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Brief Report

An uncharacterized clade in the DMSO reductase family of molybdenum oxidoreductases is a new type of chlorate reductase

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Summary

The dimethylsulfoxide (DMSO) reductase family of enzymes has many subfamilies catalysing unique biogeochemical reactions. It also has many uncharacterized subfamilies. Comparative genomics predicted one such subfamily to participate in a key step of the chlorine cycle because of a conserved genetic association with chlorite dismutase, implying they produce chlorite through chlorate or perchlorate reduction. We determined the activity of the uncharacterized enzyme by comparing strains in the phototrophic genus *Rhodoplanes* that encode either a typical perchlorate reductase or the uncharacterized enzyme. *Rpl. piscinae* and *Rpl. elegans*, which encode perchlorate reductase, grew by using perchlorate as an electron acceptor. In contrast, *Rpl. roseus*, which encodes the uncharacterized enzyme, grew by chlorate reduction but not by perchlorate reduction. This is the first report of perchlorate and chlorate being used as respiratory electron acceptors by phototrophs. When both chlorate and perchlorate were present, *Rpl. roseus* consumed only chlorate. Highly concentrated *Rpl. roseus* cells showed some perchlorate consumption, but chlorate consumption occurred at a 10-fold higher rate. Together, these genomic and physiological data define a new group of chlorate reductases. Some organisms encode both this chlorate reductase and a perchlorate reductase, raising new questions about the physiology and evolution of chlorine oxyanion respiration.

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Report

The dimethylsulfoxide reductase family consists of enzymes with a bis-molybdopterin guanine dinucleotide cofactor, many of which catalyse key reactions in biogeochemical cycles, including the reduction of dimethylsulfoxide, arsenate, nitrate, selenate, polysulfide, thiosulfate, perchlorate, and chlorate and the oxidation of dimethyl sulfide, arsenite, nitrite and formate (Leimkühler and Iobbi-Nivol, 2015). Several subfamilies within this superfamily have unknown activity, including several clades in the functionally diverse type II DMSO reductase family enzymes (Kitzinger *et al*., 2018). Bacteria that reduce perchlorate (ClO_4^-) or chlorate (ClO_3^-) in anaerobic respiration use enzymes from that group: a perchlorate reductase (Pcr) that reduces perchlorate to chlorate and chlorate to chlorite, or a chlorate reductase (Clr) that reduces only chlorate to chlorite (Youngblut *et al*., 2016. The product of Pcr and Clr, the reactive oxidant chlorite (ClO_2^-), is detoxified into chloride and oxygen by a heme-containing chlorite dismutase (Cld) (Rikken *et al*., 1996).

A distant homologue of Pcr and Clr was recently identified to be adjacent to the gene for Cld in several genomes (Barnum *et al*., 2018). This homologue, most closely related to periplasmic Nar nitrate reductase, was always associated with *cld* and formed a monophyletic clade, suggesting a specialized activity producing chlorite. We hypothesized that this enzyme was the second type of perchlorate reductase, or group 2 Pcr, based on the conservation of most active site residues with Pcr and the growth of a bacterium encoding no other perchlorate reductase in enrichments provided only perchlorate (Barnum *et al*., 2018). However, none of the other organisms with only group 2 Pcr has been directly observed to grow by perchlorate reduction, several genomes encode both group 1 and group 2 Pcr in an apparent redundancy (Barnum *et al*., 2018), and the enrichments also supported organisms reducing chlorate produced by perchlorate-reducing bacteria (Barnum *et al*., 2020). The enzyme's function remains uncharacterized.

