Chlorate and nitrate reduction pathways are separately induced in the perchlorate-respiring bacterium *Dechlorosoma* sp. KJ and the chlorate-respiring bacterium *Pseudomonas* sp. PDA

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Received 14 May 2003; received in revised form 2 October 2003; accepted 15 October 2003

**Abstract**

The effect of nitrate on perchlorate and chlorate reduction by perchlorate-respiring bacteria (PRB), and on chlorate reduction by chlorate-respiring bacteria (CRB), is not well understood, particularly with respect to the induction of pathways used to degrade these different chemicals. Based on kinetic data obtained in a series of batch tests, we determined that perchlorate respiratory enzymes were inducible (by chlorate or perchlorate) and separate from those used for denitrification by PRB strain *Dechlorosoma* sp. KJ. Aerobically grown cultures of KJ had lag times of greater than 0.3–2 days when transferred to a medium containing only perchlorate, chlorate, or nitrate as an electron acceptor. There were no lag times for transfers between identical media. Washed cells reduced very little nitrate (<10%) when grown only on chlorate or perchlorate. When grown on nitrate, they degraded little chlorate or perchlorate. The same lack of activity with these electron acceptors was also observed using cell extracts and methyl viologen as an electron carrier, indicating a lack of reactivity was not due to failure of the chemical to diffuse into the cell. Taken together, these results indicated that enzymes for perchlorate and nitrate reduction are separately expressed in strain KJ. The presence of small amounts of nitrate in contaminated groundwater may actually help to increase rates of perchlorate reduction once the nitrate is completely removed. When strain KJ was pre-grown on nitrate and perchlorate, perchlorate degradation (in the absence of nitrate) was more rapid compared to cells grown only on perchlorate. *Pseudomonas* sp. PDA was unable to degrade perchlorate or grow using nitrate, and the induction of enzymes necessary for chlorate respiration differed for strains KJ and PDA. While chlorate reductase and chlorite dismutase activity were induced in KJ by chlorate or perchlorate under anaerobic conditions, these two enzymes were constitutively expressed by PDA under anaerobic and aerobic conditions independent of the presence of chlorate. To our knowledge, this is the first report of constitutive expression of both chlorate reductase and chlorite dismutase in a bacterium.

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**Keywords:** Rocket propellent; Biodegradation; Perchlorate; Chlorate; Nitrate; Anaerobic respiration

**1. Introduction**

Perchlorate contamination of many ground waters in California and the arid southwestern US has occurred primarily as a result of the production and use of perchlorate in solid rocket propellant [1]. Perchlorate...
affects hormone production by the thyroid, and a recent health study prompted the state of California to lower its drinking water action level from 18 to 4 ppb [2]. Perchlorate can be used as an electron acceptor by bacteria found in a wide range of natural environments [3,4]. Many perchlorate-respiring bacteria (PRB) have recently been isolated that can all degrade chlorate, and most of these PRB are facultative denitrifiers [1,5–10]. Relatively fewer chlorate-respiring bacteria (CRB), that are unable to respire using perchlorate, have been isolated [1,11,12].

Respiratory enzymes used by PRB and CRB for perchlorate and chloride reduction have now been isolated [12,13], but induction, regulation and control of the respiratory enzymes used for degrading these chemicals have not been extensively studied. Perchlorate and chlorate reduction proceeds according to ClO$_4^-$ → ClO$_3^-$ → ClO$_2^-$ → Cl$^-$. O$_2$ [14]. Perchlorate reductase expressed by PRB reacts with both perchlorate and chlorate [13] while the chlorate reductase expressed by CRB is unable to degrade perchlorate [12]. The final reduction of chlorite is catalyzed by a separate non-respiratory enzyme (chlorite dismutase).

