oxygen removal rate by L-cysteine; L_1mg/L: Oxygen removal rate by L-cysteine in the presence of 1 mg/L of CN⁻.
Figure 3.5 Inhibition of CN⁻ at different concentrations on chlorite dismutation by *Dechlorosoma* sp. KJ. 1 mg/L: Chlorite dismutation by culture in the presence of 1 mg/L of CN⁻.
4. DIFFERENT ELECTRON TRANSPORT CHAINS TO MOLECULAR O\textsubscript{2} IN PERCHLORATE-RESPIRING BACTERIA

Abstract

Oxygen is a preferential electron acceptor to perchlorate for perchlorate-respiring bacteria (PRB), and high oxygen concentrations completely inhibit perchlorate reduction. However, oxygen is evolved during chlorite disproportionation and therefore perchlorate reduction occurs in the presence of low concentrations of dissolved oxygen (DO). Dissimilative perchlorate reduction and aerobic respiration were studied using pure cultures of *Dechlorosoma* sp. KJ (ATCC strain BAA-592) to establish a threshold DO level inhibiting perchlorate reduction and to identify respiratory enzymes involved in aerobic respiration and perchlorate reduction. The threshold DO concentration that inhibited perchlorate reduction was determined to be < 0.5% saturation (< 0.04 mg/L). Using oxidized-minus-reduced difference spectra of washed, whole-cell suspensions, it was determined that \(c\)-type cytochrome(s) (peaks at 521 and 552 nm) were always present in both anaerobic and aerobic cultures. \(a\)-type and \(d\)-type cytochromes (peaks at 592 and 629 nm, respectively) were found in anaerobically grown cells based on difference spectra of the cell lysate. Specific respiratory inhibitors (dicumarol, HOQNO, and cyanide) were used to inhibit respiratory enzymes, and thus to identify elements of the electron transport chain (ETC) involved in the reduction of molecular oxygen or perchlorate. At high DO concentrations (80% saturation, 6.29 mg O\textsubscript{2}/L), experimental evidence suggested a respiratory chain consisting of cytochrome \(bc_1\), cytochrome \(c\), and cytochrome \(aa_3\). HOQNO completely inhibited DO uptake at low DO concentrations (3% saturation, 0.24 mg O\textsubscript{2}/L). This suggests one branch point in the ETC to oxygen used under low DO concentrations at the quinone, connecting to cytochrome \(bd\). However, perchlorate reduction occurred in the presence of HOQNO, indicating a second branch in the ETC to perchlorate reductase at a point prior to cytochrome \(b\). These results provide direct
evidence of two branches in the ETC used by perchlorate-respiring bacteria under low DO conditions.
4.1 Introduction

Perchlorate (ClO$_4^-$) is mainly manufactured as ammonium perchlorate for use in rocket and missile propellants [1, 2]. The periodic replacement and use of solid propellant has resulted in the discharge of more than 15.9 million kg of perchlorate salts into the environment since the 1950's [3]. Recent studies have indicated that low concentrations of perchlorate significantly inhibit iodide uptake in humans and animals [4-6]. The U.S. EPA has identified perchlorate users and manufacturers in 44 states, and perchlorate releases in at least 20 states[6]. Such perchlorate releases are estimated to have affected the drinking water of 15 million people. Although there is no current federal drinking water standard for perchlorate, perchlorate has been included on the federal Contaminant Candidate List.

Since perchlorate can be used as a terminal electron acceptor by many newly isolated strains of PRB, several drinking water, wastewater, and in-situ treatment systems are being developed to biologically remove perchlorate[7-11]. The three-step biochemical pathway for perchlorate reduction is ClO$_4^- \rightarrow$ ClO$_3^- \rightarrow$ ClO$_2^- \rightarrow$ Cl$^- +$ O$_2$ [12]. In the last step, a chlorite dismutase produces molecular oxygen. Under fully aerobic conditions, perchlorate reduction by PRB is rapidly inhibited by oxygen [13, 14]. It has been suggested that PRB contain a complete high-oxygen aerobic respiratory chain including at least cytochrome $c$ oxidase, and that an additional low-oxygen enzyme is involved in perchlorate and oxygen reduction [14]. This is based on the following observations. First, almost all PRB are facultative anaerobes (except HAP-1, which is microaerobic) [15, 16]. Second, oxygen never accumulates to measurable concentration (> 0.04 mg/L) during perchlorate reduction indicating that oxygen is rapidly scavenged (chapter 3) [12]. Third, high oxygen concentrations completely inhibit perchlorate reduction [17]. Thus, it seems likely that two membrane-bound oxidases are produced by the bacteria, one for aerobic respiration under high DO concentrations and another for low DO conditions. Strain GR-1 has higher utilization rates of oxygen than perchlorate, and is therefore
able to maintain anaerobic conditions even while oxygen is evolved [12]. However, the threshold of DO needed to fully inhibit perchlorate reduction is not known. Although microbes can respire using perchlorate, little has been done to determine the relationship between perchlorate reduction and aerobic respiratory pathways.