Here we identified a gene for the uncharacterized DMSO reductase family enzyme in the publicly available genome of the purple non-sulfur bacterium *Rhodoplanes roseus* DSM 5909 (Fig. 1) (LaSarre *et al*., 2018). The genus *Rhodoplanes* contains four sequenced strains, all of which are capable of anoxygenic phototrophy (Hiraishi and Ueda, 1994; Chakravarthy *et al*., 2012; Mariñán *et al*., 2019). No bacterium in the genus *Rhodoplanes* has yet been observed to grow by dissimilatory perchlorate or chlorate reduction. However, every sequenced *Rhodoplanes* genome contains a DMSO reductase family enzyme adjacent to *cld*. Cld from these genomes formed a monophyletic clade in a phylogenetic tree, but their associated reductases differ (Fig. 1). Only *Rpl*. *roseus* encodes the uncharacterized DMSO reductase family enzyme, which is most similar (83% amino acid identity) to that of *Magnetospirillum magnetotacticum* MS-1 (Bertani *et al*., 2001). *Rpl*. *elegans* DSM 11970, *Rpl*. *piscinae* DSM 19946, and *Rpl*. sp. strain T2.26MG-98 encode a canonical perchlorate reductase most closely related (73% and 74% amino acid identity, respectively) to that of *Azospirillum* sp. TSO22-1 (Jang *et al*., 2019). The presence of different reductases in similar strains provided a natural experiment for determining the uncharacterized enzyme's function.

We tested the hypothesis that the uncharacterized enzyme has perchlorate reductase activity by comparing the ability to grow by perchlorate reduction between *Rpl*. *roseus* and two other *Rhodoplanes* strains, *Rpl*. *elegans* DSM 11970 and *Rpl*. *piscinae* DSM 19946. All strains were kindly provided by Breah Lasarre and James B. Mckinlay at Indiana University, Bloomington. Like all previously characterized organisms with perchlorate reductase and chlorite dismutase, *Rpl*. *elegans* and *Rpl*. *piscinae* were able to couple the reduction of perchlorate to the oxidation of acetate and growth in the dark (data not shown). *Rpl*. *roseus*, which encodes the uncharacterized enzyme, grew in dark with chlorate and

