

# MICROBIAL PERCHLORATE REDUCTION: ROCKET-FUELLED METABOLISM

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It is less than 7 years since perchlorate, a predominantly man-made toxic anion, was first identified as a significant water contaminant throughout the United States. Owing to its solubility and non-reactivity, bioremediation was targeted as the most promising solution for the problem of perchlorate contamination. Since 1996, concerted efforts have resulted in significant advances in our understanding of the microbiology, biochemistry and genetics of the microorganisms that are capable of reductively transforming perchlorate into innocuous chloride. The recent completion of the whole-genome sequence of the perchlorate-reducing microorganism *Dechloromonas aromatica* offers further insight into the evolution and regulation of this unique metabolic pathway. Several *in situ* and *ex situ* bioremediative processes have been engineered, and many monitoring tools that are based on immunology, molecular biology and stable isotope content are now available. As such, the rapid scientific response to this emerging contaminant offers great hope for its successful elimination from contaminated environments in the future.

## HYPOTHYROIDISM

The most common thyroid disorder and one in which the thyroid is underactive.

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In recent years, perchlorate has become a household word in the United States as concerns about its presence in water supplies have resulted in a public outcry. These concerns have been further fuelled by articles in the popular press<sup>1</sup> that recount disputes between the US Environmental Protection Agency (EPA) and the Pentagon regarding the reporting and regulation of this contaminant<sup>2,3</sup>. Furthermore, the application of newly developed highly sensitive analytical techniques<sup>4</sup> has demonstrated that the true extent of perchlorate contamination was severely underestimated<sup>5–8</sup>, and the recent identification of its presence in the main vegetable and dairy food products indicates that perchlorate might represent an even greater threat to health than was previously considered<sup>9</sup> (see [Environmental Working Group Suspect Salads Report](#) in the online links box).

## The problem

Perchlorate ( $\text{ClO}_4^-$ ) is a soluble anion that consists of a central chlorine atom surrounded by a tetrahedral array of four oxygen atoms. It is known to affect the function

of the thyroid gland in mammals<sup>10</sup> and its toxicity primarily results from the fact that it inhibits thyroid hormone output. Perchlorate binds to the sodium–iodide symporter and consequently competitively inhibits the uptake of iodide by the thyroid gland. Thyroid hormones are synthesized from iodide in the thyroid gland and are responsible for regulating mammalian metabolism. Long-term reduction in iodide uptake in an adult can ultimately result in HYPOTHYROIDISM<sup>11,12</sup>. Furthermore, because thyroid hormones are required for normal physical and mental development, exposure to thyroid inhibitors such as perchlorate can have a direct impact on foetal and infant neuropsychological development<sup>13,14</sup>. Previous studies have indicated that children of mothers suffering from maternal thyroid deficiency during pregnancy performed below average on 15 tests relating to intelligence, attention, language, reading ability, school performance and visual-motor performance<sup>15</sup>.

Before 1997, perchlorate was an unregulated compound in the United States. However, the discovery of perchlorate contamination in drinking water resources

throughout the United States, especially in the south-western states of Nevada, Utah and California, prompted the establishment of a PROVISIONAL ACTION LEVEL of  $18 \mu\text{g L}^{-1}$  in 1997 (REF. 16). The worst contamination was in the Las Vegas, Nevada area, where perchlorate has been manufactured for more than 50 years — ground-water contamination was discovered ranging from  $630,000 \mu\text{g L}^{-1}$  to  $3,700,000 \mu\text{g L}^{-1}$  (REF. 8). In 1998, perchlorate was added to the US EPA CONTAMINANT CANDIDATE LIST for drinking water supplies<sup>17</sup>. In January 2002, as a result of the publication of a US EPA draft review on toxicological and risk assessment data associated with perchlorate contamination, a revised and lowered health protective standard of  $1 \mu\text{g L}^{-1}$  was suggested (see [Perchlorate Environmental Contamination: Toxicological Review and Risk Characterization](#) in the online links box). This draft assessment, which is currently being reviewed by the US National Academy of Sciences, has been rejected by the Pentagon, which is targeting a higher benchmark of  $200 \mu\text{g L}^{-1}$ . This higher standard would markedly reduce the total cleanup costs associated with perchlorate contamination, as vast areas of sites that are known to be contaminated with perchlorate would fall below the level for regulation<sup>3</sup>.

#### The source

Perchlorate is principally a synthetic compound and its salts have a broad range of different industrial applications, ranging from pyrotechnics to lubricating oils<sup>8</sup>. Its presence in the environment predominantly results from legal historical discharge of unregulated manufacturing waste streams, leaching from disposal ponds and from the periodic servicing of military inventories<sup>18–20</sup>. So far, the only known significant natural source of perchlorate is that associated with mineral deposits found in Chile, where the perchlorate content averages as much as 0.03% of the total mineral mass<sup>21</sup>. Throughout the last century, the Chilean ore deposits were extensively mined as a source of minerals and nitrate for fertilizer manufacture, and the perchlorate often persists throughout processing and is found in low concentrations in the final product<sup>22,23</sup>. Although the full extent of the historical use of Chilean-ore-based fertilizers in the United States is unknown, their current usage represents less than 0.2% of the fertilizer consumption in the United States<sup>6</sup>. Furthermore, recent modifications to the refinement process have significantly reduced the perchlorate content of these products<sup>23</sup> and, as such, they are not thought to be a significant source of perchlorate in the environment<sup>23</sup>.

By contrast, however, the presence of perchlorate has been indicated in various other natural phosphorous-bearing minerals that are formed through evaporation processes (evaporites) and that are collected from a diversity of arid locations<sup>5</sup>. More recently, it was demonstrated that solid fertilizers that are not derived from the Chilean deposits and that are commonly used for the HYDROPONIC GROWTH of various fruits and vegetables can contain perchlorate at concentrations as high as  $350 \text{ mg kg}^{-1}$  (REF. 6). Such levels could represent a significant global health threat owing to the increasing use of

hydroponic farming techniques for the production of a wide variety of plants for human consumption throughout the world<sup>6</sup>. Studies performed on different plant species grown in soils containing perchlorate have indicated uptake<sup>24–26</sup> and, in some cases, transformation — reduction to chlorate ( $\text{ClO}_3^-$ ), chlorite ( $\text{ClO}_2^-$ ) and chloride ( $\text{Cl}^-$ ) — in plant tissues<sup>24,25</sup>. In certain plant species, such as tobacco and lettuce, perchlorate accumulates and persists during processing into the final shelf products, such as cigarettes, cigars and chewing tobacco, at concentrations as high as  $60 \text{ mg kg}^{-1}$  (REF. 26).

#### The solution

As these sorts of studies continue and more sensitive analytical methods are developed<sup>4</sup>, it is anticipated that other natural sources of perchlorate will be identified. For example, recent reports have indicated low levels of perchlorate in drinking water wells in the southern part of Texas in an area that exceeds 30,000 square miles<sup>7</sup>. This perchlorate is known not to be associated with industrial activities or the application of agricultural fertilizer, indicating that it might originate from an unidentified natural geological source<sup>7</sup>. However, anthropogenic sources such as that found in Henderson, Nevada<sup>3</sup>, which resulted in a major contaminant plume containing more than 9,000 metric tons of perchlorate<sup>3</sup> stretching down through the Las Vegas Wash into Lake Mead and the Colorado River, will probably remain the principal culprit for the presence of perchlorate in water supplies. From April 2003 onwards, perchlorate has been manufactured and used in more than 150 industrial facilities throughout the United States and more than 90 perchlorate releases have been reported in 25 states.