In order to study the branch points and enzymes in the ETCs used by PRB, dissimilative perchlorate reduction and aerobic respiration were studied using batch and chemostat modes cultures of Dechlorosoma sp. KJ (ATCC strain BAA-592), a gram-negative, heterotrophic, facultative anaerobe. Specific inhibitors were used to block respiratory enzymes in order to identify elements of the ETCs involved in the reduction of molecular oxygen and perchlorate. The chemostat system inoculated with KJ was also used to explore the threshold concentration of oxygen that inhibits perchlorate reduction. The goal of this work was to characterize the components of the ETCs for perchlorate-reducing bacteria in order to improve our understanding of biological perchlorate reduction.

4.2 Material and methods

4.2.1 Bacterium and media

Dechlorosoma sp. KJ (ATCC BAA-592), originally isolated from a perchlorate-degrading packed bed bioreactor [18], was used in all experiments. The basal medium was prepared as described by Song and Logan [14] using ultrapure water (Milli-Q system; Millipore Corp., New Bedford, Mass.) and research-grade chemicals. Sodium acetate (C\(_2\)H\(_3\)O\(_2\)–, 2 g/L) was added as the electron donor and sodium perchlorate (ClO\(_4\)–, 1.0 g/L) was added as the acceptor. The pH of the medium was adjusted to 7.0 using NaOH. Anaerobic medium was sterilized by autoclaving and degassed in an anaerobic glove box (Coy Scientific Products, Grass Lake, Mich.). Aerobic medium was prepared as above, but saturated with dissolved oxygen using air.
Cells grown anaerobically (perchlorate) or aerobically were harvested during late-log-phase growth (optical density at 600 nm (OD$_{600}$) of 0.3 to 0.4, 1.74 to 2.32 ± 0.04 × 10$^9$ cells/ml), washed once at 5,000 xg at 4°C for 10 min, and resuspended in fresh medium to the same optical density in a glove box (anaerobic culture) or a laminar flow hood (Model 36212; Labconco Corporation, Kansas City, MO.) (aerobic culture). Cultures were grown, harvested, and tested in all experiments at 30°C unless stated otherwise.

4.2.2 Effect of different levels of dissolved oxygen on ClO$_4^-$

The effect of oxygen on perchlorate removal was examined in continuous-culture experiments using a chemostat (BioFlo® 110, 3 liters; New Brunswick Scientific Co. Inc., Edison, NJ.) at 30°C. The reactor was inoculated and initially operated in batch mode until the culture became turbid. The reactor was then switched to continuous-flow mode by pumping in medium at a constant flow rate (Q). Anoxic conditions were maintained by continuous nitrogen gas sparging. Different levels of DO [saturation concentration of 0.5% (0.04 mg O$_2$/L), 1% (0.08 mg O$_2$/L), 3% (0.24 mg O$_2$/L), 5% (0.39 mg O$_2$/L), and 100% (7.86 mg O$_2$/L)] were maintained by the system software using a combination of nitrogen and air sparging. DO concentrations were constantly measured with a DO probe (InPro 6000 series O$_2$ sensor, T-96; Mettler Toledo, Woburn, MA). The minimum detection limit of the probe indicated by the manufacturer was 0.1%. Samples were obtained directly from the reactor and analyzed for OD$_{600}$ and concentrations of perchlorate. Redox potentials (E$_0$, potential relative to a normal hydrogen electrode) were measured in the reactor with a combination-redox electrode (Pt4805-DPA-S8; Mettler Toledo, Woburn, MA). The reactor was run for at least three detention times (?) before changing the dissolved oxygen concentration. Perchlorate removals were calculated at steady state, where $\mu = 1/\tau = Q/V$, and $V$ is the liquid volume in the reactor (2.0 liters). Steady state was verified by stable perchlorate concentration in the effluent (triplicate samples taken over 3?).
4.2.3 Aerobic and perchlorate reduction in the presence of respiratory inhibitors