Perchlorate reduction is induced only under anoxic conditions, and high concentrations of dissolved oxygen are known to inhibit perchlorate reduction [1,9,15,16] and chlorate reduction [11]. However, oxygen is produced by both CRB and PRB as an intermediate during perchlorate reduction. This oxygen is rapidly consumed by PRB and CRB so that it does not accumulate in solution to high levels [17]. Perchlorate reductase is sensitive to oxygen, and the enzyme has been found to have a half-life of approximately 2–3 days with oxygen [13,14]. Similarly, Wolterink et al. [12] found that chlorate reductase was “sensitive” to oxygen and therefore they purified the enzyme under anoxic conditions. Chlorite dismutase activity is unaffected by oxygen, but expression of this enzyme is not constitutive in PRB under aerobic conditions even in the presence of perchlorate or chlorate [14,18,19].

It is not known whether any respiratory enzymes used for chlorate and perchlorate reduction are shared with denitrifying enzymes in PRB capable of nitrate reduction. Nitrate is sequentially degraded via the pathway: NO$_3^-$ → NO$_2^-$ → NO → N$_2$O → N$_2$ [20], and complete denitrification requires four enzymes (nitrate reductase, nitrite reductase, NO reductase, and N$_2$O reductase). It is believed that membrane-bound nitrate reductase is involved in denitrification, and this nitrate reductase is also known to reduce chlorate [29]. In early studies it was suggested that both perchlorate and nitrate reduction were catalyzed by the same nitrate reductase in bacteria because perchlorate degradation was found to be inhibited by nitrate [21–23]. If true, this may imply that the failure of many denitrifiers to grow using perchlorate could result from chloride toxicity due to a lack of chlorite dismutase.

Recent studies support the existence of completely separate pathways for perchlorate and nitrate reduction, but they have not completely eliminated the potential of shared enzymes used for both chloride and nitrate reduction in some PRB. Perchlorate-grown cells of strain GR-1 cannot reduce nitrate [14], but perchlorate reductase purified from strain GR-1 has a higher activity with nitrate than perchlorate [13]. Therefore, it was stated by Kengen et al. [13] that “whether GR-1 contains a separate nitrate reductase when grown on nitrate is not known.” The PRB Dechloromonas agitata CKB cannot respire using nitrate; however, it has nitrate reductase activity when growing on perchlorate and nitrate [15]. The CRB Pseudomonas chloritidismutans also cannot grow using nitrate as the sole electron acceptor [11], and the chlorate reductase isolated from this microorganism does not react with nitrate [12].

A better understanding of the factors that regulate the expression of the enzymes involved in perchlorate, chlorate, and nitrate reduction are important as many perchlorate-contaminated groundwaters that are being considered for bioremediation using PRB contain high concentrations of nitrate [24,25]. However, there has been no systematic comparison of the rates of perchlorate and nitrate reduction by PRB, no direct determination if the nitrate degradation pathway is constitutive or inducible in PRB, and no reports on whether the chlorate degradation pathway is inducible in CRB. Such information would be useful in understanding the potential for natural attenuation of perchlorate and the performance of engineered bioremediation systems when both nitrate and perchlorate are present.

In order to find out more about the factors that control perchlorate reduction by PRB, we investigated the relative rates of perchlorate, nitrate, and chlorate reduction by cells acclimated to only one of these electron acceptors using the PRB strain Dechloromonas sp. KJ. We contrast these results to similar studies conducted using a CRB (Pseudomonas sp. PDA) that cannot respire using perchlorate.

2. Methods

2.1. Media

Experiments with different electron acceptors were conducted using VG medium [19] containing 1 g/L of acetate (C$_2$H$_3$O$_2$). The pH was adjusted to 7.0 using NaOH. Electron acceptors (sodium salts) were added separately at the same high concentration (500 mg/L) that should produce maximum growth rates for these chemicals [8], or 5.0 mM perchlorate (ClO$_4^-$), 6.0 mM chlorate (ClO$_3^-$), and 8.1 mM nitrate (NO$_3^-$). In some
experiments both perchlorate and nitrate were used, each at a concentration of 500 mg/L. All anaerobic media were degassed in an anaerobic glove box (Coy Scientific Products, Grass Lake, Mich.). Aerobic medium was prepared by air saturation without addition of other electron acceptors.