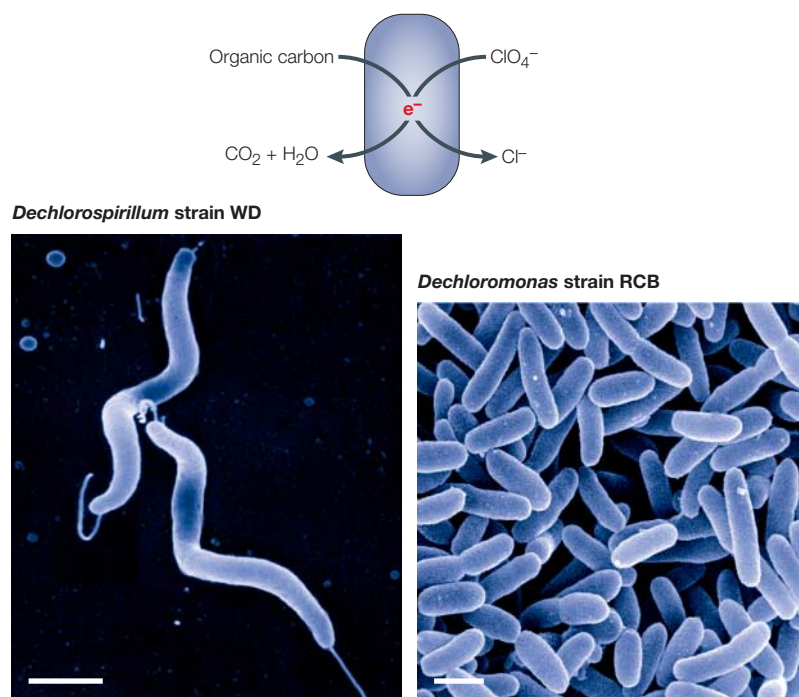
Ammonium perchlorate ( $\text{NH}_4\text{ClO}_4$ ) represents approximately 90% of all perchlorate salts manufactured<sup>8</sup>. It is predominantly used by the munitions industry and the US Department of Defence as an energetics booster or oxidant in solid rocket fuels<sup>8,18,27,28</sup>. Although a powerful oxidant, under most environmental conditions perchlorate is highly stable and non-reactive owing to the high energy of activation associated with its reduction<sup>18,19</sup>. Owing to the large molecular volume and single anionic charge, perchlorate also has a low affinity for cations and, as a result, perchlorate salts, such as ammonium perchlorate, are generally highly soluble and completely dissociate into  $\text{NH}_4^+$  and  $\text{ClO}_4^-$  in aqueous solution. Furthermore, perchlorate does not adsorb to any significant extent to soils or sediments and, in the absence of any biological interactions, its mobility and fate are largely influenced by the hydrology of the environment<sup>29</sup>.

Owing to its unique chemical stability and high solubility, remediation efforts for perchlorate contamination have focused primarily on microbial processes<sup>18</sup> and many novel bioremediative technologies are currently being developed<sup>30</sup>. In 2001, a report published by the Ground Water Remediation Technologies Analysis Center<sup>28</sup> outlined 65 different case studies of perchlorate-treatment technologies to target contaminated wastewater, surface water, groundwater and soils. Most (45 case studies) were either *in situ* or *ex situ* biological

**PROVISIONAL ACTION LEVEL**  
A contamination level set by each state in the United States that is used to protect consumers until a federal maximum concentration level is defined by the US EPA.

**CONTAMINANT CANDIDATE LIST**  
The primary source of priority contaminants for evaluation by the EPA's drinking water programme; updated every five years.

**HYDROPONIC GROWTH**  
Growing plants in water containing dissolved nutrients instead of soil.



**Figure 1 | Microbial perchlorate reduction.** Perchlorate-reducing bacteria grow by the complete oxidation of organic carbon or various alternative inorganic electron donors ( $\text{H}_2$ ,  $\text{H}_2\text{S}$  or  $\text{Fe}^{2+}$ ) coupled to the reduction of perchlorate in anoxic environments. Perchlorate-reducing bacteria are phylogenetically, physiologically and morphologically diverse, as shown here for the  $\alpha$ -Proteobacterium *Dechlorospirillum* strain WD and the  $\beta$ -Proteobacterium *Dechloromonas* strain RCB. Both scale bars = 1  $\mu\text{m}$ .

treatment technologies based on the unique ability of some microorganisms to reductively respire perchlorate completely to innocuous chloride in the absence of oxygen<sup>28</sup> (FIG. 1). Other physical/chemical technologies, such as adsorption by activated charcoal or REVERSE OSMOSIS, have proved difficult, inapplicable or have failed because of rapid saturation of active sites or the high costs, especially the costs that are associated with the processing of surface or groundwater contamination where large volumes containing low levels of perchlorate can require treatment. Although ion-exchange technologies do show promise, they are non-selective and still require subsequent disposal of the removed perchlorate.

### The microorganisms

It has been known for more than 50 years that microorganisms can reduce oxyanions of chlorine such as chlorate ( $\text{ClO}_3^-$ ) and perchlorate ( $\text{ClO}_4^-$ ) (referred to here as (per)chlorate) under anaerobic conditions<sup>31</sup>. The high reduction potential of chlorate and perchlorate ( $\text{ClO}_4^-/\text{Cl}^- E^0 = 1.287 \text{ V}$ ;  $\text{ClO}_3^-/\text{Cl}^- E^0 = 1.03 \text{ V}$ ) makes them ideal electron acceptors for microbial metabolism<sup>32</sup>. Early studies indicated that unknown soil microorganisms rapidly reduced chlorate that was applied as a herbicide for thistle control<sup>31</sup>, and the application of this reductive metabolism was later proposed for the measurement of biological oxygen demand in sewage and wastewater<sup>33,34</sup>. Initially, it was thought that chlorate reduction was mediated by nitrate-respiring microorganisms in the environment, with chlorate

uptake and reduction simply being a competitive reaction for the nitrate reductase system in these bacteria<sup>35–37</sup>. This was supported by the fact that many nitrate-reducing microorganisms in pure culture were also capable of reducing (per)chlorate<sup>35,37,38</sup>. Furthermore, early studies demonstrated that membrane-bound respiratory nitrate reductases and assimilatory nitrate reductases could alternatively reduce chlorate<sup>39</sup> and presumably also perchlorate. For many years, selection for chlorate resistance has been used as a screening tool to obtain mutants that are unable to synthesize the molybdenum cofactor that is required for nitrate reduction<sup>40</sup>. However, in all cases, chlorite ( $\text{ClO}_2^-$ ) was produced as a toxic end product of (per)chlorate reduction by nitrate-reducing bacteria and no evidence was provided that these microorganisms could grow using this metabolism.

It is now known that specialized microorganisms have evolved that can grow by the anaerobic reductive dissimilation of (per)chlorate into innocuous chloride. More than 40 dissimilatory (per)chlorate-reducing bacteria are now in pure culture<sup>20,41–51</sup> and this number is rapidly increasing, as evidenced by the fact that a further 16 microorganisms were described as this article went to press<sup>52</sup>. (Per)chlorate-reducing microorganisms have been isolated from a broad diversity of environments, including both pristine and contaminated soils and sediments<sup>20,41–47</sup>. This was unexpected owing to the supposed limited natural abundance of (per)chlorate. However, the diverse metabolic capabilities of these microorganisms could account for their presence in environments in which (per)chlorate is not found. Phenotypic characterization revealed that the known dissimilatory (per)chlorate-reducing bacteria (DPRB) exhibit a broad range of metabolic capabilities, including the oxidation of hydrogen<sup>20,49</sup>, simple organic acids and alcohols<sup>41–43,46,47,50</sup>, aromatic hydrocarbons<sup>48</sup>, hexoses<sup>46</sup>, reduced humic substances<sup>42,48,53</sup>, both soluble and insoluble ferrous iron<sup>41–43,54–56</sup>, and hydrogen sulphide<sup>41,42</sup>. All of the known DPRB are facultatively anaerobic or microaerophilic, which is reasonable in light of the fact that molecular oxygen is produced as a transient intermediate of the microbial reduction of (per)chlorate<sup>20,41–43,47</sup>. Some, but not all, DPRB alternatively respire nitrate<sup>41,42</sup>. So far, all microorganisms that are capable of perchlorate reduction can alternatively use chlorate; however, the same is not necessarily true of chlorate-reducing bacteria, and there are now several chlorate-reducing microorganisms in pure culture that are incapable of the reductive respiration of perchlorate.