Three respiratory inhibitors (dicumarol [19-21], HOQNO (2-Heptyl-4-Hydroxy-Quinoline N-oxide) (Sigma Chemical Co.) [20, 22], and cyanide) were used to identify elements of the ETCs involved in DO uptake. A list of chemical inhibitors, concentration ranges, selected inhibition sites, and inhibitor solvents is provided in Table 4.1. Dicumarol was dissolved in deionized water by adding NaOH [19, 20] to final pH as 8.5. Dicumarol was then added to growth medium at final concentrations of 0.34, 3.36, 33.63, and 336.30 mg/L, respectively. HOQNO was added to the test solution at concentrations of 0.1, 0.5, 1, 5, 10, and 20 mg/L [20, 22]. Cyanide was added at final concentration of 1 mg/L as described in Chapter 3.

Cells were grown under anaerobic and aerobic batch conditions with perchlorate or oxygen, as the electron acceptors, by serial transfers (three times) in anaerobic and aerobic media, respectively. Cells used in uptake experiments were harvested from the third anaerobic and aerobic transfers during late log growth and washed once by centrifuging (5000 × g, 4°C) for 10 min in phosphate buffer (10 mM) at pH 7.0, and resuspended to a final OD$_{600}$ = 0.3 unless indicated otherwise. DO uptake rates of cultures in the presence of different inhibitors were calculated from DO measurements taken over time using a YSI Clark-type DO probe (YSI Model 5300 biological oxygen monitor, Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio). The probe was calibrated with an air-saturated phosphate buffer solution at 30°C before each experiment. Cell suspensions (triplicate) (0.6 ml, 1.72×10$^9$ cells/ml) were added to a 20-ml chamber containing 2.4 ml of phosphate buffer with perchlorate (0.5 g/L) and acetate (2g/L), and constantly stirred. Inhibitors were added (1.5 to 30 µl) at final concentrations given above. DO concentrations were taken every 15 seconds for 3 to 6 minutes.

To test the effect of inhibitors on perchlorate reduction, washed cell suspensions were transferred in the anaerobic glove box to 23-mL test tubes (final volume 15 mL) containing
acetate (200 mg/L) and perchlorate (100 mg/L) in 10 mM phosphate buffer at pH 7.0. Dicumarol and HOQNO were added as described above. In some tests with HOQNO, Oxyrase™ was added at 0.02ml/ml as recommended by the manufacturer. Abiotic controls were run to ensure that HOQNO did not inhibit Oxyrase™. Cell suspensions (triplicate) were incubated in the glove box at 30°C and sampled for perchlorate concentration every hour for 5 hours.

4.2.4 Cytochromes assays

Cytochrome c was assayed by reduced–minus-oxidized spectra using whole cells [23]. Cell suspensions grown in batch were harvested during late exponential growth, while aerobic cultures were obtained directly from the chemostat operating at steady state (100% DO saturation). Cells were washed as above twice and concentrated to 4% of the original volume in phosphate buffer solution. Reduced–minus-oxidized spectra were obtained using a dual-beam spectrophotometer (Shimadzu model UV1601) at room temperature (20ºC), using sodium dithionite as reductant and potassium ferricyanide as oxidant [19, 24].