2.2. Bacteria

Dechlorosoma sp. KJ (ATCC BAA-592) and Pseudomonas sp. PDA were originally isolated from a laboratory-scale perchlorate-degrading packed bed bioreactor, and a wastewater treatment plant, respectively [8]. Frozen (−80°C) cultures preserved in 20% glycerol were revived in shake flask in aerobic medium (150 rpm, 29°C) and transferred (5%) to fresh aerobic medium. Cells were then grown (25°C) under anaerobic conditions with chlorate, perchlorate, or nitrate, as the electron acceptor by serial transfer (three times) in anaerobic media. Cells from the third anaerobic transfer in perchlorate medium were further used to inoculate medium containing both perchlorate and nitrate to obtain a cell suspension acclimated to both of these electron acceptors. Cells used in uptake experiments were harvested from these cultures during late log growth and were washed once by centrifugation (10,000 × g) in phosphate buffer (10mM) at pH 7.0, and resuspended to a final OD600 = 0.2 (1-cm cuvette) unless indicated otherwise.

2.3. Reduction of perchlorate, chlorate, and nitrate by acclimated cell suspensions

To test the rates of reduction of different electron acceptors, washed cell suspensions were transferred in an anaerobic glove box to 23-mL test tubes (final volume 10mL) containing acetate (2mM) and one or more electron acceptors (1mM of perchlorate, chlorate, or nitrate) in the 10mM phosphate buffer (pH 7.0). Cell suspensions (triplicate) were incubated in the glove box at 25°C and sampled every hour for 5h.

2.4. Measurement of chlorite dismutation rates

Chlorite dismutation rates were measured based on chloride evolution, according to the chloride-probe method of Xu and Logan [8]. Washed cell suspensions were diluted to OD600 = 0.03 in 100-mL bottles containing 50 mL of 10 mM phosphate buffer at pH 7.0 at 25°C. Chlorite (1 mM final concentration) was added to the bottles (triplicate). Samples (1 mL) were withdrawn every 10 s for 1 min and combined with 10 µL of NaOH (25% w/w) to halt enzyme activity [28].

2.5. Reduction of perchlorate, chlorate, and nitrate by cell extracts

PDA and KJ were grown as indicated above, washed twice with 0.1 M Tris buffer containing 0.03 M NaCl at pH 8.0, and resuspended in 0.1 M Tris containing 40% sucrose at the same pH. Cells were treated with 0.2 mg/mL lysozyme for 4 min, and then combined with EDTA (2.5 mM) at 26°C for 1 h. Cells were broken with a Branson Sonifier 450 (VWR Scientific) set at a duty of 20% and output of 2 for 2 min. After centrifugation at 14,000 × g for 10 min, the supernatant was recovered and used as the cell extract. Enzyme activities were measured in a 10 mM phosphate buffer at pH 7.0 at 26°C by a method based on methyl viologen (MV) [12,13]. The MV method is based on the ability of MV to donate electrons directly to respiratory enzymes when they are coupled to the appropriate electron acceptor. One unit of activity is defined as the amount of enzyme required to oxidize 2 µmol of reduced MV per min. All measurements of MV activity with electron acceptors were corrected for non-respiratory (background) rates of MV reduction by subtraction of rates of controls (samples prepared without the electron acceptor). Protein concentrations were measured with the Bradford reagent (Cat No. B6916, Sigma). All rate measurements were performed in an anaerobic glove box.

2.6. Chemical analysis

Perchlorate, chlorate, chlorite, nitrate and nitrite concentrations were measured using an ion chromatograph (IC; Dionex-500) equipped with an AS-11 column and guard column (Dionex Corp.). A high concentration of NaOH (100mM) was used as eluent for perchlorate analysis, while a low concentration of NaOH (10 mM) was used for chlorate, chlorite, nitrite and nitrate analysis. Nitrogen gas and nitrous oxide were measured by gas chromatography (SRI model 310, Torrence, CA) using a Porapak Q 80/100 column (Alltech, Deerfield, CA), thermal conductivity detector, and helium as a carrier gas. A chloride selective electrode (P/N CL01502, pHoenix Electrode Co., Houston, Texas) was used for chloride concentrations in chlorite dismutase measurements.

