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Behavioral response of dissimilatory perchlorate-reducing bacteria to different electron acceptors

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Abstract The response behavior of three dissimilatory perchlorate-reducing bacteria to different electron acceptors (nitrate, chlorate, and perchlorate) was investigated with two different assays. The observed response was species-specific, dependent on the prior growth conditions, and was inhibited by oxygen. We observed attraction toward nitrate when *Dechloromonas aromatica* strain RCB and *Azospira suillum* strain PS were grown with nitrate. When *D. aromatica* and *Dechloromonas agitata* strain CKB were grown with perchlorate, both responded to nitrate, chlorate, and perchlorate. When *A. suillum* was grown with perchlorate, the organism responded to chlorate and perchlorate but not nitrate. A gene replacement mutant in the perchlorate reductase subunit (*pcrA*) of *D. aromatica* resulted in a loss of the attraction response toward perchlorate but had no impact on the nitrate response. Washed-cell suspension studies revealed that the perchlorate grown cells of *D. aromatica* reduced both perchlorate and nitrate, while *A. suillum* cells reduced perchlorate only. Based on these observations, energy taxis was proposed as the underlying mechanism for the responses to (per)chlorate by *D. aromatica*. To the best of our knowledge, this study represents the first investigation of the response behavior of perchlorate-reducing bacteria to environmental stimuli. It clearly demonstrates attraction toward chlorine oxyanions and the unique ability of these organisms to distinguish structurally analogous compounds, nitrate, chlorate, and perchlorate and respond accordingly.

Keywords *Dechloromonas* · *Dechlorosoma* · *Azospira* · Chemotaxis · Perchlorate

Introduction

Perchlorate (ClO_4^-), a predominantly synthetic compound manufactured in the form of ammonium salt, is used primarily as an energetics booster or oxidant in solid rocket fuels and munitions (Motzer 2001). However, it has been known to inhibit mammalian thyroid hormone production by blocking iodide uptake (Stanbury and Wyngaarden 1952). Recently, it was demonstrated that perchlorate was translocated through the Na+/I− symporter, which can be found in thyroid and lactating breast tissues and subsequently caused iodine deficiency and accumulation of perchlorate in milk (Dohan et al. 2007). Long-term exposure to perchlorate has been implicated in thyroid hormone deficiency, a known cause of impaired neuropsychological fetal and infant development (Porterfield 1994; Howdeshell 2002). Because of historical legal discharge of unregulated manufacturing waste streams, disposal pond leachate, and the periodic servicing of military inventories, the widespread presence of perchlorate in the environment poses a significant health threat.

Perchlorate is unique in its chemical stability and high solubility. Therefore, remediation efforts for perchlorate contamination have primarily focused on either in situ or ex situ biological treatment technologies (Urbansky 1998;

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Coates and Achenbach 2004; Coates and Jackson 2008) based on the ability of some bacteria to reductively respire perchlorate completely to innocuous chloride in the absence of oxygen. Many dissimilatory perchlorate-reducing bacteria (DPRB) are now in pure culture with the dominant species in mesophilic environments belonging to the *Dechloromonas* and *Azospira* genera of the Betaproteobacteria (Coates and Achenbach 2004). All known DPRB are non-fastidious and exhibit a broad range of metabolic capabilities. They are facultative anaerobes or microaerophiles, and most of them alternatively respire nitrate (Coates and Achenbach 2004).

Although DPRB are found to be ubiquitous (Coates et al. 1999), little is known of their abilities to access perchlorate in their natural environment. Three pure cultures from the environmentally dominant *Dechloromonas* and *Azospira* genera were chosen in this study. *Dechloromonas aromatica* strain RCB and *Dechloromonas agitata* strain CKB were previously isolated from aquatic sediment (Coates et al. 2001) and paper mill waste, respectively, (Bruce et al. 1999), while *Azospira suillum* strain PS was isolated from swine waste lagoon sludge (Achenbach et al. 2001). All three organisms are non-fermentative, motile, facultative anaerobes that reduce chlorate and perchlorate [(per)chlorate] coupled to the oxidation of simple organic acids and alcohols at circum-neutral pH. These organisms are unable to utilize hydrogen as electron donor or Fe(III) and sulfate as alternative electron acceptors (Bruce et al. 1999; Achenbach et al. 2001; Coates et al. 2001). In contrast to *D. aromatica* and *A. suillum*, *D. agitata* does not grow by dissimilatory nitrate reduction (Bruce et al. 1999; Chaudhuri et al. 2002). As a continuation of our ongoing studies into this unique form of metabolism, we investigated the behavioral response of these three organisms to structurally analogous electron acceptors and identify their ability to distinguish between nitrate, chlorate, and perchlorate.