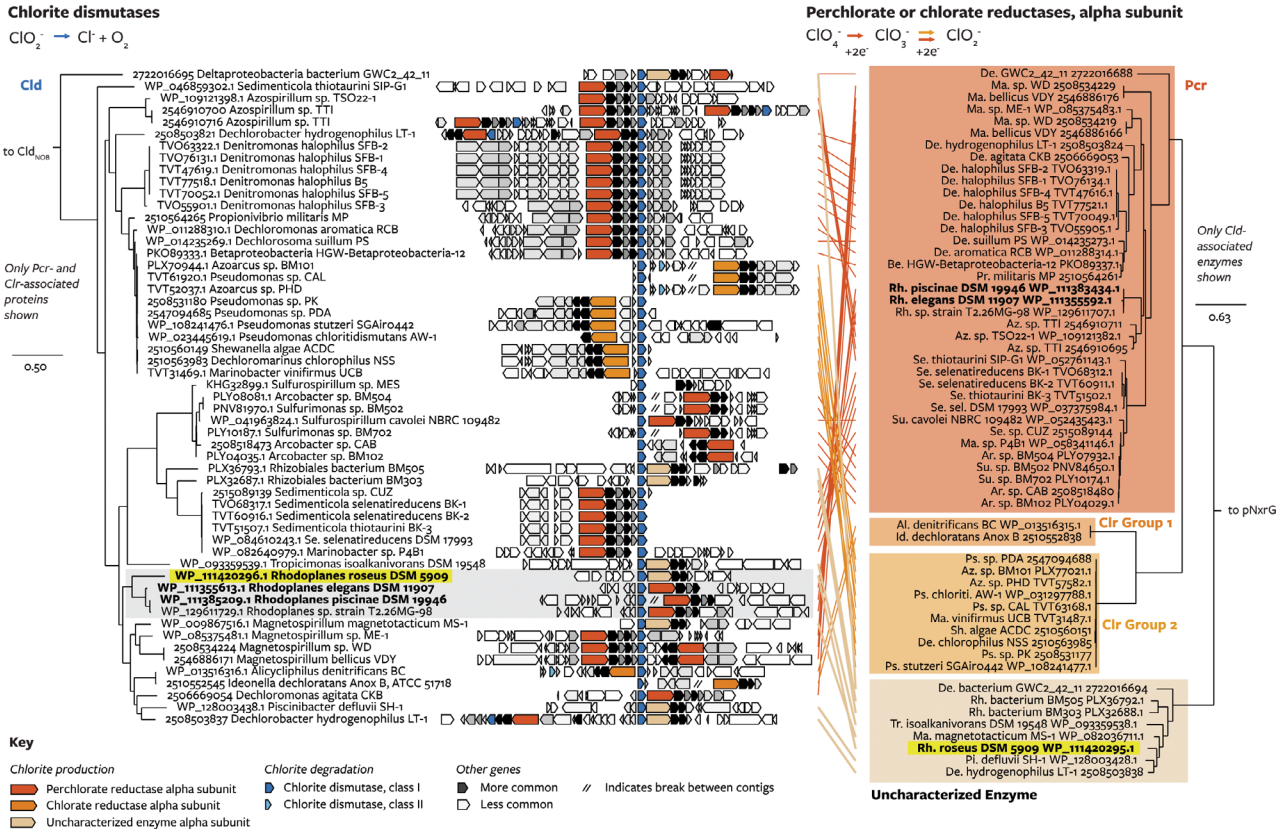

Fig. 1. One member of the genus *Rhodoplanes* encodes an uncharacterized DMSO reductase family enzyme instead of perchlorate reductase near chlorite dismutase (Cld). The genes for dissimilatory perchlorate and chlorate reduction (centre) sorted by the phylogeny of chlorite dismutase and centred on chlorite dismutase. Key genes are coloured, whereas all other genes are shaded by the frequency of their occurrence near (per)chlorate reduction genes across organisms. Maximum phylogenetic trees are shown for Cld (left) and perchlorate or chlorate reductases (right), with lines connecting the proteins to indicate horizontal gene transfer. The Cld tree was rooted to Cld proteins from *Nitrospira* spp., and the (per)chlorate reductase tree was rooted to periplasmic nitrite oxidoreductases from *Nitrospira* spp. For simplicity, proteins not involved in perchlorate or chlorate reduction were excluded from the tree; of particular note is that the different groups of perchlorate and chlorate reductases are more separated by evolution than suggested by the phylogenetic tree shown.