The presence of cytochrome a, or d was assayed using cell lysate. Batch and chemostat cultures were harvested as described above, washed twice with 0.1 M Tris-HCl buffer at pH 8.0, and resuspended to 4% of the original volume in 0.1 M Tris-HCl buffer containing lysozyme (0.25 mg/ml cell), EDTA (10 mM), a protease inhibitor cocktail (0.5 ml/ml cell), and Triton-X100 (0.1%) at the same pH. Cells were incubated with shaking at 50 rpm for 2 hrs at 30°C. After centrifugation at 14,000 × g for 10 min, the supernatant was recovered and used as the cell lysate [24]. Reduced–minus-oxidized spectra were recorded on the spectrophotometer as above at room temperature (20ºC). Reduced spectra were obtained by adding TMPD (N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride) [25] to the sample and reference cuvettes, and oxidized spectra were obtained by adding potassium ferricyanide to both cuvettes.
4.2.5 Chemostat DO uptake kinetics

To verify that low $k_m$ cytochrome d is the terminal oxidase for uptake of DO in low concentration, DO uptake rates were measured using chemostat cultures harvested at different DO levels. Cells were obtained from reactors operating at steady state with 0%, 0.5%, 1%, 3%, 5%, and 100% of DO saturation, respectively, washed once at 5,000 $\times$ g (4°C) for 10 min in phosphate buffer (10 mM) at pH 7.0, and resuspended to a final $\text{OD}_{600} = 0.3$ unless indicated otherwise. DO uptake rates were tested under high DO (80% saturation) and low DO (3% saturation) using a DO probe (YSI Clark type), as described above. Cell suspensions (triplicate) (0.6 ml) were added to a 20-ml chamber containing 2.4 ml of phosphate buffer with perchlorate (1.0 g/L) and acetate (2 g/L), and constantly stirred. DO concentrations were taken every 15 seconds for 6 minutes.

4.2.6 Analytical Techniques

Protein concentrations were measured by the Bradford Brilliant blue G method with bovine serum albumin as a standard (Sigma, MI). Cell dry weight (DW) was measured using polycarbonate membrane filters (25 mm, 0.2-µm pore diameter; Osmonics Corp., Minnetonka, Minn.) and a microbalance (± 0.1 µg; duplicate samples; Mettler Toledo UMT2, Greifensee, Switzerland). Concentrations of perchlorate and acetate were determined with an ion chromatograph (DX500; Dionex, Sunnyvale, Calif.) equipped with an AS11 column and guard column, a self-regenerating suppressor, and an autosampler. Perchlorate ion was measured using 100 mM NaOH eluent. For chlorate, chlorite, acetate and chloride measurements, a 10 mM NaOH eluent was used. The minimum detection limit for perchlorate was 4 µg/L.
4.3 Results

4.3.1 Perchlorate removal as a function of DO concentration

Complete perchlorate removal (100%) was achieved when no DO was introduced into the reactor (nitrogen gas sparging only, $E_0 = 158$ mV, $\tau = 7.6$ h) (Fig. 4.1). When the DO level was increased to 0.5% saturation (0.04 mg O$_2$/L), only 4% of perchlorate in the influent was degraded, and the redox potential ($E_0$) increased to ca. 220 mV under steady state conditions (Fig. 4.1). After 178 hours at 0.5% DO saturation, anoxic conditions were restored (nitrogen gas sparging only) and perchlorate removal was immediately restored to 100% (Fig. 4.1).

Note in Fig 4.1 that the redox potential varied even though the DO concentration was stable. Because the redox potential is proportional to the logarithm of the oxygen pressure ($\log pO_2$), a very small change in oxygen concentration causes the redox potential to vary by a large amount [26]. Because the DO concentration for the chemostat was set based on DO concentration measured by the computer-controlled software, the redox potential tended to fluctuate at low DO concentrations.

The ability of the system to completely degrade perchlorate following exposure to elevated DO levels depended on the concentration of DO and the exposure time. Complete perchlorate removal was recovered after 178 hours at 0.5% DO saturation (0.04 mg O$_2$/L) or 50 hours at a 3% DO saturation (0.24 mg O$_2$/L). However, the culture lost the capability to completely reduce perchlorate after 59 hours at a DO level at a 5% saturation (0.39 mg O$_2$/L) (data not shown). Previous work (Chapter 2) showed that cells were unable to degrade perchlorate after 12 hours of exposure to DO under near-saturated conditions.