Materials and methods

Strains and culturing

All cultures were grown on phosphate-buffered (pH 7) basal freshwater medium containing (per liter): NH_4Cl (0.25 g), K_2HPO_4 (1.07 g), KH_2PO_4 (0.52 g), a vitamin stock solution (10 mL), and a mineral stock solution (10 mL). The vitamin stock solution contained the following (per liter): biotin (2 mg), folic acid (2 mg), pyridoxine HCl (10 mg), riboflavin (5 mg), thiamine (5 mg), nicotinic acid (5 mg), pantothenic acid (5 mg), vitamin B12 (0.1 mg), *p*-aminobenzoic acid (5 mg), and thioctic acid (5 mg). The mineral stock solution contained (per liter) the following: nitrilotriacetic acid disodium salt (1.5 g), $MgSO_4.7H_2O$ (3.0 g), $MnSO_4.H_2O$ (0.5 g), NaCl (1.0 g), $FeSO_4.7H_2O$ (0.1 g), $CaCl_2.2H_2O$ (0.1 g), $CoCl_2.6H_2O$ (0.1 g), ZnCl (0.13 g), $CuSO_4.5H_2O$ (0.01 g), $AlK(SO_4)_2.12H_2O$ (0.01 g), H_3BO_2 (0.01 g), $Na_2MoO_4.2H_2O$ (0.025 g), $NiCl_2.6H_2O$ (0.024 g), $Na_2WO_4.2H_2O$ (0.025 g), and Na_2SeO_4 (0.02 g). Acetate was the sole electron donor, and one of oxygen, nitrate (10 mM), chlorate (10 mM), or perchlorate (10 mM) was used as the sole electron acceptor as required. *D. aromatica* was grown at 30°C, while *D. agitata* and *A. suillum* were grown at 37°C, the respective temperature optima for these organisms.

Microbial perchlorate reduction is accomplished with the action of several enzymes, including perchlorate reductase (Pcr) encoded by the *pcrABCD* operon and chlorite dismutase (Cld) encoded by a single open reading frame *cld*. A deletion mutation in the *pcrA* gene was made as previously described (Bender et al. 2005). Briefly, a 357-bp region upstream of *pcr*A start codon was amplified by polymerase chain reaction (PCR) and cloned in between *Sac*I and *Spe*I sites in pBluescript II KS+ (Stratagene), creating pPCRA1. A 323-bp region downstream of *pcr*A stop codon was amplified by PCR and cloned in between *Eco*RI and *Xho*I in pPR1, resulting in pPCRA2. Finally, the knockout construct pPCRA3 was generated by cloning the 1.6-kb tetracycline resistance cassette from pBBR322 into *Spe*I and *Eco*RI sites in pPR2. The deletion of *pcrA* gene in the knockout mutant was confirmed by PCR.

The resultant mutant that is incapable of growth or reduction of perchlorate or chlorate (Bender et al. 2005) was used to determine the role of perchlorate reductase in taxis towards (per)chlorate and nitrate. Each of the *pcrA* mutant and wild-type *D. aromatica* culture was grown with limited amounts of oxygen as the electron acceptor in phosphate-buffered (10 mM, pH 7) basal freshwater media in sealed serum bottles supplemented with 20 mM acetate as the electron donor and 2 mM perchlorate to induce the perchlorate reduction pathway. The limiting amount of oxygen was supplied as 10 mL of air injected through a sterile 0.22-μm nylon membrane filter into a sealed serum bottle (100 mL) containing 50 mL of liquid culture. Inoculated bottles were horizontally agitated overnight at 30°C to maximize oxygen diffusion. Induction of the perchlorate reduction pathway was confirmed by reverse transcription PCR (RT-PCR) determination of the presence of transcript for chlorite dismutase (*cld*) in the *pcrA* mutant grown with perchlorate relative to a control grown in media without perchlorate (Supplementary Fig. 1).

Reverse transcriptase PCR

Cells were collected by filtration onto a 0.2-μm filter. Total RNA was prepared directly from the filters using the TRIzol

reagent (Invitrogen, number 15596) following manufacturer's protocol. For each sample, 0.2 μg of total RNA extract was used for complementary DNA (cDNA) synthesis of *pcrA* and *cld* transcripts using reverse primers (5′-CGCCGATGTATCTCTTCATGTTCAC-3′ for *pcrA* and 5′-TGAATGGTTCCGAGCGTTGTCGGAC-3′ for *cld*) and the Moloney murine leukemia virus reverse transcriptase (Promega, number M1701) following manufacturer′s protocol. Then, 1 μl of cDNA synthesis product was used as template in 20 cycles of PCR amplifications for each gene of interest. Parallel reactions lacking the reverse transcriptase were set up for each sample as controls for amplification from genomic DNA contamination.