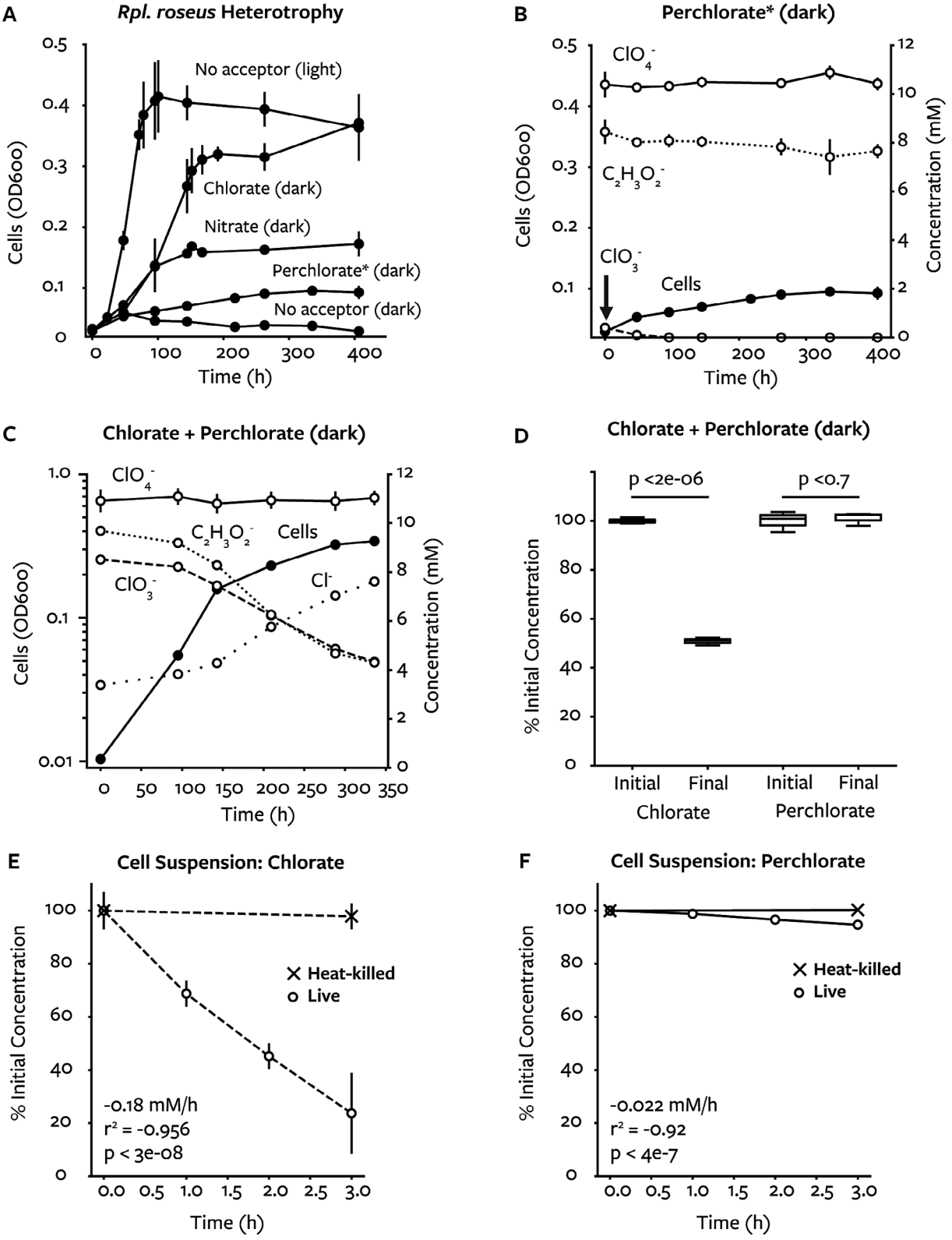

Fig. 2. Legend on next page.

nitrate as the electron acceptor, but limited growth was observed in the no acceptor control and in the perchlorate condition (Fig. 2A). The limited growth could be explained by residual chlorate from the inoculum, and no perchlorate was reduced over the course of 400 h (Fig. 2B). Previously characterized bacteria with perchlorate reductases reduce both perchlorate and chlorate throughout growth (Youngblut *et al*., 2016), while bacteria with chlorate reductases reduce only chlorate (Barnum *et al*., 2020). When *Rpl*. *roseus* was provided with ~10 mM of both chlorate and perchlorate, it reduced chlorate but not perchlorate (Fig. 2C and D). Thus, at the concentrations tested, perchlorate reduction was neither a metabolism nor a co-metabolism of *Rpl*. *roseus*.

Activity towards chlorate and perchlorate was further characterized using washed whole-cell suspensions of chlorate reduction-grown *Rpl*. *roseus*, concentrated to an optical density (600 nm) of 4.51 (Fig. 2E and F). Live *Rpl*. *roseus* cells reduced chlorate at a rate of 0.18 millimolar per hour (linear least-squares regression, $r^2 = 0.956$, $p < 3e^{-8}$) (Fig. 2E). In contrast, perchlorate was reduced at a dramatically slower rate: 0.02 mM h^{-1} (linear least-squares regression, $r^2 = 0.92$, $p < 4e^{-7}$) (Fig. 2F). No reduction of chlorate or perchlorate was observed in heat-killed controls (Fig. 2E and F). Chlorate reductase activity was approximately 10-fold higher than perchlorate reductase activity. These data are consistent with the uncharacterized enzyme in *Rpl*. *roseus* functioning as a chlorate reductase at high densities and laboratory concentrations of chlorate or perchlorate.

