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Overview of organohalide-respiring bacteria and a proposal for a classification system for reductive dehalogenases

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Organohalide respiration is an anaerobic bacterial respiratory process that uses halogenated hydrocarbons as terminal electron acceptors during electron transport-based energy conservation. This dechlorination process has triggered considerable interest for detoxification of anthropogenic groundwater contaminants. Organohalide-respiring bacteria have been identified from multiple bacterial phyla, and can be categorized as obligate and non-obligate organohalide respirers. The majority of the currently known organohalide-respiring bacteria carry multiple reductive dehalogenase genes. Analysis of a curated set of reductive dehalogenases reveals that sequence similarity and substrate specificity are generally not correlated, making functional prediction from sequence information difficult. In this article, an orthologue-based classification system for the reductive dehalogenases is proposed to aid integration of new sequencing data and to unify terminology.

1. Organohalide respiration

Organohalide respiration is the preferred term for the energy-conserving respiratory process wherein a halogen–carbon bond is broken and the halogen atom is liberated as a halide. The removal of halogens from these compounds may reduce or eliminate toxicity or render the compound more biodegradable, making this process important for the remediation of contaminated sites [1]. Organohalide-respiring bacteria (OHRB) are micro-organisms capable of deriving energy for growth from dehalogenation of aromatic and/or aliphatic halogenated compounds. These bacteria are of environmental importance because they reduce anthropogenic halogenated compounds, many of which are significant contaminants in groundwater systems that pose hazards to human health and the environment [2,3]. Beyond bioremediation, the activities of OHRB are part of the global halogen cycle [4].

OHRB have been identified from diverse bacterial phyla, including the Proteobacteria, Firmicutes and Chloroflexi [5–8]. The known OHRB can be grouped as either obligate or non-obligate organohalide respirers [9]. The proteobacterial OHRB, including *Geobacter*, *Desulfuromonas*, *Anaeromyxobacter* and *Sulfurospirillum*, are all non-obligate organohalide respirers with versatile metabolisms encoded on relatively large genomes [9]. In contrast, the currently known organohalide-respiring Chloroflexi are all obligate organohalide respirers, meaning they are niche specialists with a very restricted metabolism. The Firmicutes contain non-obligate organohalide-respiring *Desulfitobacterium* spp. as well as metabolically restricted *Dehalobacter* OHRB [9]. It has recently

been shown that some *Dehalobacter* spp. are able to ferment dichloromethane [10,11]. There is little to no correlation between phylogenetic affiliation and chlorinated substrate specificities: aliphatic and aromatic substrates are used by taxonomically diverse organisms [12].

Genome sequences exist for several OHRB, including five *Dehalococcoides mccartyi* strains [13–16] and *Dehalogenimonas lykanthroporepellens* strain BL-DC-9 [17] within the Chloroflexi; *Geobacter lovleyi* strain SZ [18] and *Anaeromyxobacter dehalogenans* strains 2CP-C and 2CP-1 [19] from the Proteobacteria; and *Desulfitobacterium hafniense* strains Y51 and DCB-2 [6,20], *Desulfitobacterium dehalogenans* (NC_018017), *Desulfitobacterium dichloroeliminans* (NZ_AGJE00000000) and most recently two *Dehalobacter* strains [21] from the Firmicutes. Given the low cost and broad availability of sequencing from pure or mixed cultures, the number of OHRB genomes will increase rapidly. Consistent with this expectation, a draft genome for *Dehalobacter* sp. E1 was recently elucidated based on metagenome analysis of a defined co-culture [22]. The availability of OHRB genomes has allowed comparative genomic studies within genera [15,23] as well as across phyla [6]. Genome sequences of the *Dehalococcoides* have revealed specific metabolic requirements, including corrinoid auxotrophy for the majority of sequenced strains [13–15].

The diversity of OHRB is not yet fully described. Moving forward, novel strains, species and even new genera with organohalide-respiring activity will probably be discovered, enriched and isolated from environments where suitable halogenated electron acceptors, either of natural or anthropogenic origin, are available.

Metagenomes of defined mixed cultures and entire microbial communities are currently being unravelled, allowing determination of metabolic interactions within organohalide-respiring consortia. Metagenomes were sequenced from consortia such as (i) the KB-1 culture, containing populations of *Dehalococcoides*, *Geobacter*, *Methanosarcina*, *Spirochaeta* and *Sporomusa* [24,25]; (ii) the ANAS tetrachloroethene (PCE)-dechlorinating mixed culture containing *Dehalococcoides*, fermentative bacteria and two methanogenic populations, among other organisms [26,27]; and (iii) a highly stable and efficient dechlorinating bioreactor community containing *Dehalococcoides* [26,28]. In addition, a defined coculture of *Dehalobacter* sp. E1 and *Sedimentibacter* sp. B4 that reductively dechlorinates beta-hexachlorocyclohexane has been sequenced [22].