Swarm plate assay

A standard swarm plate assay (Adler 1966) was adapted to establish the ability of all three organisms to navigate in a gradient of the electron donor, sodium acetate. Plastic Petri dishes containing 25 mL of phosphate buffered (pH 7) basal freshwater medium amended with 1 mM sodium acetate and 0.3% Noble agar were prepared. Cells of *D. aromatica, D. agitata*, and *A. suillum* were inoculated in the middle of the agar plates and incubated aerobically at 30°C for *D. aromatica* and at 37°C for *D. agitata* and *A. suillum*. The size of swarm ring was recorded 48 h after inoculation.

Agar plate-based assay

Cells were grown to mid-log phase and harvested by centrifugation at 6,000 rpm for 10 min in centrifuge bottles that were flushed with nitrogen gas. The cell pellet was washed and resuspended in fresh anoxic basal media without any electron donor or acceptor. All liquid transfers were performed on the bench top under a stream of nitrogen gas to avoid any contact with atmospheric oxygen. The prepared cell suspensions were transferred using N_2 gas-flushed syringes into 25 mL anaerobic phosphate-buffered basal media with 1% Noble agar in the molten state to give a final cell count of approximately 1×10^{10} cells·ml^{-1}. The inoculated molten agar bottles were amended with either an electron donor (10 mM) or acceptor (10 mM) as required and an appropriate concentration of chloramphenicol to inhibit de novo protein synthesis (Shaw 1983) and growth during the assay incubation. Growth studies with each organism amended with a range of chloramphenicol concentrations indicated that the optimum working concentrations for *D. aromatica*, *D. agitata*, and *A. suillum* were 2.0, 1.0, and 2.5 mg·L^{-1}, respectively (data not shown). At these concentrations, cell motility, as observed by phase-contrast microscopy, remained unaffected, indicating the presence of active metabolism to support flagellar rotations. In contrast, growth of these cells, as measured by optical density (data not shown), was completely inhibited.

The chloramphenicol-amended cell suspensions were poured into sterile Petri dishes in an anaerobic glove bag under a N_2–H_2 (95:5, vol/vol) atmosphere. Once solidified, sterile filter paper discs (Whatman number 2, 6 mm in diameter) saturated with a sterile anaerobic aqueous stock (1 M) of the chemical of interest were placed on the surface of the agar, allowing the development of a concentration gradient through diffusion of the chemical into the agar. The prepared plates were incubated at room temperature anaerobically inside the glove bag or aerobically on the bench top. A response was identified by the formation of a tan/white cell-dense halo (Supplementary Fig. 2) around the discs after 2 h or overnight incubation for the electron donor and electron acceptor assays respectively caused by the attraction of the motile cells toward the chemical. The presence of a visible halo around the filter disc was scored as a positive result (denoted by + in Table 1), while the absence of a halo in the same time frame was scored as a negative result (denoted by − in Table 1).

Palleroni chamber bioassay

A capillary-based assay using the Palleroni chamber (Palleroni 1976) was also adapted to provide quantitative measurement of the behavioral response. Anaerobic cell suspensions were prepared as described above and added to the Palleroni chamber including the channel and both wells. A pre-cut capillary (3-cm length, 1.46-mm outer diameter, 1.12-mm inner diameter) was filled with chemical of interest at a final concentration of 10 mM and carefully placed in the channel connecting both wells allowing cells to enter the capillaries from both open ends. Each chemical was tested in two sets of triplicates such that one set of capillaries was immediately removed from the chamber to establish a baseline, while a second set of capillaries was removed 15 min later. Once removed from the chamber, each capillary was rinsed carefully to remove cells adhered to the exterior of the tube and purged of its content into a sterile microcentrifuge tube. The amount of cell mass in the microcentrifuge tube was estimated by measuring protein content with a micro-bicinchoninic acid assay kit (Pierce, number 23235), which allows for measurement of protein concentration in the micromolar range. A positive response was identified by an increase in the measured protein content inside the capillary after 15 min of incubation relative to the protein content in the capillary tubes with brief exposure to the cells.

Resting cell suspension experiments

Cultures of *D. aromatica* and *A. suillum* grown with acetate (10 mM) and perchlorate (10 mM) were anaerobically

Table 1 Taxis response of *Dechloromonas aromatica, D. agitata*, and *Azospira suillum* using agar plate-based assay

	D. aromatica	*A. suillum*	*D. agitata*	Wild type	*pcrA* mutant
Anaerobic taxis response when grown with acetate (10 mM) and nitrate (10 mM)					
Acetate	−	−			
Chlorate	−	−			
Perchlorate	−	−			
Nitrate	+	+			
Anaerobic taxis response when grown with acetate (10 mM) and (per)chlorate (10 mM)					
Acetate	−	−	−		
Chlorate	+	+	+		
Perchlorate	+	+	+		
Nitrate	+	−	+		
Anaerobic taxis response when grown with limited oxygen and perchlorate (2 mM)					
Acetate				−	−
Chlorate				+	−
Perchlorate				+	−
Nitrate				+	+