The (per)chlorate-reducing bacteria that have been isolated so far are phylogenetically diverse<sup>20,41,43</sup> with members in the  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\epsilon$  subclasses of the Proteobacteria phylum (FIG. 2)<sup>20,41,43,57</sup>. As such, the metabolic capability of (per)chlorate reduction is widespread throughout the Proteobacteria, which has some interesting evolutionary implications in light of the assumed limited geographical distribution of natural sources of chlorate and perchlorate. Several of the known (per)chlorate-reducing isolates are representatives of previously defined genera (*Pseudomonas*,

#### REVERSE OSMOSIS

A process in which purified water is obtained from a salt solution.

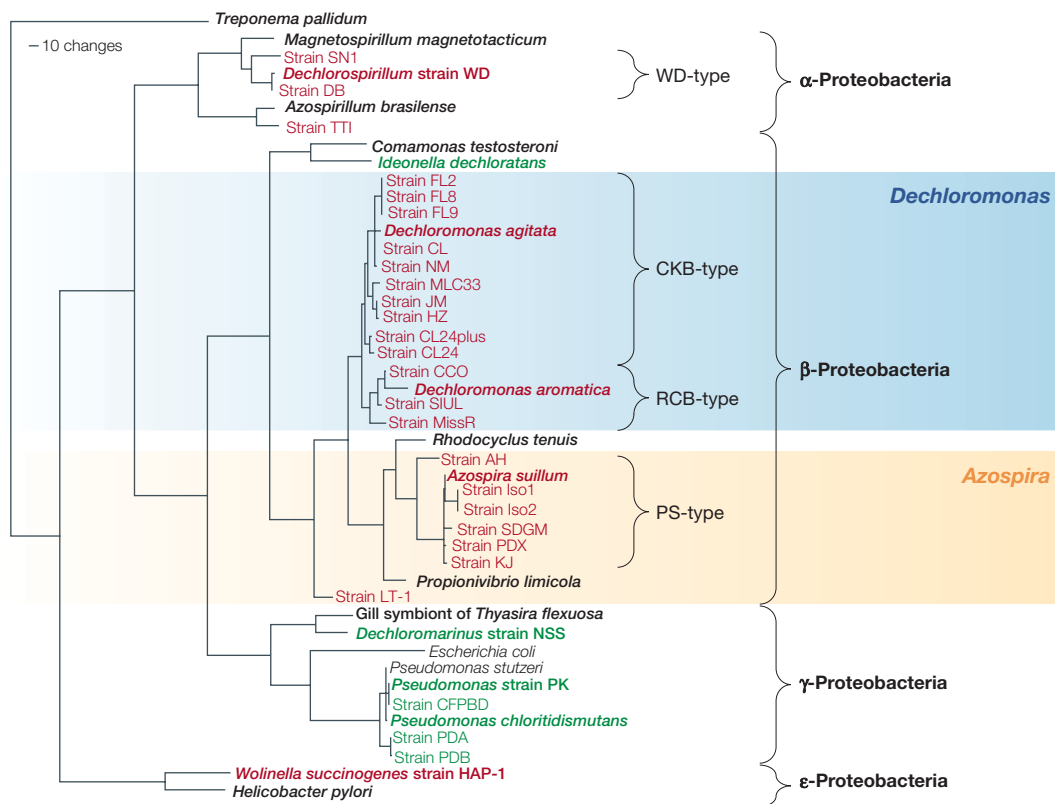


Figure 2 | **Phylogenetic distribution of (per)chlorate- and chlorate-reducing microorganisms.** (Per)chlorate-reducing bacteria (in red) and chlorate-reducing bacteria (in green) are found in four ( $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\epsilon$ ) of the five subclasses of the Proteobacteria. The (per)chlorate-reducing populations in the environment are predominantly members of the *Dechloromonas* and *Azospira* species of the  $\beta$ -subclass of the Proteobacteria. The *Dechloromonas* species can be subdivided into the CKB-type and the RCB-type based on signature nucleotide sequences in the small subunit of the ribosomal RNA gene (16S rRNA). These, and similar signature nucleotide sequences in the 16S rRNA of the *Azospira* species and the *Dechlorospirillum* species in the  $\alpha$ -Proteobacteria, have allowed the development of specific molecular probes for the rapid screening of environmental samples for the presence of these microorganisms.

*Magnetospirillum* and *Wolinella*)<sup>20,41</sup> that were not recognized for their ability to respire (per)chlorate. However, most of the known (per)chlorate-reducing bacteria are closely related to each other and to the bacterial species *Rhodocyclus tenuis* and *Ferribacterium limneticum* in the  $\beta$ -subclass (FIG. 2). In general, the known close relatives of the (per)chlorate-reducing isolates do not grow by (per)chlorate respiration, regardless of the similarity of their 16S rDNA sequence, thereby making predictions of metabolic functionality based on phylogenetic relatedness futile<sup>58</sup>. For example, *R. tenuis* is a phototrophic non-sulphur purple bacterium that contains bacteriochlorophyll and is found on soil surfaces and in shallow waters that are exposed to sunlight, whereas *F. limneticum* is an obligate anaerobic, non-fermenting, dissimilatory Fe(III) reducer<sup>59</sup>. Although the (per)chlorate-reducing bacteria are closely related to these microorganisms — often with a 16S rDNA sequence divergence of less than 1%<sup>41,48,57,58</sup> — they exhibit distinct physiologies. None of the (per)chlorate-reducing isolates can grow by phototrophy or Fe(III)-reduction. By the same token, *F. limneticum* does not grow by phototrophy or by the reduction of (per)chlorate, and

*R. tenuis* cannot grow by anaerobic respiration with a broad range of electron acceptors, including perchlorate or Fe(III)<sup>42</sup>.

The (per)chlorate-reducing species in the  $\beta$ -subclass of the Proteobacteria represent two novel genera with monophyletic origin — the *Dechloromonas* and *Azospira* (formerly *Dechlorosoma* (BOX 1)) species<sup>57,60</sup> (FIG. 2). The genus *Dechloromonas* can be further subdivided into the RCB-type and CKB-type on the basis of signature nucleotide sequences in the 16S rRNA gene sequence (FIG. 2). Members of both the *Dechloromonas* and *Azospira* genera are ubiquitous<sup>41</sup> and have been identified and isolated from nearly all environments that have been screened, including pristine and contaminated field samples<sup>41,61–63</sup>, and even in soil and lake samples collected from Antarctica<sup>62</sup>. As such, these two groups are considered to represent the dominant (per)chlorate-reducing bacteria in the environment<sup>41,57,64</sup>. A third group — the *Dechlorospirillum* species — which are closely related to the MAGNETOTACTIC *Magnetospirillum* species in the  $\alpha$ -subgroup of the Proteobacteria, are also commonly found in contaminated soils and bioreactors that are used to treat groundwater contaminated with perchlorate (J.D.C.,

MAGNETOTACTIC  
Magnetotactic bacteria are motile, mostly aquatic prokaryotes that can swim along geomagnetic field lines.