From these results, we argue that the uncharacterized DMSO reductase family enzyme should be categorized as a chlorate reductase. That classification is supported by the presence of this reductase near perchlorate reductase in some organisms (Fig. 1)—a redundant arrangement if this reductase had the same function. While further evidence may show the enzyme's apparent limited perchlorate reductase activity to be sufficient to provide energy over long periods of time or at lower concentrations of perchlorate, its function at experimentally relevant durations and concentrations is as a chlorate reductase. We propose the term group 3 chlorate reductase to distinguish this protein and its orthologs from the phylogenetically dissimilar group 1 (serA-like) and group 2 (ddhA-like) chlorate reductases (Fig. 1), named in order of discovery.

The new chlorate reductase has other substantial differences to previously characterized chlorate reductases. Bacteria isolated by *selection* for chlorate reduction so far encode only group 1 Clr or group 2 Clr, suggesting some differences are consequential for the metabolism. Group 3 ClrA has active site residues that more resemble those of PcrA than ClrA, with the main difference to PcrA being the substitution of a key non-polar aromatic tryptophan residue with a polar aromatic tyrosine residue (Barnum *et al*., 2018). Group 3 ClrA proteins have lower shared amino acid identity (Fig. 1) and are not closely related to other types of reductases (Barnum *et al*., 2018), whereas other ClrA are very similar within groups and are closely related to selenate reductase (SerA) or dimethyl sulfide dehydrogenase (DdhA) (Clark *et al*., 2013). Similarly, Cld proteins associated with group 3 ClrA have much greater phylogenetic diversity than those associated with group 1 or group 2 ClrA, which tend to be nearly identical within each group (Fig. 1). Group 3 *clrA* can be found in genomic regions lacking markers of recent recombination, whereas group 1 and group 2 *clrA* are contained within chlorate reduction composite transposon islands (Clark *et al*., 2013). Group 3 *clr* have conserved synteny with *cld*, with *cld* always preceding group 3 *clrA*, while group 1 and 2 *clr* do not (Fig. 1). In summary, group 3 Clr is unique in its active site composition, phylogenetic diversity, and a more conserved relationship with Cld and host genome.

FIG. 2. Patterns of cellular growth and enzymatic activity in *Rhodoplanes roseus* establish the uncharacterized DMSO reductase family enzyme as a chlorate reductase. A, B. Growth of *Rhodoplanes roseus* DSM 5909 through anaerobic phototrophy (light) or anaerobic respiration with different electron acceptors (dark) and with acetate as the electron donor and carbon source. The concentration of perchlorate (ClO_4^-), chlorate (ClO_3^-), chloride (Cl^-) and acetate ($C_2H_3O_2^-$) is shown in the perchlorate growth condition specifically (B). Error bars represent the standard deviation of three biological replicates. The growth medium was modified from RM2 medium (Hiraishi and Kitamura, 1984) and consisted of (g L^{-1}) ammonium sulfate (1.0), potassium phosphate monobasic (1.0), magnesium chloride hexahydrate (0.2), sodium chloride (0.2), calcium chloride dihydrate (0.045) and sodium bicarbonate (2.5). Vitamins and minerals were added as described previously (Coates *et al*., 1999). The medium was made anoxic by sparging with a gas mixture consisting of 80% molecular nitrogen and 20% carbon dioxide. Yeast extract (0.01% w/v, BD Bacto™) was added to stimulate growth (LaSarre *et al*., 2018). Inoculum consisted of a 1:10 dilution of cells in the late-log phase grown anaerobically with chlorate. Cultures were incubated at 30°C without shaking either in the dark or in the light (continuous, Agrobrite High Output T5 24W 6400K bulb). Concentrations of anions were measured using ion chromatography and cell density was measured by absorbance at 600 nm. C, D. Growth of *Rpl*. *roseus* in the dark with both chlorate and perchlorate present in the growth medium. The percent of initial chlorate or perchlorate consumed between the initial (0 h) and final (336 h) time points was compared using an independent t-test. E, F. Chlorate and perchlorate reduction by washed whole-cell suspensions of *Rpl*. *roseus*. Error bars represent the standard deviation of four biological replicates. A linear regression for chlorate reduction and perchlorate reduction by live cells is shown. Cultures in the late-log phase were supplied 100 μg ml^{-1} chloramphenicol to inhibit gene expression then centrifuged at 4°C at 10 000*g* for 30 min, washed with media lacking donor or acceptor, centrifuged again and re-suspended at 10-fold their original concentration in media. To these washed cell suspensions were added approximately 1 mM acetate and 1 mM of either perchlorate or chlorate and monitored ion consumption during incubation at 30°C in the light and a molecular nitrogen atmosphere. A control for abiotic reactions was produced by autoclaving cells for 20 min.