Positive (+) responses represent the presence of cell-dense halos around the filter discs after overnight incubation at room temperature. Negative (−) responses represent the absence of visible cell-dense halos around the filter discs after overnight incubation at room temperature

harvested at late log phase by centrifugation. Cell pellets were washed with anaerobic phosphate buffer (10 mM, pH 7) twice and resuspended in the same phosphate buffer. The suspensions were added to anaerobic phosphate buffer amended with 10 mM acetate and 10 mM of electron acceptor (nitrate, chlorate, or perchlorate) and incubated at 30°C and 37°C for *D. aromatica* and *A. suillum*, respectively. Samples were taken out at different time points, and concentrations of anions were analyzed.

Anion analyses

Concentrations of perchlorate, chlorate, chloride, nitrate, and nitrite were analyzed by ion chromatography as previously described (Chaudhuri et al. 2002).

Results

Genomic potential

The recently finished genome of *D. aromatica* (NC_007298) (http://genome.jgi-psf.org/finished_microbes/decar/decar.download.html) revealed the genetic potential for a motility behavioral response in perchlorate-reducing bacteria. Based on the annotated genome, *D. aromatica* contains three sets of gene clusters for flagella biosynthesis suggesting flagella-based motility. Three sets of gene clusters located on chromosome (bp# 790489–810487, 1244490–1254769, and 4194360–4191281) were annotated to encode chemotaxis signal transduction proteins, including multiple copies of *cheA*, *cheB*, *cheD*, and *cheR*. In addition, 22 putative chemotaxis sensory transducer genes were apparent, five of which were located in the three chemotaxis gene clusters. The remaining chemotaxis sensory transducer genes were dispersed throughout the genome occasionally in gene clusters unrelated to motility or chemotaxis.

Swarm plate assay

Phase-contrast microscopy revealed that all three organisms were motile when grown under planktonic conditions either aerobically or anaerobically with nitrate or (per)chlorate as electron acceptors. To initially investigate the ability of these organisms to respond to a chemical gradient, a traditional swarm plate assay was utilized with acetate (1 mM) as the limiting growth substrate in the agar under aerobic conditions. Swarm rings were visible around the inoculation site 24 h after inoculation. Within 48 h, migration of swarm rings had advanced to a diameter of 1.02±0.08 cm for *D. aromatica*, 0.94±0.05 cm for *D. agitata*, and 2.68±0.33 cm for *A. suillum*, indicating a motility response in these organisms to a substrate concentration gradient.

Agar plate-based assay

In the agar plate-based assay, appropriate concentrations of chloramphenicol were amended to ensure the observed cell-dense halo was a result of bacterial accumulation from migration in the agar instead of growth. Different concentrations of perchlorate were tested with *A. suillum* grown anaerobically on acetate as the sole electron donor and perchlorate as the electron acceptor to determine assay efficiency. The size of the halo was dependent on the

concentration of perchlorate used in the filter discs with a lower limit of approximately 50 mM for a detectable response (Supplementary Fig. 3). As a result, all subsequent plate-based assays were performed with 1 M stock solution of the chemical of interest onto the filter discs for the most pronounced observable result. Initial studies indicated that inclusion of an electron donor such as acetate in the agar plate was a prerequisite for a motility response toward an electron acceptor, suggesting that active cell metabolism was required. No taxis response was observed with sulfate (Supplementary Fig. 2), which is not an electron acceptor utilized by these organisms (Bruce et al. 1999; Achenbach et al. 2001; Coates et al. 2001). No taxis response toward any of the anaerobic electron acceptors (nitrate, perchlorate, chlorate) was observed if agar plates were prepared anaerobically but incubated aerobically on the bench top. However, each of the DPRB tested in this study was attracted toward the electron donor acetate under aerobic incubation regardless of the prior growth conditions (data not shown).