Box 1 | **Dechlorosoma versus Azospira**

Recently, the genera *Azoarcus*, *Azospira*, *Azovibrio* and *Azonexus* were identified. These genera form phylogenetically distinct branches within the *Rhodocyclus* cluster in the  $\beta$ -subclass of the Proteobacteria<sup>94</sup>. Phylogenetic analysis indicated that *Dechlorosoma suillum*, the type strain of the formerly identified *Dechlorosoma* genus, and *Azospira oryzae* shared 99.9% 16S rRNA gene sequence identity<sup>60</sup>, indicating that these microorganisms belong to the same genus. Furthermore, DNA–DNA hybridization studies showed more than 90% homology between *D. suillum* and two strains of *A. oryzae*, and whole-cell SDS–PAGE profiles of SDS-soluble proteins of strains *D. suillum* and *A. oryzae* were almost identical<sup>60</sup>. Phenotypic comparison also indicated very similar metabolic capabilities, except for the lack of growth of *A. oryzae* with perchlorate as the terminal electron acceptor, the feature by which *D. suillum* was isolated<sup>32,57</sup>. As such, *D. suillum* was recently renamed *Azospira suillum*.

The *Azospira* genus consists of nitrogen-fixing bacteria that occur as endophytes of grass roots, especially rice<sup>95</sup>, and in association with fungi<sup>94</sup>. The presence of a *nifH* gene and nitrogenase activity, a key feature of *Azospira*, were also detected in *A. suillum* by Southern hybridization and by the acetylene-reduction assay, respectively, indicating that this microorganism can also fix nitrogen<sup>60</sup>. Whether *A. suillum* can also grow as an endophyte is unknown; however, such a capability is tantalizing to consider in light of a possible role in the reduction of perchlorate in plants in which perchlorate is actively taken up from the environment and transported by transpiration to the leaf surface. Further investigation of this possibility could offer insight into the mechanistic application of phytoremediation to perchlorate-contaminated soils and surface waters.

## MAGNETOSOMES

Intracellular structures in magnetotactic bacteria that contain magnetic mineral crystals.

unpublished observations). The *Magnetospirillum* genus has been described on the basis of its ability to form MAGNETOSOMES — an intracellular form of magnetite — when grown microaerophilically on iron-based media, which confers a unique magnetotactic characteristic on these microorganisms. The best described of the *Dechlorospirillum* species is *Dechlorospirillum anomalous* strain WD, which shows almost 97% 16S rDNA sequence identity to *Magnetospirillum gryphiswaldense*<sup>43</sup>. Like *M. gryphiswaldense*, *D. anomalous* strain WD is a microaerophile; however, in contrast to *M. gryphiswaldense*, *D. anomalous* does

not produce magnetosomes<sup>43</sup>. Interestingly, a recent genome-screening study<sup>65</sup> revealed that the closely related magnetotactic species *Magnetospirillum magnetotacticum* did contain a gene encoding chlorite dismutase — an enzyme that is unique to (per)chlorate-reducing bacteria; however, phenotypic studies indicated that this microorganism could not reduce (per)chlorate in growth cultures<sup>43</sup> and did not dismutate chlorite (J.D.C., unpublished observations).

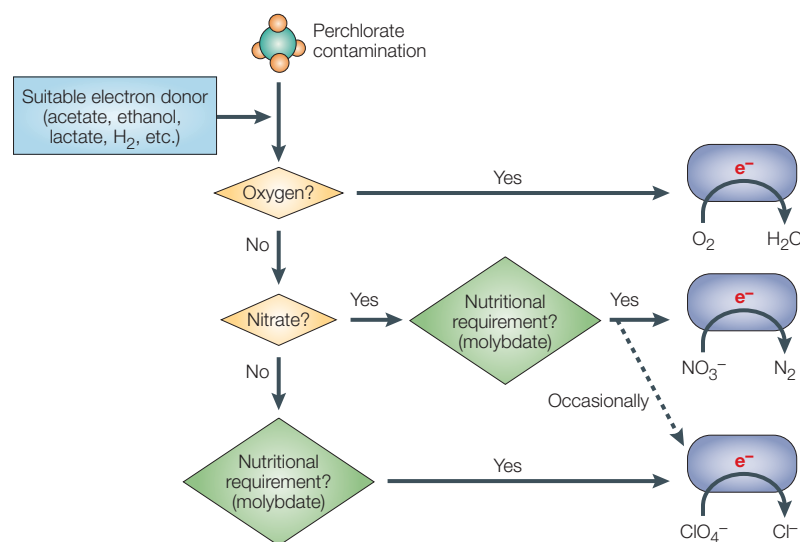
**The controlling parameters**

Pure-culture studies have demonstrated that members of the *Dechloromonas* and *Azospira* genera can grow in a broad range of environmental conditions. These microorganisms are generally not nutritionally fastidious, although molybdenum is a required trace element for perchlorate reduction<sup>66</sup>. Although most pure-culture studies have been performed in media that is supplemented with a defined or undefined vitamin source, it has been demonstrated for at least one microorganism, *Dechloromonas agitata*, that vitamin supplementation was unnecessary for growth in minimal media<sup>42</sup>. The *Dechloromonas* and *Azospira* species generally grow optimally at pH values near neutrality in freshwater environments<sup>41–43</sup>. Even so, recent field studies have shown that related deep-branching members of these genera often predominate in sites of adverse pH or salinity, with some species being capable of growth and perchlorate respiration at pH values as low as pH 5 (REF. 67). Although studies have demonstrated microbial perchlorate reduction in salt brines that are as concentrated as 11% NaCl (REF. 68), so far no isolated microorganism has been shown to grow by perchlorate respiration in salinities greater than 2%. One putative perchlorate reducer, *Citrobacter* sp., has been shown to partially reduce perchlorate in salt concentrations as high as 7.5%; however, neither growth coupled to perchlorate reduction nor complete reduction of perchlorate to chloride was demonstrated for the microorganism<sup>51</sup>.

By contrast, one chlorate-reducing microorganism, *Dechloromonas chlorophilus*, isolated from a marine sediment, can grow and reduce chlorate in salinities greater than 5% (REF. 69). This microorganism, however, does not reduce perchlorate<sup>69</sup>.

A recent investigation of the environmental factors that control reduction of perchlorate by the dissimilatory perchlorate-reducer *Azospira suillum* showed that perchlorate reduction only occurred under anaerobic conditions in the presence of perchlorate<sup>66</sup> (FIG. 3). Perchlorate reduction was also dependent on the presence of the genetically regulated enzyme chlorite dismutase. Anaerobic conditions alone were not enough to induce expression of an active form of this enzyme, and activity was only observed under anaerobic conditions in the presence of perchlorate. Dissolved oxygen concentrations less than 2 mg L<sup>-1</sup> were enough to inhibit perchlorate reduction by *A. suillum*<sup>66</sup>.

Similarly to oxygen, nitrate also negatively regulates the production of active chlorite dismutase and inhibits perchlorate reduction by *A. suillum*<sup>66</sup>. If present, nitrate was used preferentially, even if the cultures had previously



**Figure 3 | Environmental factors affecting the activity of perchlorate-reducing bacteria.** The activity of perchlorate-reducing bacteria is affected by several environmental factors. Microbial perchlorate reduction is dependent on the presence of biologically available molybdenum, and is inhibited by oxygen and, depending on the perchlorate-reducing microorganism, nitrate.