3. Results

3.1. Growth using different electron acceptors

When aerobically grown cultures of strain KJ were first transferred to an anaerobic medium, there were lag periods of about 2 days prior to growth using perchlorate, 0.3 days for chlorate, and 1 day for nitrate (Fig. 1). There was no lag phase when cells were
subsequently transferred to an identical medium. In contrast, aerobically grown PDA did not show any lag phase when transferred between an aerobic medium and chlorate medium (Fig. 1) suggesting that enzymes necessary for chlorate reduction were constitutively expressed in this strain. PDA was unable to grow using nitrate. However, PDA cultures growing on a medium containing nitrate and chlorate (5 mM each) were able to partially reduce nitrate to nitrite (30% reduction) and all chlorate to chloride within 1 day (data not shown). Lack of growth on nitrate and perchlorate has also been found for strains CKB and *P. chloritidismutans* [6,11,15].

The chlorate reductase isolated from the CRB *P. chloritidismutans* does not react with nitrate, suggesting that strain PDA may contain a separate nitrate reductase (but not a complete denitrification pathway) that was expressed in the presence of nitrate [12]. However, additional studies would be needed to confirm the existence of a nitrate reductase in strain PDA.

### 3.2. Rates of perchlorate and chlorate reduction by strain KJ and PDA acclimated to different electron acceptors

Perchlorate reduction by strain KJ was not induced by nitrate. There was no significant perchlorate reduction by nitrate-grown KJ cell suspensions using acetate over a 5-h period (95% C.I.) when washed cells were transferred to a medium containing only perchlorate as an electron acceptor (Fig. 2A). When grown on nitrate, cells produced N₂ gas without the accumulation of intermediates (NO₂ or N₂O) (data not shown) indicating strain KJ has a complete denitrification pathway. Perchlorate-grown cells were initially unable to grow using nitrate. The lack of perchlorate reduction by nitrate-acclimated bacteria provides direct evidence that perchlorate reduction does not involve any enzymes used for denitrification.

If strain KJ is grown in a medium containing both perchlorate and nitrate, both chemicals can be used as electron acceptors, but they are used in separate pathways. When bacteria were transferred from a medium containing both perchlorate and nitrate to a medium containing only one of these chemicals, cells were immediately able to degrade the chemical and grow. However, if they were grown on only one of the chemicals (nitrate or perchlorate), there was a lag in growth when they were transferred to a medium containing only the other chemical as an electron acceptor. There was no lag observed for transfers with
the same electron acceptor. The observation of lag periods when cells were transferred to media containing a different electron acceptor indicates that different enzymes are used for denitrification and perchlorate reduction. Surprisingly, perchlorate degradation rates were higher by cells acclimated to the medium containing both perchlorate and nitrate than when acclimated to a medium containing only perchlorate (Fig. 2A). This suggests that rate-limiting enzymes or co-enzymes needed for perchlorate reduction may be shared or promoted by both the denitrification and perchlorate reduction pathways.

Chlorate uptake by washed cell suspensions of KJ acclimated to different electron acceptors is shown in Fig. 2B. Chlorate uptake rates were the same when bacteria acclimated to either chlorate or perchlorate were given only chlorate as an electron acceptor. Other trends in chlorate uptake are similar to those obtained for perchlorate uptake for this strain. Bacteria acclimated to nitrate did not have significant uptake of chlorate over the 5-h incubation period, and chlorate uptake rates were highest for cells originally acclimated to both perchlorate and nitrate. These results for chlorate indicate that chlorate reduction is induced by chlorate or perchlorate, but not nitrate, in strain KJ.

For washed cells of strain PDA, the rate of chlorate disappearance was similar for aerobically grown cultures and cells grown under anaerobic conditions using chlorate (Fig. 3). Therefore, chlorate reductase is constitutively expressed in PDA. However, chlorate-grown (Fig. 3) or aerobically grown (data not shown) suspensions of strain PDA cells did not reduce perchlorate.

### 3.3. Rates of nitrate reduction by washed cells of KJ and PDA

Washed cells of strain KJ grown on perchlorate or chlorate were capable of only a low level of nitrate reduction compared to those grown on nitrate or nitrate and perchlorate (Fig. 2C). The rate of nitrate degradation was less than 10% of that observed when cells were previously acclimated to the nitrate medium. These results suggest that there is some small amount of nitrate reductase activity present under anoxic growth conditions with terminal electron acceptors other than nitrate. Washed cells of PDA grown on chlorate were unable to reduce nitrate during the 5 h incubation (Fig. 3).