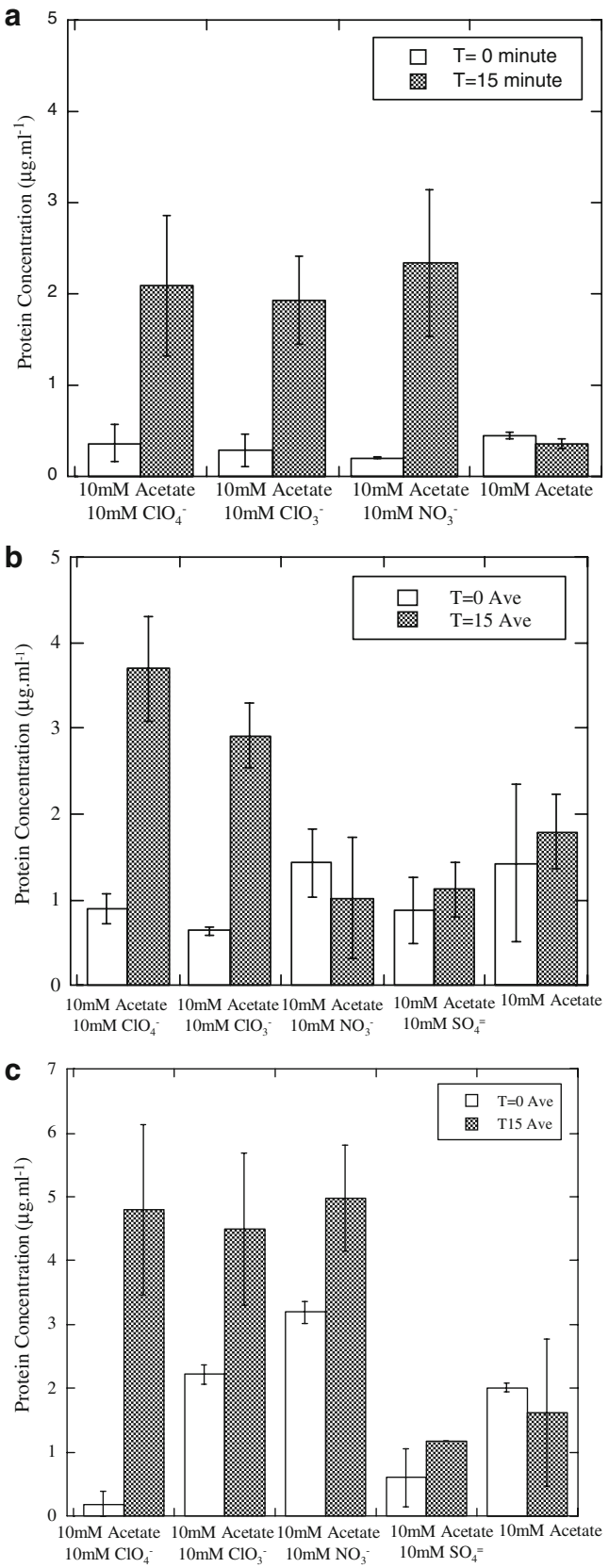
From the agar plate-based assay, both *D. aromatica* and *A. suillum*, grown under nitrate-reducing conditions, showed acetate-dependent motility toward nitrate but not (per)chlorate (Table 1). When grown with perchlorate, both organisms were attracted toward both chlorate and perchlorate, while perchlorate-grown *D. aromatica* was also attracted to nitrate (Table 1). Surprisingly, while *D. agitata* is incapable of growth by nitrate reduction (Bruce et al. 1999), this organism also responded readily toward nitrate when grown with perchlorate (Table 1). In contrast to both the *Dechloromonas* species, perchlorate-grown *A. suillum* did not respond to nitrate (Table 1) demonstrating the unique ability of *A. suillum* to distinguish between structurally analogous nitrate and (per)chlorate when establishing a physiological response. When acetate in the agar was replaced by an electron acceptor and was added instead onto the filter discs, similar behavioral patterns were observed: perchlorate-grown *A. suillum* responded toward acetate only when (per)chlorate was amended in the agar, while perchlorate-grown *D. aromatica* responded toward acetate when nitrate or (per)chlorate was amended in the agar. Identical results were observed when each organism was grown under chlorate-reducing conditions.

Palleroni chamber assay

In the capillary-based assay using the Palleroni chamber (Palleroni 1976), 10 mM acetate was amended in the chamber and the capillary tube in order to observe a behavioral response. Acetate-dependent responses were observed using 10 mM of electron acceptor in the capillary tube after 15 min of incubation. As such, no chloramphenicol was amended because of the short incubation time, which is lower than the minimum doubling time of approximately 90 min observed for any of these organisms (Chaudhuri et al. 2002). Acetate-only samples were included as a negative control to account for random migration of cells into the capillary tube. The capillary-based assay using the Palleroni chamber yielded similar results as the agar plate-based assay. In the case of *D. aromatica* grown with perchlorate, a fivefold increase in protein content inside the capillary tube filled with either (per)chlorate or nitrate was apparent compared to time zero and acetate-only samples (Fig. 1a). This confirms that perchlorate grown *D. aromatica* cells were unable to distinguish these anions and were attracted to each of the three electron acceptors (Fig. 1a). In contrast, when *A. suillum* was grown under identical conditions with perchlorate as the sole electron acceptor, the protein content within the capillary tube filled with nitrate did not increase relative to the control. However, increases in protein content were observed if the capillary tube was filled with (per)chlorate (fourfold and sixfold for perchlorate and chlorate, respectively) further demonstrating the ability of this organism to distinguish these analogous compounds (Fig. 1b). Similar to agar plate-based assay, perchlorate-grown *D. agitata* also responded to nitrate and (per)chlorate even though the organism does not grow by dissimilatory nitrate reduction nitrate (Fig. 1c).