Over a range of 0.5% to 5% DO saturation (0.04 to 0.39 mg/L), the inhibition of perchlorate removal was a function of DO concentration (Fig. 4.2). At a 1% DO saturation (0.08 mg O$_2$/L) or a redox potential of 253 mV, only 3% of perchlorate in the influent was removed (Fig. 4.2). As the DO level was increased to 3% (0.24 mg O$_2$/L) and 5% (0.39 mg O$_2$/L), the
perchlorate removals decreased to 2% and 1%, respectively, with the redox potentials of 334 mV and 384 mV, respectively. Under completely aerobic conditions (100% DO saturation, 7.86 mg O$_2$/L), no perchlorate was reduced (data not shown).

The redox potential is essentially a more accurate reflection of DO at steady state operating conditions under low DO conditions [26, 27]. Redox potential data obtained over a range of 0.04 to 0.39 mg/L in this study were, therefore, fitted by nonlinear regression analysis to obtain DO concentrations below those accurately measured with a DO probe. A redox potential of 150 mV is therefore calculated to be equal to a DO concentration of 0.2% DO saturation or 0.02 mg/L (Fig. 4.2). This 150 mV point is obtained for the chemostat operating without introducing air to the system (nitrogen gas sparging only).

4.3.2 Effect of respiratory inhibitors on aerobic and perchlorate reduction

Dicumarol was added to see if aerobic respiration could be inhibited in the ETC at the quinone. However, dicumarol did not inhibit aerobic respiration, as indicated by DO uptake by aerobically grown cells under both high and low DO concentration levels. When cells were grown anaerobically, dicumarol also had no effect on perchlorate reduction. Controls lacking dicumarol showed the same DO uptake and perchlorate reduction rates as those of cultures with dicumarol (data not shown). Dicumarol inhibits only vitamin K-type quinones [19]. This lack of inhibition by dicumarol in aerobic or anaerobic respiration indicates either that cells possess a dicumarol-resistant quinone reductase, or they do not use a vitamin K-type quinone for electron transport using perchlorate or oxygen.

HOQNO was tested to see if a $b$-type cytochrome was a component in the ETC with oxygen as a terminal electron acceptor. HOQNO at low concentrations (0.1 and 0.5 mg/L) did not inhibit aerobic respiration by aerobically grown cultures at 80% DO saturation (6.29 mg
HOQNO above 1 mg/L, however, reduced DO uptake by 9, 18, 30, and 45% at HOQNO concentration of 1, 5, 10, and 20 mg/L, respectively (Fig. 4.3A).

Although HOQNO partially inhibited aerobic respiration at higher DO concentrations, it completely inhibited aerobic respiration at low DO concentrations (Fig. 4.3B). For anaerobic batch cultures, HOQNO (20 mg/L) decreased DO uptake by 12% at 80% DO saturation (6.29 mg O\textsubscript{2}/L) (Fig. 4.3B), while this inhibitor at the same concentration completely inhibited DO utilization at 3% saturation (0.24 mg O\textsubscript{2}/L) by both aerobic and anaerobic batch cultures (Fig. 4.3B). Because HOQNO inhibits cytochrome \(b\) activity, these results indicate that cytochrome \(b\) was involved in aerobic respiration, but in different forms or complexes in the ETCs at high and low DO levels.

HOQNO was also tested to see if the ETC to perchlorate was branched off the aerobic ETC at cytochrome \(b\). HOQNO at 20 mg/L completely inhibited perchlorate reduction. However, by adding Oxyrase\textsuperscript{TM}, perchlorate reduction was restored with the same amount of HOQNO (Fig. 4.4). Thus, inhibition of perchlorate reductase was due to accumulation of DO not HOQNO inhibition. This result with HOQNO indicates that the ETC to perchlorate was branched off the ETC to oxygen prior to cytochrome \(b\).

Cyanide was added to inhibit terminal oxidases of the ETCs to oxygen at different DO levels. Cyanide (1 mg/L) inhibited DO uptake at low (3% DO saturation, 0.24 mg O\textsubscript{2}/L) and high (80% DO saturation, 6.29 mg O\textsubscript{2}/L) DO concentrations for anaerobic and aerobic batch culture, respectively (Fig. 4.5). This inhibition at both DO concentrations indicates that the terminal oxidase(s) were heme-based cytochromes.

4.3.3 Whole cell and cell lysate spectra

Dithionite-reduced–minus-ferricyanide-oxidized spectra were obtained using washed whole cell suspensions of both anaerobic and aerobic batch cultures and aerobic chemostat
culture. All scans showed peaks at 521 and 552 nm, indicating the presence of type c cytochrome(s) (Fig. 4.6).