### 3.4. Perchlorate, chlorate and nitrate reduction by cell extracts of KJ and PDA

The lack of perchlorate or chlorate reduction by strain KJ when grown only on nitrate, and the inability of strain PDA to grow using perchlorate or nitrate, could result from the failure of these chemicals to be transported across the cell membrane under certain growth conditions. We therefore measured chemical reduction rates using cell extracts of strains KJ and PDA to confirm that the above results were due solely to a lack of enzyme activity.

Cell extracts from nitrate-grown cells of strain KJ had substantially greater nitrate reductase activities than extracts from chlorate-grown cells. Chlorate and perchlorate reduction was much higher with cell extracts from chlorate-grown cells than with cell extracts from nitrate-grown cells (Table 1). These findings are consistent with those presented above for whole cells.

Similarly, the results obtained using cell extracts of strain PDA agreed with the results described above using whole cells. Cell extracts from chlorate-grown PDA cells demonstrated no perchlorate reduction, and nitrate reductase activity was only 0.5% of chlorate reductase activity (Table 1), in agreement with washed cell uptake experiments (Fig. 3). Thus, the lack of perchlorate reduction by strain PDA cannot be due to a lack of perchlorate transport across the cell membrane.

### Table 1

<table>
<thead>
<tr>
<th>Activities (U/C₃/mg)</th>
<th>NO₃⁻ grown KJ</th>
<th>ClO₃⁻ grown KJ</th>
<th>ClO₄⁻ grown PDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO₃⁻ reductase</td>
<td>1.19</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>ClO₃⁻ reductase</td>
<td>0.09</td>
<td>1.20</td>
<td>5.85</td>
</tr>
<tr>
<td>ClO₄⁻ reductase</td>
<td>0.04</td>
<td>0.12</td>
<td>ND *</td>
</tr>
</tbody>
</table>

*ND: not detectable.
3.5. Chlorite dismutation by washed cells of KJ and PDA

Previous studies have not reported whether chlorite dismutase activity by PRB is associated only with chlorate and perchlorate reduction or if it is also induced under denitrifying conditions. We therefore tested whether cells acclimated to the same electron acceptor conditions examined above had appreciable amounts of chlorite dismutase activity (Fig. 4A). Chlorite dismutase activity, measured directly in terms of chlorite degradation, was small, but measurable, for aerobically grown and nitrate-grown cell suspensions (based on the 95% C.I. of rate data). Chlorite degradation rates were much larger, but not significantly different from each other, for cells acclimated to either chlorate or perchlorate. These results indicate that chlorite dismutase activity was induced by the presence of perchlorate or chlorate, but not by oxygen or nitrate.

For strain PDA, chlorite dismutation rates were the same for aerobically grown and anaerobically grown cultures (Fig. 4B). However, chlorite dismutase activity has been found to be inducible in other chlorate-respiring bacteria examined to date [15,19]. Strain PDA is therefore the only bacterium identified to date that expresses a constitutive chlorate reductase and chlorite dismutase.

4. Discussion

Although many PRB can respire using both perchlorate and nitrate, the results obtained here indicate that perchlorate degradation and denitrification pathways are separate in Dechlorosoma sp. KJ. Batch growth tests, kinetic tests using whole cells, and kinetic tests using cell extracts all support the existence of separate and inducible perchlorate and nitrate respiratory enzymes in strain Dechlorosoma sp. KJ (Figs. 1, 2 and 4; Table 1). When grown on nitrate, KJ was able to completely reduce nitrate to nitrogen gas. However, there was little nitrate utilization by cell suspensions grown on only perchlorate or chlorate, and little chlorate, perchlorate, or disproportionation of chlorite when cells were grown on nitrate.