Response of *D. aromatica pcrA* mutant

As part of a previous study on the genetics of perchlorate reductase, a *D. aromatica* mutant in which the gene coding for the alpha subunit of perchlorate reductase, *pcrA*, was replaced by a kanamycin-resistance cassette (Bender et al. 2005). The *pcrA* mutant lacks a functional perchlorate reductase and thus was unable to grow anaerobically with (per)chlorate but was still able to grow aerobically and anaerobically with nitrate (Bender et al. 2005). To determine if perchlorate reductase is necessary for taxis toward (per)chlorate, motility behavior between wildtype and *pcrA* mutant was compared by growing both strains overnight with a limited supply of oxygen and 2 mM perchlorate for inducing expression of the perchlorate reduction pathway after the depletion of oxygen. Mobility of both strains grown under these conditions was confirmed with phase-contrast microscopy and induction of the (per)chlorate reduction pathway was confirmed by RT-PCR of the chlorite dismutase gene (*cld*) (Supplementary Fig. 1). Using the agar plate-based assay where acetate was amended in the agar, wild-type *D. aromatica* was attracted toward nitrate and (per)chlorate, while *pcrA* mutant was only attracted toward nitrate but not (per)chlorate (Table 1), suggesting that an active perchlorate reductase is necessary for taxis to (per)chlorate.



◀ **Fig. 1** Capillary assay in Palleroni chamber using **a** *Dechloromonas aromatica*, **b** *Azospira suillum*, and **c** *Dechloromonas agitata* grown anaerobically with acetate (10 mM) and perchlorate (10 mM). The results depicted are the average of triplicate samples

(Per)chlorate and nitrate reduction by active cell suspensions

Washed cell suspension experiments were performed to test the inherent ability of *D. aromatica* and *A. suillum* to reduce nitrate and (per)chlorate under non-growth conditions. When grown with perchlorate, suspensions of *D. aromatica* readily reduced approximately 4.2 mM perchlorate to 1.1 mM chlorate and 3.3 mM chloride (Fig. 2a). Additionally, these same cell suspensions completely reduced 10 mM nitrate to equimolar nitrite within 4 h (Fig. 2b), supporting the inability of this organism to distinguish between perchlorate and nitrate when grown with perchlorate. In contrast, when grown with nitrate, suspensions of *D. aromatica* did distinguish between the alternative electron acceptors and only reduced nitrate but not (per)chlorate (Fig. 2c). Unlike *D. aromatica*, suspensions of perchlorate-grown *A. suillum* did not reduce nitrate but readily reduced (per)chlorate (Fig. 2d), again demonstrating the ability of this organism to discriminate between these two compounds regardless of growth conditions.

Discussion

These studies provide evidence that DPRB and microorganisms of any type can sense and show a behavioral response to a concentration gradient of chlorine oxyanions in their environment. Furthermore, these results also demonstrate that the response is species-specific. Bacterial motility response toward oxygen as an electron acceptor has been studied extensively in multiple organisms, and transducers such as Aer have also been identified (Taylor et al. 1999). However, the phrase "electron acceptor taxis" was first coined for attraction toward nitrate and fumarate in *Escherichia coli* and *Salmonella typhimurium* after anaerobic induction of the corresponding reduction pathway and the repelling effects from some respiratory inhibitors and uncouplers (Taylor et al. 1979). Subsequently, more organisms were reported to be attracted toward anaerobic electron acceptors. For example, *Rhodobacter sphaeroides* 2.4.3 showed a nitrite reductase-dependent attraction toward nitrate and nitrite (Lee et al. 2002). *Shewanella oneidensis* strain MR-1 was also attracted toward many diverse electron acceptors out