This is the first report of dissimilatory perchlorate or chlorate reduction in organisms capable of phototrophy. It is unclear whether the ability of *Rhodoplanes* species to perform both metabolisms is related. Another DMSO reductase family enzyme, nitrite oxidoreductase, enables the phototrophic oxidation of nitrite to nitrate by providing electrons to photosystem II (Griffin *et al*., 2007; Hemp *et al*., 2016). It is unlikely that chlorate and chlorite serve as electron donors for phototrophy, however, because the reduction potential of the half-couple reactions are higher than that of photosystem II (Hemp *et al*., 2016). Additionally, while some DMSO reductase family enzymes have been observed to perform both forward and reverse oxidoreductase activity, the enzyme Cld only performs the forward reaction of separating chlorite into chloride and oxygen, which would favour the reduction of perchlorate, chlorate and chlorite. A more likely reason for *Rhodoplanes* species to encode chlorate and perchlorate reduction genes is that anoxygenic phototrophs experience large fluctuations in oxygen concentrations and light (Franks and Stolz, 2009). Such environmental conditions would select for the ability to respire diverse substrates available at the Earth's surface, which includes atmospherically produced perchlorate and chlorate (Dasgupta *et al*., 2005).

With the identification of the new chlorate reductase gene, it is clear that genes for perchlorate reduction and chlorate reduction can be found in the same genome, for example, *Dechlorobacter hydrogenophilus* LT-1. Cells with both Pcr and Clr might express the reductases in different conditions, alternating between perchlorate reduction and chlorate reduction, or perhaps the presence of both reductases in the same genomic region is an intermediate state in horizontal gene transfer in which the organism transitions completely from perchlorate reduction to chlorate reduction or vice versa. Some evidence in support of the latter hypothesis is that the group 3 *clr* in *Rhizobiales* bacterium BM505 is co-located with a *pcrA* pseudogene containing a premature stop codon (locus tag 0606_13310). Yet another explanation is that Clr helps perchlorate-reducing bacteria compete for respiratory pathways intermediates. Because Pcr can reduce both perchlorate and chlorate, large amounts of perchlorate lead to the accumulation of chlorate (Nerenberg *et al*., 2006). Perchlorate-reducing bacteria must compete with other strains and with chlorate-reducing bacteria, which can grow to large population sizes in perchlorate-reducing cultures by stealing chlorate (Barnum *et al*., 2020). Encoding a chlorate reductase in addition to a perchlorate reductase may help perchlorate-reducing bacteria minimize the loss of chlorate to competing cells. Studying bacteria capable of both perchlorate reduction and chlorate reduction will likely provide additional insights into the physiology, regulation and evolution of perchlorate and chlorate metabolism.

Identification of this new chlorate reductase was made possible by the observation of a conserved association of an enzyme with an accessory enzyme across a relatively small number of genomes (Barnum *et al*., 2018). We predict that as many more genomes are sequenced, more uncharacterized subfamilies in the DMSO reductase family will be associated with specific accessory genes, providing further opportunities for targeted characterization of these biogeochemically important oxidoreductases.

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