TMPD-reduced-minus-ferricyanide-oxidized spectra were obtained using the cell lysate of cultures described above. The spectra of both anaerobic and aerobic batch cultures showed evidence of $a_1$- and $d$- type cytochromes (Fig. 4.6; peaks at 592 and 629 nm, respectively). The spectrum of cell lysate from aerobic chemostat cultures, however, did not show evidence of cytochrome $d$ or $a_1$ in the cells.

4.3.4 DO uptake kinetics at low DO concentrations

DO uptake kinetics were obtained using chemostat cultures harvested at different DO concentrations to verify that a low $k_m$ cytochrome $d$ is the terminal oxidase for aerobic respiration at low DO concentrations. DO uptake at low DO concentration (3% saturation, 0.24 mg O$_2$/L) by anoxic chemostat cultures was greater than that by aerobic cultures harvested at different DO levels (0.5, 1, 3, 5, and 100% saturation) (Fig. 4.7). The maximum observed DO uptake rate by the anoxic chemostat culture was about 43% higher than those of aerobic cultures harvested at 0.5, 1, and 3% saturation, indicating that low $k_m$ cytochrome $d$ was predominant in the terminal oxidases in the anaerobic culture. DO uptake rates by cultures harvested at these low DO conditions (0.5, 1, and 3%) were similar (Fig. 4.6), but 17% and 23% higher than that by culture harvested at 5% and 100% DO saturation, respectively. For the culture harvested at 100% of saturation, DO uptake rate at a low DO concentration (3% saturation, 0.24 mg O$_2$/L) was similar to that at a high DO concentration (80% saturation, 6.29 mg O$_2$/L) (Fig. 4.7).
4.4 Discussion

4.4.1 Threshold DO Concentration for Perchlorate Reduction

The results of this study demonstrated that oxygen inhibits perchlorate reduction over a DO range of 0.5% to 5% DO saturation (0.04 to 0.39 mg O₂/L). At DO concentrations as low as 0.5% saturation, only 4% of perchlorate was reduced (Fig. 4.1), indicating that the threshold DO concentration to inhibit perchlorate reduction is less than < 0.5%. Although a minimum DO concentration could not accurately be measured below 0.5% of DO saturation, a redox potential of 150 mV obtained under the anoxic conditions suggests that a DO level of 0.2% of saturation (0.02 mg O₂/L) did not affect perchlorate degradation. Therefore the threshold DO level to inhibit perchlorate reduction is somewhere in the range of 0.2 to 0.5% of saturation (0.02 to 0.04 mg O₂/L).

4.4.2 ETC for Aerobic Respiration (High DO)

Inhibition studies and whole cell and cell lysate spectra to identify respiratory enzymes support the existence of two branches in the ETC involved in aerobic respiration for Dechlorosoma sp. KJ as shown in Fig. 4.8. The presence of cytochrome bc₁ and cytochrome c in the pathway used under high DO concentrations (> 80% saturation, 6.29 mg O₂/L) is deduced from inhibition studies and spectra. Difference spectra of whole cells grown aerobically showed the presence of cytochrome c, but cytochrome d was absent (Fig. 4.6). HOQNO inhibition studies supported the presence of cytochrome complex bc₁. HOQNO, which is an inhibitor to electron transfer from cytochrome b to cytochrome c₁ in a cytochrome bc₁ complex [19, 20, 28], partially inhibited DO uptake at high DO concentrations (Fig. 4.3A and B). Thus it was concluded that cells grown aerobically possessed cytochrome bc₁. The presence of cytochrome aa₃ is partly based on observations for the other bacteria that ETCs containing cytochrome bc₁
and cytochrome c are followed by cytochrome \textit{aa}_3 as the terminal oxidase (Fig. 1.1) [29]. The other evidence for the presence of cytochrome \textit{aa}_3 is based on the action of cyanide on heme-based enzymes such as cytochrome \textit{aa}_3. Cyanide inhibited oxygen utilization at high DO concentrations, a result which is consistent with the presence of cytochrome \textit{aa}_3. These findings therefore suggest that cytochrome \textit{bc}_1 transfers electrons from a quinone to cytochrome c, and that cytochrome \textit{aa}_3 then delivers electrons from cytochrome c to molecular oxygen at high DO levels (80% saturation, 6.29 mg O_2/L) [29, 30]. Furthermore, the presence of cytochrome c in both anaerobic and aerobic batch cultures, and aerobically grown chemostat cultures (Fig. 4.6), suggests that the ETC involved in aerobic respiration at high DO concentrations is constitutively expressed.