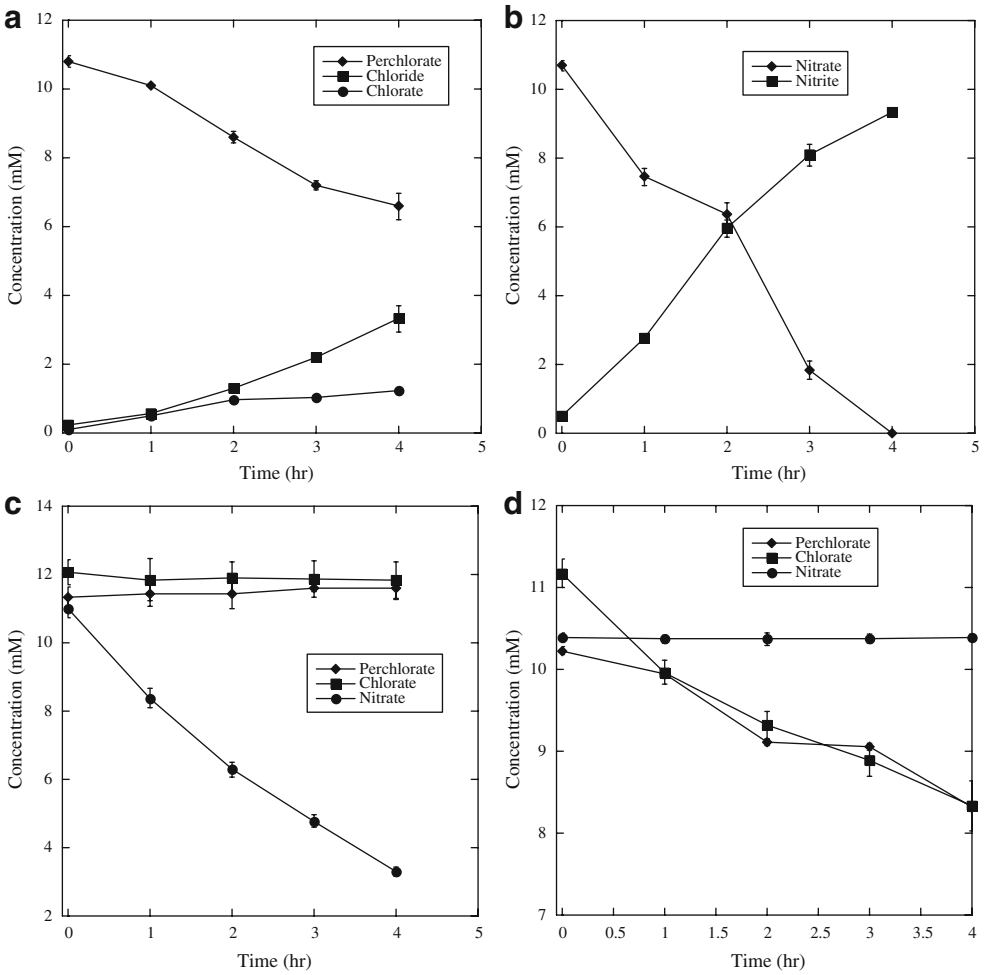


Fig. 2 Reduction of perchlorate and nitrate in washed cell suspensions of (**a**, **b**, **c**) *Dechloromonas aromatica* and (**d**) *Azospira suillum* grown anaerobically with acetate (10 mM) as the electron donor and 10 mM perchlorate (**a**, **b**, **d**) or 10 mM nitrate (**c**) as the electron acceptor. The results depicted are the average of triplicate samples

of more than a dozen that the organism is capable of utilizing for growth (Nealson et al. 1995; Bencharit and Ward 2005). It was established in *R. sphaeroides* that motility response to light, oxygen, and the alternative electron acceptor dimethyl sulfoxide (DMSO) was dependent on the electron transport. It was proposed that an electron transport intermediate potentially served as the response signal instead of an actual chemical attractant (Armitage et al. 1985; Gauden and Armitage 1995). Such responses were categorized as "energy taxis" because the electron transport chain is part of a cellular energy-generating system. In energy taxis, bacteria sense the stimuli through the internal redox state reflected by changes in the electron transport chain and transduce the signal through the common chemotaxis signaling pathway (Taylor and Zhulin 1998; Alexandre and Zhulin 2001). Alexandre et al. conclusively demonstrated electron transport as the stimulus for energy taxis in the diazotrophic *Azospirillum brasilense* (Alexandre et al. 2000) and described criteria to identify this type of response (Alexandre and Zhulin 2001). First, chemicals interacting directly with the electron transport should elicit a response. Second, there should be a correlation between the magnitude of the response with the physiology of electron transport chain. Third, mutations that surrender activity in a terminal reductase for a particular substrate will disrupt the electron flow leading to the substrate, subsequently resulting in a loss of the attraction response.

A brief survey in the genome of *D. aromatica* revealed strong potential for this organism to display taxis behavior such as the presence of flagellar biosynthesis genes and multiple copies of genes known to be involved in chemotaxis signal transduction. In this study, several lines of evidence led us to propose energy taxis as the basis for the observed responses by *D. aromatica*. First, the requirement of an electron donor such as acetate for any behavioral response to electron acceptors suggests a metabolism-dependent behavior where reduction of electron acceptors is necessary to elicit a response. Exchanging the placement of acetate and electron acceptors tested between the agar and the filter discs in the agar plate-based assay yielded the same behavioral pattern, suggesting the involvement of the electron transport such that both the input of reducing equivalents and the output of electrons

onto acceptors can directly or indirectly serve as response signals. The inhibition of oxygen on the response to alternative electron acceptors excludes the possibility of nitrate or (per)chlorate-specific chemoreceptors and further suggests the involvement of the electron transport chain in which oxygen was interrupting electron transport onto nitrate or (per)chlorate by inhibiting nitrate and perchlorate reductases, respectively (Chaudhuri et al. 2002; Coates and Achenbach 2004).