Box 2 | **Difference spectra for the detection of cytochromes**

Type-*c* cytochromes are common redox active components that are involved in the transfer of electrons in the respiratory electron-transport chain of many organisms. These compounds absorb light differently in the oxidized and reduced state. Subtraction of the absorbance spectrum of the oxidized type-*c* cytochromes from that of the reduced type-*c* cytochromes gives characteristic absorbance maxima at 425, 525 and 552 nm. Difference spectra can similarly be performed on whole cells that have been either exposed to O<sub>2</sub> (oxidized) or degassed with H<sub>2</sub> (reduced). Under anaerobic conditions, reoxidation of the H<sub>2</sub>-reduced type-*c* cytochromes of washed whole cells in the presence of physiological electron acceptors such as perchlorate is diagnostic of a role for these cytochromes in the transfer of electrons to this electron acceptor.

been grown on perchlorate. However, this is not predictable and some notable exceptions do exist<sup>50,66</sup>. For example, nitrate had no significant inhibitory effect on perchlorate reduction by the perchlorate reducer *D. agitata* strain CKB. *D. agitata* is the only perchlorate reducer described that is incapable of growth by dissimilatory nitrate reduction<sup>42,64</sup>. Interestingly, although *D. agitata* does not grow by nitrate reduction, during perchlorate reduction the nitrate in the culture medium was concomitantly reduced to nitrite, which accumulated in the culture broth, indicating that the nitrate is co-reduced by the perchlorate reductase<sup>66</sup>. Previous studies performed on the DPRB strain *perc*lace also indicated that nitrate was concomitantly reduced with perchlorate by this DPRB<sup>50</sup>. However, in contrast to *D. agitata*, strain *perc*lace could grow by nitrate reduction and nitrite did not accumulate in the culture broth<sup>50</sup>.

**The biochemistry**

Although there is still relatively little known about the biochemistry of (per)chlorate reduction, some recent studies have yielded important information. Initial investigations have demonstrated the presence of *c*-type cytochrome(s) in (per)chlorate-reducing bacteria and their involvement in the reduction of (per)chlorate<sup>41,42</sup>. Difference-spectra studies (BOX 2) revealed that the H<sub>2</sub>-reduced *c*-type cytochrome content of different DPRB was readily reoxidized in the presence of chlorate

or perchlorate but was unaffected by non-physiological electron acceptors (such as sulphate, fumarate or Fe(III)) for these microorganisms, indicating their specific involvement in the transfer of electrons to (per)chlorate<sup>41</sup>.

More recently, a single oxygen-sensitive perchlorate reductase enzyme was purified and partially characterized from a (per)chlorate reducing strain, strain GR-1 (REF. 70). This enzyme is located in the periplasm of the microorganism, is a heterodimer in an α<sub>3</sub>β<sub>3</sub> configuration, has a total molecular mass of 420 kDa and contains iron, molybdenum and selenium<sup>70</sup>. In addition to perchlorate, this perchlorate reductase also catalyses the reduction of chlorate, nitrate, iodate and bromate<sup>70</sup>. Perchlorate and chlorate are reduced to chlorite. Subsequent phenotypic studies showed that although selenium can be replaced with alternative cations for effective perchlorate reduction by DPRB (J.D.C., unpublished observations), molybdenum has a prerequisite functional role in the reduction of perchlorate<sup>66</sup>.

The quantitative dismutation of chlorite into chloride and O<sub>2</sub> is now known to be a central step in the reductive pathway of (per)chlorate that is common to all (per)chlorate-reducing bacteria<sup>41</sup> (FIG. 4). Chlorite dismutation by DPRB is mediated by a highly conserved chlorite dismutase<sup>41,71–73</sup>. Studies with washed whole-cell suspensions demonstrated that the chlorite dismutase is highly specific for chlorite and none of a broad range of alternative analogous anions tested served as substrates for dismutation<sup>42</sup>. The purified chlorite dismutase from *D. agitata* strain CKB is a homotetramer with a molecular mass of 120 kDa and a specific activity of 1,928 μmol chlorite dismutated per mg of protein per minute<sup>41</sup>. This is similar to the molecular mass and specific activity observed for the chlorite dismutase that was previously purified from DPRB strain GR-1 (REF. 71) and subsequently from *Ideonella dechloratans*<sup>72</sup>. Studies with an immunoprobe that is specific for chlorite dismutase indicated that chlorite dismutase is present on the outer membrane of all DPRB and is highly conserved among these microorganisms, regardless of their phylogenetic affiliation<sup>73</sup>.

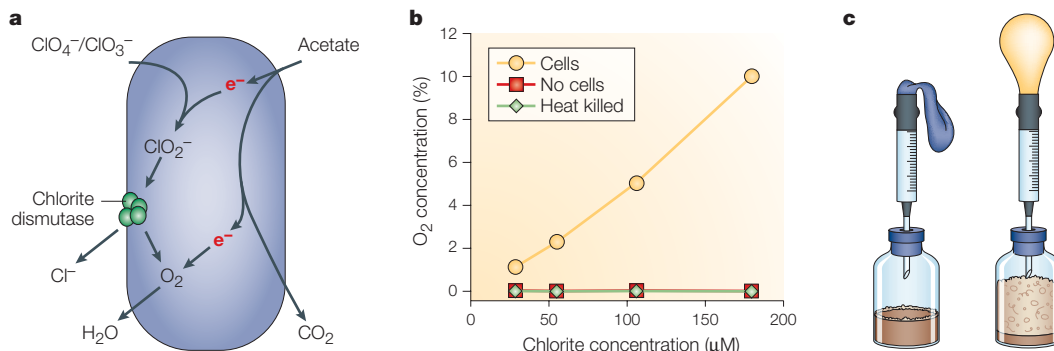
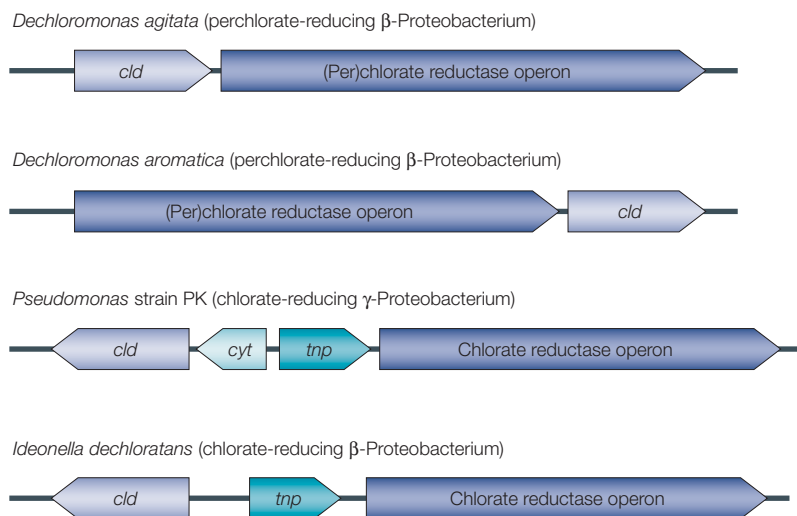


Figure 4 | **Microbial chlorite dismutase.** Chlorite dismutase is a key enzyme involved in the reduction of (per)chlorate and mediates the rapid dismutation of chlorite (ClO<sub>2</sub><sup>-</sup>) into chloride (Cl<sup>-</sup>) and molecular oxygen (a). Through the activity of this enzyme, washed whole-cell suspensions of (per)chlorate-reducing bacteria rapidly and quantitatively evolve oxygen on the addition of chlorite (b), producing frothing of the culture and enough O<sub>2</sub> from 10 mmol chlorite to partially inflate a balloon (c). This enzyme is a homotetramer and is highly conserved at the amino-acid and nucleotide level amongst the phylogenetically diverse (per)chlorate-reducing bacteria.