It is well known that oxygen inhibits perchlorate reduction and that chlorite dismutase is not constitutively expressed under aerobic growth conditions by PRB [7,9,15,19]. However, we found that Pseudomonas sp. PDA, a chlorate-respiring bacterium unable to degrade perchlorate, constitutively expressed enzymes necessary for chlorate and chlorite reduction under aerobic and anaerobic conditions independent of the presence of chlorate. To our knowledge, this is the first report of the constitutive expression of chlorate reductase and chlorite dismutase in a bacterium.

Strain PDA has previously been reported to be unable to respire using perchlorate [8], but this is the first time this strain has been shown not to grow using nitrate. Most, but not all, PRB can grow on nitrate [1,3,9]. Dechloromonas agitata CKB, for example, is a PRB strain unable to grow using nitrate. However, PRB strains CKB and PDA can both reduce nitrate to nitrite [15]. Strain PDA appears to have characteristics similar to that of the only other CRB to be studied, Pseudomonas chloritidismutans, because both CRB strains are unable to grow using nitrate [11,12]. Additional CRB will need to be studied to determine whether this inability of CRB to degrade nitrate is common among bacteria unable to respire using perchlorate.

The separation of denitrification and perchlorate-reduction pathways found for strain KJ cannot be generalized to all PRB. For example, washed cells of strain perchlacle grown on perchlorate could reduce nitrate, and that nitrate-grown cells reduced perchlorate [26]. When Dechlorosoma saillum grown on nitrate or perchlorate was transferred to a medium containing both perchlorate and nitrate, nitrate was always completely reduced prior to perchlorate degradation.
[15]. These findings for strain perclace and *D. suillum* could be explained by a single enzyme that is responsible for both perchlorate and nitrate reduction in these bacteria. A (per)chlorate reductase obtained from strain GR-1 did in fact catalyze both perchlorate and nitrate reduction, and the rate of nitrate reduction by this enzyme was even higher than that found for perchlorate [13]. The complete inhibition of perchlorate reduction by nitrate for *D. suillum*, versus nitrate reduction with complete perchlorate reduction in strain KJ, suggests that the perchlorate reductase enzyme differs between these two strains. The lack of perchlorate reduction by strain PDA, and recent findings that the chlorate reductase in CRB does not react with perchlorate, supports the presence of a completely different enzyme in CRB such as PDA versus those in PRB such as strain KJ. Additional biochemical research will be necessary to confirm whether the different perchlorate reductase and chlorate reductase enzymes found for strain GR-1 and *P. chloritidismutans* can be generalized to other PRB and CRB such as KJ and PDA.

4.1. Implications for bioremediation of perchlorate-contaminated waters

Most groundwaters contaminated with perchlorate also contain nitrate [24,25]. The experiments conducted here have shown that when perchlorate and nitrate are both present in the water, separate pathways for perchlorate and nitrate reduction are induced as evidenced by the reduction of both chemicals. Although we have used relatively high concentrations of perchlorate in our studies (500 mg/L), perchlorate reduction is known to be induced by PRB even at low concentrations typically found in perchlorate contaminated groundwaters (ca. 100 μg/L) [27]. Although it was not examined here, several previous studies have shown that perchlorate and nitrate are simultaneously degraded by perchlorate-respiring bacteria [24,25,27]. It has been shown here that cells of strain KJ pre-grown on nitrate and perchlorate show better perchlorate degradation than cells pre-grown only on perchlorate. Therefore, the presence of small amounts of nitrate in the water will improve the perchlorate reduction rate in bioreactors treating contaminated groundwaters, once the nitrate is completely removed.

5. Conclusions

The perchlorate-degrading bacterium *Dechloromonas* sp. KJ contains separate pathways for the complete degradation of perchlorate and nitrate. These pathways are individually induced only by the respective compounds (perchlorate or chlorate, and nitrate) and are not constitutive. In contrast, *Pseudomonas* sp. PDA is unable to degrade either perchlorate or nitrate, but the complete pathway for chlorate and chlorite degradation is constitutive. This represents the first time that a bacterium has been shown to constitutively degrade chlorate under aerobic conditions.

Acknowledgements

We thank Y. Song for her help with preliminary experiments. This research was supported by National Science Foundation Grant BES-0001900.

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