Second, chemicals that elicit positive motility response were also the physiological substrates for each DPRB tested in washed cell suspensions under non-growth conditions. Nitrate was the only electron acceptor to elicit a positive motility response in *D. aromatica* grown with nitrate and the only electron acceptor that was reduced by nitrate-grown *D. aromatica* in washed cell suspensions. Similarly, both nitrate and (per)chlorate elicited a positive response in *D. aromatica* grown with perchlorate, and in washed cell suspensions, (per)chlorate was reduced to chloride and nitrate was readily reduced to nitrite. Accumulation of nitrite in the suspension suggested the absence of an active nitrite reductase in perchlorate grown cells and also suggested that perchlorate reductase from *D. aromatica* was responsible for overlapping reduction of (per)chlorate and nitrate. A similar dual activity of the perchlorate reductase was previously observed in *D. agitata*, which cannot grow on nitrate (Chaudhuri et al. 2002) and was further demonstrated in this study by the behavioral response of *D. agitata* to nitrate and (per)chlorate. In fact, perchlorate reductase purified from *D. agitata* is known to be capable of reducing both nitrate and (per) chlorate (Coates and Heinnickel, unpublished data) further supporting this conclusion.

The same correlation between behavioral response and physiology (i.e., reduction of specific electron acceptors) was also evident in *A. suillum*. When grown with nitrate, *A. suillum* was attracted toward nitrate but not (per)chlorate and reduced only nitrate in washed cell suspensions. When grown with perchlorate, *A. suillum* was attracted toward (per)chlorate and reduced only (per)chlorate but not nitrate in washed cell suspensions. If perchlorate reductase was central in mediating both the motility response and reduction in cell suspensions, then *A. suillum* harbors a unique homolog of perchlorate reductase, which can successfully differentiate between nitrate and (per)chlorate and reduce (per)chlorate only. Interestingly, a PCR primer set recently designed based on known *pcrA* sequences to detect DPRB in the environment failed to amplify *pcrA* gene in *A. suillum* (Nozawa-Inoue et al. 2008), supporting the distinctiveness of *A. suillum* perchlorate reductase apart from the known *Dechloromonas* perchlorate reductases. Moreover, the difference could be reflected in unique amino acid residues attributing to the loss of nitrate-reducing capability and failure of detection by the canonical primer set. Nevertheless, the connection between behavioral response and physiological reduction capacity of each DPRB tested in this study strongly supported energy taxis as the underlying mechanism in which reduction of nitrate and (per)chlorate gradients by corresponding terminal reductases coupled to oxidation of acetate created the signal to elicit the response.

Finally, to demonstrate the necessity of terminal reductases in the observed behavior, the response of a *D. aromatica* perchlorate reductase mutant to (per)chlorate was tested. Unable to grow on (per)chlorate, the *pcrA* mutant was grown with a limited amount of oxygen in the presence of (per)chlorate to induce the perchlorate reduction pathway. Using agar plate-based assay, the mutant cells failed to show attraction to (per)chlorate while the wild-type cells under the same conditions were readily attracted to (per)chlorate, further supporting the requirement of an active perchlorate reductase to establish a motility response toward (per)chlorate in *D. aromatica*. Interestingly, it was observed that the perchlorate reductase mutant was attracted to nitrate suggesting that the nitrate reductase was possibly induced when the culture growth conditions became anaerobic even though nitrate was not present.

Together, these results demonstrate that DPRB are capable of metabolism-dependent movement toward (per)chlorate with energy taxis as the potential underlying mechanism in *D. aromatica*. These results also suggested for the first time a possible difference in substrate range for perchlorate reductases in different DPRB. While *D. aromatica* and *D. agitata* have perchlorate reductases that reduce nitrate and (per)chlorate, *A. suillum*, however, has a unique homolog of perchlorate reductase that can potentially distinguish between these electron acceptors and reduce (per)chlorate but not nitrate. Phylogenetic comparisons between perchlorate reductases with or without nitrate-reducing capability can provide important insight into key residues in (per)chlorate reduction as well as in the evolutionary history of perchlorate reductases in the DMSO reductase family. Moreover, the specificity of recognition may offer a unique opportunity to develop a (per)chlorate-specific biosensor using perchlorate reductase from *A. suillum* that avoids interferences from nitrate. Ultimately, this study demonstrates the potential for DPRB to seek out perchlorate in the environment during active perchlorate reduction, strengthening the support for using DPRB in the treatment of perchlorate contamination.

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