**Figure 5 | Genomic organization of the chlorite dismutase gene *cld*, the perchlorate reductase operon and the chlorate reductase operon in selected isolates.** A gene encoding a c-type cytochrome (*cyt*) lies between *cld* and a transposase gene (*tnp*) in the *Pseudomonas* strain PK. A different transposase gene is located upstream of *cld* in *Ideonella dechloratans*. The arrows indicate gene location and the direction of transcription.

### The genetics

The chlorite dismutase gene was recently isolated and characterized from both a (per)chlorate-reducing microorganism, *D. agitata* strain CKB<sup>65</sup>, and a chlorate-reducing microorganism, *I. dechloratans*<sup>74</sup>. In the case of *D. agitata*, sequence analysis identified an open-reading frame of 834 base pairs that encodes a mature protein with an amino-terminal sequence that is identical to that of the chlorite dismutase protein previously purified from *D. agitata*<sup>65</sup>. The predicted protein product of the *D. agitata* chlorite dismutase gene comprises 277 amino acids, including a leader peptide of 26 amino acids that targets the protein to the cell membrane. Northern blot analysis with purified RNA indicated that the chlorite dismutase gene is basally expressed under aerobic conditions but, as indicated by the previous physiological observations, its transcription is upregulated when the cells are grown under perchlorate-reducing conditions. By contrast, chlorite dismutase expression is constitutive in the chlorate-reducing microorganisms *Pseudomonas* strain PDA<sup>75</sup> and *Pseudomonas* strain PK (J.D.C. and L.A.A., unpublished observations). SLOT-BLOT hybridization studies demonstrated the high degree of sequence conservation of this gene among the (per)chlorate-reducing bacteria and no protein in the available databases showed >24% homology, emphasizing the unique nature of this metabolic protein<sup>65</sup>.

Directly downstream of the *D. agitata* chlorite dismutase gene, another open-reading frame containing molybdopterin domains and encoding the  $\alpha$ -subunit of perchlorate reductase showed significant sequence similarities at the amino-acid level with subunits of known redox enzymes, such as nitrate reductase and selenate reductase<sup>76</sup>. Although regulated separately from the chlorite dismutase gene, expression of the

perchlorate reductase gene was also induced under anaerobic conditions in the presence of either chlorate or perchlorate<sup>76</sup>. In contrast to the chlorite dismutase gene, expression of the perchlorate reductase gene was completely inhibited if either nitrate or oxygen were present<sup>76</sup>. Recent studies have indicated that this gene is conserved in all DPRB, but the gene was absent or altered beyond recognition and detection in those strains capable of reducing only chlorate and not perchlorate, indicating that a different enzyme is involved in reductive chlorate respiration<sup>76</sup>. Purification and analysis of the chlorate reductase from the chlorate-reducing microorganisms *I. dechloratans*<sup>46</sup> and *Pseudomonas chloritidismutans*<sup>77</sup> revealed that, unlike perchlorate reductase, this enzyme is a heterotrimer ( $\alpha_1\beta_1\gamma_1$ ) and was able to use chlorate but not perchlorate as a substrate<sup>78,79</sup>. Identification and characterization of the genes encoding this enzyme from *I. dechloratans* and the *Pseudomonas* strain PK revealed a gene order of *chrABDC* ( $\alpha$ -subunit,  $\beta$ -subunit, chaperone protein and  $\gamma$ -subunit)<sup>76,78</sup>. This differs from the gene order for perchlorate reductase genes, which are organized *pcrABCD* ( $\alpha$ -subunit,  $\beta$ -subunit,  $\gamma$ -subunit and chaperone protein)<sup>76</sup>. The significance of the differences of the perchlorate reductase and chlorate reductase gene order is unknown. Whereas the *pcrABCD* arrangement matches the gene order of the closely related ethylbenzene dehydrogenase, the *chrABDC* arrangement is more common to the selenate reductase and dimethyl sulphide dehydrogenase DMSO enzymes.

### An insight into the past

Analysis of several DPRB indicates that not only are (per)chlorate-reducing bacteria phenotypically and phylogenetically diverse, but they are also diverse on the genomic level. The organization and transcriptional direction of the genes involved in (per)chlorate reduction differ from strain to strain independently of phylogenetic similarity, with the chlorite dismutase gene *cld* being the most capricious at the organizational level (FIG. 5). For example, the organization of the genes from the closely related DPRB *D. agitata* and *D. aromatica* is identical except for the location of the *cld* gene. In *D. agitata*, the *cld* gene is located upstream and transcribed in the same direction as the (per)chlorate reductase genes, whereas in *D. aromatica*, the *cld* gene is downstream of the (per)chlorate reductase genes (FIG. 5). Similarly, the *cld* gene in the chlorate reducers *Pseudomonas* strain PK and *I. dechloratans* is located upstream of the chlorate reductase genes. However, the *cld* gene seems to be transcribed in the opposite direction to the chlorate reductase. Unique to these two chlorate reducers is the presence of a transposase gene that is downstream of the *cld* gene. Both transposases are transcribed in the opposite direction from the *cld* gene and possess little sequence identity. As the *cld* gene is monocistronic and, as such, controlled by a separate promoter region, the organization and transcriptional direction of the *cld* gene does not seem to depend on either the location or direction of the chlorate reductase genes. The

#### SLOT-BLOT

A technique that is used to determine whether an organism possesses a specific gene of interest. Genomic DNA is extracted and transferred to a solid membrane with a slot-array apparatus. After denaturation of the DNA, the membrane is hybridized with a labelled probe that targets a specific DNA sequence.

varied arrangements of the genes involved in perchlorate and chlorate reduction could be due to separate pathway evolution or could be an artefact of horizontal gene transfer.

Recently, a comparison of a phylogenetic tree based on 16S rDNA sequence analysis with a tree developed from the *cld* gene sequence from 11 diverse DPRB was found to support evolution through horizontal gene transfer<sup>62</sup>. Although, as expected, DPRB representatives of the  $\alpha$ ,  $\beta$  and  $\gamma$  subclasses of the Proteobacteria formed distinct monophyletic units on the 16S rDNA tree, this was not the case for the *cld* gene tree<sup>62</sup>. For example, the *cld* genes from the  $\gamma$ -Proteobacteria *Dechloromonas* strain NSS and *Pseudomonas* strain PK are most closely related to the *cld* genes from the  $\beta$ -Proteobacteria *D. aromatica*, *A. suillum* and strain CR. In addition, the *cld* genes from two members of the same genus, *D. aromatica* and *D. agitata*, are distinct from one another and reside in different CLADES on the *cld* gene tree. The incongruent tree topologies generated from the 16S rRNA and chlorite dismutase gene sequence data sets suggest a role for horizontal gene transfer in the evolution of the chlorite dismutase gene.

#### The toolbox

As outlined above, in the past few years phenotypic characterization studies have demonstrated that the known (per)chlorate-reducing bacteria exhibit a broad range of metabolic capabilities and can thrive in adverse environments<sup>64,67</sup>. Similarly, significant advances have been made in the biochemistry and genetic systems that are involved in microbial (per)chlorate reduction and the environmental factors that affect their activity<sup>64–66,76</sup>. As such, the applicability of this metabolism offers great potential for the bioremediation of perchlorate-contaminated environments. Several tools based on unique signature molecules that are characteristic of DPRB and novel metabolic capabilities are now available to monitor the effectiveness of a bioremediative strategy in field environments<sup>65,67,73,76,80</sup>.