4.4.3 ETCs Involved in Anaerobic Respiration (Low DO)

The findings reported here suggest that the ETC for perchlorate reduction branches off the high DO-pathway used for aerobic respiration at the quinone. Although HOQNO inhibited perchlorate reduction by anaerobic cells, the inhibition was reversed by addition of Oxyrase™ (Fig. 4.4), indicating that apparent HOQNO inhibition of perchlorate reduction was actually due to inhibition by accumulation of dissolved oxygen. Similar rates of dissimilative perchlorate reduction by cultures containing both HOQNO and Oxyrase™, and a biotic control (neither chemical present) (Fig 4.4), suggest that the ETC to perchlorate is branched off the aerobic respiration ETC prior to cytochrome \textit{b}. The conclusion that the ETC for perchlorate reduction is branched from the ETC used for aerobic respiration at the quinone is therefore based on a more general observation for anaerobic ETCs in bacteria that reductases deliver electrons from quinone to terminal electron acceptors (Fig. 1.3) [29, 30].

Experimental evidence also supports a second branch point in the ETC under low DO concentrations (3% DO saturation, 0.24 mg/L). The branch also likely occurs at the quinone,
where electrons then transferred to cytochrome \( bd \). Cell lysate spectra showed the presence of cytochrome \( d \) in anaerobically grown cells, but not in the aerobically grown cells (Fig. 4.6). This finding suggests that a cytochrome \( bd \)-type terminal oxidase, possessing an unusually high affinity for oxygen, is present in bacteria under low DO conditions. This conclusion is consistent with the observed inhibition of cell respiration in the presence of HOQNO, an effective inhibitor of cytochrome \( bd \) binding by the quinone [22]. HOQNO completely inhibited DO uptake under low DO conditions (Fig. 4.3B) demonstrating that the electrons are shuttled from the quinone to cytochrome \( bd \). Further evidence for the presence of cytochrome \( bd \) is provided by experiments using cyanide. Cytochrome \( bd \), a heme-based metalloenzyme, was inhibited by cyanide (Fig. 4.5). In addition, either an additional enzyme that uses oxygen as an electron acceptor was used or additional cytochrome \( c \) was produced under low DO conditions. DO uptake under low DO concentration by anaerobically grown cells was two times faster than DO uptake in high DO concentration by aerobically grown cells (Fig 4.7). Taken together, increased DO uptake and effects of the inhibitors (HOQNO and cyanide) demonstrate the presence of an additional branch in the ETC to a terminal oxidase which is likely cytochrome \( bd \).

The ETC for DO uptake at low DO concentrations was associated with the ETC used for perchlorate reduction. Anaerobically grown cultures showed the highest concentration of cytochrome \( d \) based on difference spectra (Fig. 4.6). The expression of a low \( k_m \) cytochrome oxidase having a high affinity for oxygen would be critical to endowing the cell with a mechanism for scavenging oxygen and preventing oxidative damage to the respiratory \( \text{O}_2 \)-labile enzyme, perchlorate reductase [17].

In conclusion, these data support the presence of two branches off the ETC used by *Dechlorosoma* sp. KJ (ATCC BAA-592) for aerobic growth under high DO concentrations (Fig. 4.8). However, further work will be needed to confirm the presence and expression of respiratory enzymes identified here. This information will be useful in understanding how bacteria, such as
Dechlorosoma sp. KJ, can maintain perchlorate respiration in the presence of low DO concentrations even when the enzyme needed for perchlorate reduction is inactivated by oxygen.