**Probes to the 16S rRNA gene.** It has long been recognized that comparison of the gene sequence that encodes the small ribosomal RNA subunit (16S rRNA) can be used to measure the relationship between any two microorganisms<sup>81</sup>. From this information, certain limited conclusions can be drawn regarding the metabolic capabilities of unknown microorganisms<sup>82</sup>. However, such genetic comparisons cannot be used to identify the metabolic capability of a microorganism categorically, particularly (per)chlorate reduction, owing to the high 16S rRNA gene sequence similarity between many of the known DPRB and their closest non-(per)chlorate-reducing relatives<sup>82</sup>. Even so, molecular probes specific to the 16S rRNA genes of the *Dechloromonas* (both CKB- and RCB-type), *Azospira* (PS-type) and *Dechlorospirillum* (WD-type) (FIG. 2) genera have been designed (TABLE 1) and proven to be useful in the rapid pre-screening of environmental samples for the presence of these bacteria<sup>67</sup> or to monitor the health

of a known (per)chlorate-reducing population in soils or bioreactors<sup>61</sup>. When used in conjunction with enumeration techniques for DPRB, such as MOST PROBABLE NUMBER counts (MPNs) or real-time PCR, specific 16S rDNA molecular probes can be used to monitor population shifts in response to particular stimuli that are introduced as part of a bioremediative process from which the effectiveness of the strategy can be inferred. A recent study that used this approach to investigate the perchlorate-reducing population associated with an active permeable barrier treating perchlorate- and radionuclide-contaminated surface waters in Los Alamos, New Mexico, indicated that the perchlorate-reducing population was dominated by species of the *Dechloromonas* RCB-type and that the relative size of this population responded directly to perchlorate concentrations and water volume treated within a 6-month period (J.D.C., unpublished observations).

**Chlorite dismutase gene primer sets.** The identification of several genes involved in the reduction of perchlorate and chlorate now makes it possible to use several different molecular approaches to assist bioremediation efforts. For example, because chlorite dismutase is a highly conserved enzyme unique to organisms capable of (per)chlorate reduction<sup>41,73</sup>, the gene encoding this protein is an ideal target for detecting the presence of any (per)chlorate-reducing bacteria in the environment, regardless of their phylogenetic affiliation<sup>62,65</sup>. Using this approach, detection of the chlorite dismutase gene from an environmental sample can be accomplished in a short time using specific molecular probes and can be used to determine whether the indigenous bacterial population is capable of (per)chlorate reduction<sup>62</sup>. One potential error in this method is the fact that the chlorite dismutase gene might be present in microorganisms that are not capable of (per)chlorate reduction, which would result in false-positives. This has already been demonstrated for the non-(per)chlorate-reducing microorganism *Magnetospirillum magnetotacticum*<sup>65</sup> and might also occur for other, as-yet-unidentified microorganisms. However, it is unlikely that such microbial species would be dominant in an environment contaminated with perchlorate. To account for such false-positives, a combined approach incorporating the detection of chlorite dismutase genes with hybridization analysis using a gene encoding one of the subunits of perchlorate reductase allows definitive identification of microorganisms that are capable of (per)chlorate reduction.

This approach has already been successfully used to identify the presence of (per)chlorate-reducing microbial populations in soils and groundwaters collected from several diverse environments, including perchlorate-contaminated soils and ground waters collected in Los Alamos, New Mexico, and pristine sediments and lake waters collected from Antarctica<sup>62</sup>.

In addition, by extending the use of these primer sets to environmental RNA samples through reverse transcriptase PCR, it is also possible to determine if

#### CLADE

A group of organisms consisting of a single species and its descendants.

#### MOST PROBABLE NUMBER

A method that uses dilution cultures to determine the approximate number of viable cells. It is useful when samples contain too few organisms for agar plates to be used or when organisms will not grow on agar.

Table 1 | Specific 16S rDNA primers used to detect dissimilatory perchlorate-reducing bacteria

Target organism	Primer name	Primer sequence	Size of PCR product
<i>Dechloromonas agitata</i> CKB-type	CKB.495F CKB.850R	5'-CCGGGAAGAAAATCGCATCAGC-3' 5'-ACCCAACACCTAGTTGACATC-3'	397 bp
<i>Dechloromonas aromatica</i> RCB-type	RCB.495F CKB.850R	5'-CCGGGAAGAAAACWCGCATGGGT-3' 5'-ACCCAACACCTAGTTGACATC-3'	397 bp
<i>Azospira suillum</i> PS-type	PS.681F PS.1035R	5'-GAACTGCGTTTTGTGACTGCGA-3' 5'-CCATCTCTGGAAAGTTCCCTGG-3'	396 bp
<i>Dechlorospirillum</i> <i>anomalous</i> WD-type	WD.1038F WD.1260R	5'-ATCCTTCACTTCGGGTGGGT-3' 5'-TAGCTAACTCTCGCGAGCTC-3'	262 bp

active (per)chlorate reduction is taking place in a particular environment. Although this method can be interfered with by basal level expression of the chlorite dismutase in most perchlorate-reducing bacteria, its expression in the population is significantly upregulated by the presence of perchlorate, which results in an easily measurable signal<sup>65</sup>. Alternatively, interference can arise from constitutive chlorite dismutase expression, such as that observed in the chlorate reducers *Pseudomonas* strain PDA<sup>75</sup> and *Pseudomonas* strain PK (J.D.C. and L.A.A., unpublished observations). However, these microorganisms are unlikely to represent a significant population in environments contaminated with perchlorate.

This technique is currently being used to assess the efficacy of various approaches to perchlorate bioremediation in the US Army Ammunition Plant, Longhorn, Texas (J.D.C. and L.A.A., unpublished observations). Other applications include profiling the metabolic activity of DPRB in a bioreactor, and analysing the effect of changing environmental parameters on (per)chlorate reduction *in situ*. Furthermore, by combining the chlorite dismutase gene-detection methods with hybridization analysis using a gene encoding one of the subunits of perchlorate reductase, it is now possible to distinguish at the molecular level those microorganisms that are capable of (per)chlorate reduction from those that are only able to reduce chlorate.

**Chlorite-dismutase-specific immunoprobe.** An alternative probe for (per)chlorate-reducing bacteria was recently developed based on the ability of antibodies to target and attach to specific antigenic structures in a compound. Owing to the highly conserved nature of the chlorite dismutase enzyme at the amino-acid level amongst all DPRB regardless of their phylogenetic affiliation<sup>41,65,73</sup> and the uniqueness of this enzyme to these microorganisms<sup>65</sup>, the chlorite dismutase protein represents an ideal target for a DPRB-specific immunoprobe. In addition, this probe is unaffected by non-(per)chlorate-reducing microorganisms, such as *M. magnetotacticum*, which carry the *clt* gene but do not produce an active chlorite dismutase enzyme.

A recent study demonstrated the effectiveness of this approach by raising polyclonal antisera against the purified chlorite dismutase from *D. agitata* strain CKB<sup>73</sup>. Characterization studies indicated that the antisera had a high affinity for the chlorite dismutase enzyme and activity was observed in dilutions as low

as  $1 \times 10^{-6}$  of the original antisera. The antisera was active against both cell lysates and whole cells of all DPRB tested, regardless of phylogenetic affiliation, but only if the cells were grown on (per)chlorate. Little or no cross-reactivity was observed with closely related non-(per)chlorate-reducing relatives<sup>73</sup>. With this immunoprobe as a basis, a rapid enzyme-linked immunosorbent assay (ELISA) was developed that is specific for DPRB that are actively metabolizing (per)chlorate, and cell populations as low as 100 DPRB cells  $\text{ml}^{-1}$  can readily be detected in aqueous samples colorimetrically (J.D.C., unpublished observations). This assay allows the rapid screening of environmental samples for actively metabolizing (per)chlorate-reducing bacteria.

**Stable isotope analysis.** Although both molecular and immunological tools based on unique signature molecules are now available to monitor the microbial populations associated with perchlorate reduction in the environment<sup>65,73,76</sup>, monitoring the effectiveness of a bioremediative strategy in field environments is often difficult owing to the complex nature of environmental samples. The results can often be tainted by many abiotic factors, including adsorption, dilution or chemical reactivity of the target contaminant. One potential strategy for overcoming these shortcomings is to follow the changes in stable isotope composition of the molecule of interest. The stable isotopic signature of a molecule can also be used as a means of fingerprinting and locating the source of a compound. Variations of the stable isotope ratios of many elements have been used for a long time to give valuable information about elemental sources and biogeochemical processes occurring in the environment<sup>83-85</sup>. Many atoms can exist in two or more forms that are chemically identical but which differ in mass. The relative abundances of the stable (non-radioactive) isotopes are effectively constant for each element. Chlorine has two stable isotopes — <sup>35</sup>Cl and <sup>37</sup>Cl — with a natural abundance of approximately 75% and 25%, respectively. There are relatively few examples of natural physical or chemical fractionation processes for chlorine, although some do exist. The largest fractionation effect is likely to be attributable to aqueous diffusion of dissolved chloride in marine pore waters in low-permeability rocks (originally ~0‰, parts per thousand), which results in relative depletion of <sup>37</sup>Cl from the brines and an isotopic ratio of ~-0.9‰ at the diffusion front (BOX 3). By contrast,

Box 3 | **Stable isotope calculations**

Stable isotope ratios are normally reported in  $\delta$  notation, giving the difference in measurements between a standard and the sample in question. Positive  $\delta$  values are obtained when the sample contains more of the heavy isotope than the standard, negative  $\delta$  values are obtained when the sample has less of the heavy isotopes than the standard. Stable isotope ratios follow a Rayleigh fractionation process described by:

$$\delta^{37}\text{Cl}^- = \left[ \frac{R_{\text{sample}}}{R_{\text{standard}}} \right] \times 1000$$

Where, for perchlorate fractionation:

$R_{\text{sample}}$  is the ratio of the  $^{37}\text{Cl}^-$  to  $^{35}\text{Cl}^-$  in the perchlorate content of the sample

$R_{\text{standard}}$  is the ratio of the  $^{37}\text{Cl}^-$  to  $^{35}\text{Cl}^-$  in Standard Mean Ocean Chloride

$\delta$  values are denoted ‰ (parts per thousand)

significantly larger changes can result from chemical manufacturing processes by which, for example, chlorinated hydrocarbon solvents produced from natural sodium chloride (~0‰) can show a range of isotopic signature values from -3‰ to +4‰ (REF. 86), depending on the processes used. As such, the stable isotopic content of anthropogenic perchlorate will be dependent on the original source of chloride in the perchlorate and the manufacturing process used and might be distinguishable from that of naturally occurring perchlorate.

Microbial processes are known to make small but significant changes to isotopic compositions of many elements, such as carbon or sulphur, by preferentially using the lighter isotope<sup>87-92</sup>. Similarly, it was recently

shown that the perchlorate-reducing bacterium *A. suillum* preferentially uses perchlorate containing the lighter isotope ( $^{35}\text{Cl}$ ), resulting in a significant fractionation (-15‰) of the isotopic content of the perchlorate as the organism grows in pure culture<sup>80</sup> (FIG. 6). A subsequent study demonstrated similar isotopic fractionation of the chlorine content of perchlorate when DPRB were grown in natural sediments<sup>93</sup>. The results of these studies indicate that isotope-signature tracing can be successfully applied to monitor the microbial reduction and removal of perchlorate in environmental samples that are being treated for perchlorate contamination. Currently, ongoing field studies are evaluating the applicability of this technology (J.D.C. and M. L. Coleman, unpublished observations).

**Genomics and the future**

The field of microbial perchlorate reduction has clearly advanced significantly in a very short period from a poorly understood metabolism to a burgeoning scientific field of discovery. As outlined above, there is now a much greater appreciation of the microbiology, biochemistry and genetic systems involved and this knowledge is being applied to the successful treatment of contaminated environments. Overall, the future is promising; however, this field is still in its infancy. Nothing is known of the evolutionary timeline of this metabolism. From a biogeochemical perspective, a better understanding of how perchlorate is formed in the natural environment and what geochemical conditions are required for its formation might give some insight into this. From a microbial perspective, it will be important to look for this metabolism in more extreme environments, such as hypersaline or hyperthermophilic environments, to obtain DPRB isolates across a broader phylogeny to establish a broad-base molecular chronometer.

The recent completion of the first draft genome sequence of a (per)chlorate-reducing organism, *D. aromatica* strain RCB, by the Joint Genome Institute, USA, offers exciting new avenues of research (see *Dechloromonas* draft genome sequence in the online links box). In addition to its ability to reduce (per)chlorate, *D. aromatica* possesses several other unique metabolic capabilities that have direct application to the bioremediative treatment of several contaminants, including non-aromatic hydrocarbons, heavy metals and radionuclides. The availability of this genome sequence has already allowed the development of genome chips containing the entire genome of this organism (L.A.A. and J.D.C., unpublished observations). Such microarrays are being used to obtain data on the environmental factors that affect the activity of DPRB and how these microorganisms function together with other members of a microbial community during the treatment of a contaminated site. Furthermore, continual genome annotation will allow the investigation of protein-protein interactions and substrate channelling during (per)chlorate reduction and also protein-DNA interactions, which will give a deeper insight into the regulatory mechanisms involved.

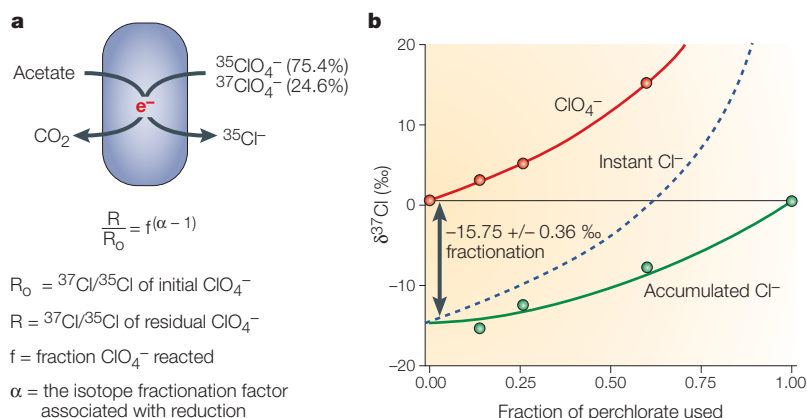


Figure 6 | **Stable isotope fractionation.** Perchlorate-reducing bacteria can distinguish between the chlorine isotope content of perchlorate in their environment and will preferentially use the perchlorate containing the lighter isotope of chlorine. This process is known as stable isotope fractionation and is calculated from the ratio of the chlorine isotopic signature of the residual perchlorate ( $R$ ) to the chlorine isotopic signature of the initial perchlorate ( $R_0$ ) as perchlorate reduction occurs (**a**). **b** | Chlorine isotope compositions of chloride and residual perchlorate during microbial reduction by *A. suillum*. Instant  $\text{Cl}^-$  indicates the calculated isotopic composition of chloride produced from the remaining perchlorate, as though unmixed with previously produced chloride. ‰, parts per thousand. Panel **b** is reproduced with permission from REF. 80 © (2003) American Society for Microbiology.

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#### Competing interests statement

The authors declare that they have no competing financial interests.

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