4.5 Acknowledgement

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4.6 References


Table 4.1 Respiration-inhibiting chemicals used

<table>
<thead>
<tr>
<th>Chemical inhibitor</th>
<th>Conc range (mg/L)</th>
<th>Solvent</th>
<th>Inhibition site or action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dicumarol</td>
<td>0.34 – 336.30</td>
<td>NaOH + H₂O</td>
<td>Quinones (Vitamin K)</td>
<td>18-20</td>
</tr>
<tr>
<td>HOQNO</td>
<td>0.1 – 20.0</td>
<td>Ethanol</td>
<td>Cytochrome $b$</td>
<td>19, 21</td>
</tr>
<tr>
<td>NaCN</td>
<td>1</td>
<td>H₂O</td>
<td>Cytochrome oxidase</td>
<td>Chapter 3</td>
</tr>
</tbody>
</table>

* Inhibitors were dissolved in as little solvent as possible to minimize the chances of secondary effects attributable to solvent addition. Controls were run to ensure that solvents were not themselves inhibitory at levels added.
Figure 4.1 Effect of introducing a low DO concentration at 0.5% saturation (0.04 mg/L) into chemostat on perchlorate removal by *Dechlorosoma* sp. KJ culture. $E_0$: redox potential relative to a normal hydrogen electrode.
Figure 4.2 Perchlorate removals by chemostat *Dechlorosoma* sp. KJ culture and redox potentials at steady states of the chemostat running under different DO concentrations: 0% to 5% saturation (0.02 mg O$_2$/L to 0.39 mg O$_2$/L). The regression line for redox is based on the closed symbols. The open symbols are based on the DO concentration calculated from the regression line.
Figure 4.3 Inhibition of HOQNO on DO uptake by *Dechlorosoma* sp. KJ. (A) Effect of HOQNO on aerobic respiration at 80% saturation (6.29 mg O$_2$/L) by aerobic batch culture. “0”: aerobic control without HOQNO; numbers refer to HOQNO (mg/L). (B) Effect of HOQNO at 20 mg/L on aerobic respiration at different DO levels: low as 3% (0.24 mg O$_2$/L) and high as 80% saturation. “A”: aerobic culture; “N”: anaerobic culture; “H”: high DO; “L”: low DO; “C”: Control without HOQNO; “20”: HOQNO at 20 mg/L.
Figure 4.4 Effect of HOQNO at 20 mg/L on perchlorate reduction by *Dechlorosoma* sp. KJ.
Figure 4.5 Inhibition of CN⁻ at 1 mg/L on DO uptake by anaerobic and aerobic cultures. ?: DO uptake at high DO (80%) by aerobic culture; ?: DO uptake at low DO (3%) by anaerobic culture. Solid symbols: control without CN⁻; open symbols: CN⁻ at 1 mg/L.
Figure 4.6 Difference spectra of whole cell and cell lysate of *Dechlorosoma* sp. KJ recorded at room temperature: dithionite-reduced–minus-ferricyanide-oxidized spectra of washed whole cell at 500-565 nm and TMPD-reduced–minus-ferricyanide-oxidized spectra of cell lysate at 565-700 nm (approximately 2 mg of protein per ml). Aerobic chemostat culture was harvested at 100% saturation.
Figure 4.7 DO uptake at low DO level as 3% saturation (0.24 mg O₂/L) by chemostat cultures (except sample 100_H). Numbers refer to “%” of DO saturation when cultures were harvested. 100_H: DO uptake at high DO level (80%) by culture harvested at 100% of DO saturation.
Figure 4.8 Schematic representation of electron transport to O\textsubscript{2} or ClO\textsubscript{4}\textsuperscript{-} in Dechlorosoma sp. KJ.

A dehydrogenase complex removes electrons from an electron donor and transfers these to a quinone. The electrons are transferred to an oxidase complex or a perchlorate reductase (PR) via a branched pathway. The main pathway used for aerobic respiration consists of quinone, cytochrome \textit{bc}\textsubscript{1}, cytochrome \textit{c}, and cytochrome \textit{aa}\textsubscript{3} (a letter in box refers to a complex; elements in square brackets are assumptions as described in the text). Two branches exist off the aerobic pathway under low DO concentrations: both pathways branch from the quinone; one through PR to perchlorate; the other branch through cytochrome \textit{bd} to oxygen is used under low DO concentrations in order to scavenge DO evolved from chlorite disproportionation to oxygen and chloride.
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