2. Reductive dehalogenases

Organohalide respiration reactions are catalysed by reductive dehalogenases. The first reductive dehalogenase that was biochemically characterized was the 3-chlorobenzoate reductive dehalogenase of *Desulfomonile tiedjei* strain DCB-1 [29]. The first gene sequences encoding reductive dehalogenases were identified using classical reverse genetics approaches based on partial amino acid sequences of purified enzymes [30,31]. PCR and genome studies have since identified new sequences with high sequence identity to the characterized genes [6,13,14,32–42]. Reductive dehalogenase genes (or reductive dehalogenase homologous genes (*rdh*) if the encoded protein has not yet been biochemically characterized) typically comprise an operon containing *rdhA*, the gene for the catalytically active enzyme (RDase if characterized, otherwise RdhA), *rdhB*, a gene encoding a putative membrane-anchoring protein [34], and sometimes one or more members of *rdhTKZECD*, genes associated with reductive dehalogenase genes [43]. For some, but not all, of the predicted gene products, function in transcription regulation and maturation of the holoenzyme has been experimentally confirmed [44,45].

The nomenclature of the *rdh* genes as homologues is based on the presence of specific conserved motifs [46]. RdhA proteins have several conserved features, including two iron–sulfur cluster-binding motifs and a twin-arginine signal motif for translocation to or across the cell membrane [34]. Many, if not all, characterized RdhAs contain corrinoid co-factors (derivatives of vitamin B12), including the PCE dehalogenase from *Sulfurospirillum multivorans* (formerly *Dehalospirillum*), which was shown to contain the very specific, previously unknown corrinoid norpseudo-B_{12} [47]. Involvement of a Co(I) corrinoid in the catalytic activity of reductive dehalogenases has been demonstrated by the reversible inactivation of corrinoid using propyl iodides for a number of enzymes, and by the fact that the lack of the corrinoid cofactor resulted in loss of the PCE-dechlorination ability in *S. multivorans* and *Dehalobacter restrictus* PER-K23 [38,48]. Six chlorophenol and four PCE/trichloroethene (TCE) reductive dehalogenases have been purified from different *Desulfitobacterium* strains, and the majority of reductive dehalogenases that have so far been tested for the presence of a corrinoid prosthetic group contained such a group [49]. These corrinoid co-factors are thought to be essential for enzymatic function, and show some specificity: cocultures of *Geobacter* strains and *Dehalococcoides mccartyi* strains revealed cobamides produced by the OHRB *Geobacter lovleyi* strain SZ supported dechlorination by the *Dehalococcoides* strain, while cobamides produced by the non-OHRB *Geobacter sulfurreducens* did not [50].

Although the identity of several *rdhA* genes has been confirmed based on partial (N-terminal) amino acid sequences of purified enzymes with proven activity, the three-dimensional structure of an RdhA protein has not yet been determined, nor has an active site been identified. Furthermore, identification of most *rdhA* genes, especially those encountered in genome sequences of OHRB and other bacteria, has been based entirely on sequence similarity and the presence of the above-mentioned motifs. It is, in addition, not certain if all RdhA proteins are homologous (i.e. share a common evolutionary origin). To this end, we are maintaining the nomenclature of *rdhA* genes as homologues based on high sequence similarity along the entire length of the genes, conservation of shared motifs, and an absence of evidence of convergent evolution.

Reductive dehalogenase encoding genes have been identified in a wide variety of strictly anaerobic bacteria, including *Sulfurospirillum* [51], *Desulfitobacterium* [1,31,52,53], *Dehalobacter* [7] and *Dehalococcoides* [13–15,54], and in microaerophilic bacteria as in the case of *Anaeromyxobacter* [19], and others (figure 1) [18,58]. Only one archaeal putative reductive dehalogenase gene has been identified to date from a *Ferroglobus* species [59], but this organism has not been demonstrated to conserve energy via organohalide respiration. Most, but not all, of these *rdhA* gene-carrying organisms are known to reduce halogenated organic compounds. Within the known dehalogenating organisms, some do not use halogenated substrates for energy conservation in a respiratory process; for example, a strain of *Dehalobacter* has been shown to ferment dichloromethane [11].

The reductive dehalogenases present within an organism, enrichment culture or contaminated site dictate the range of

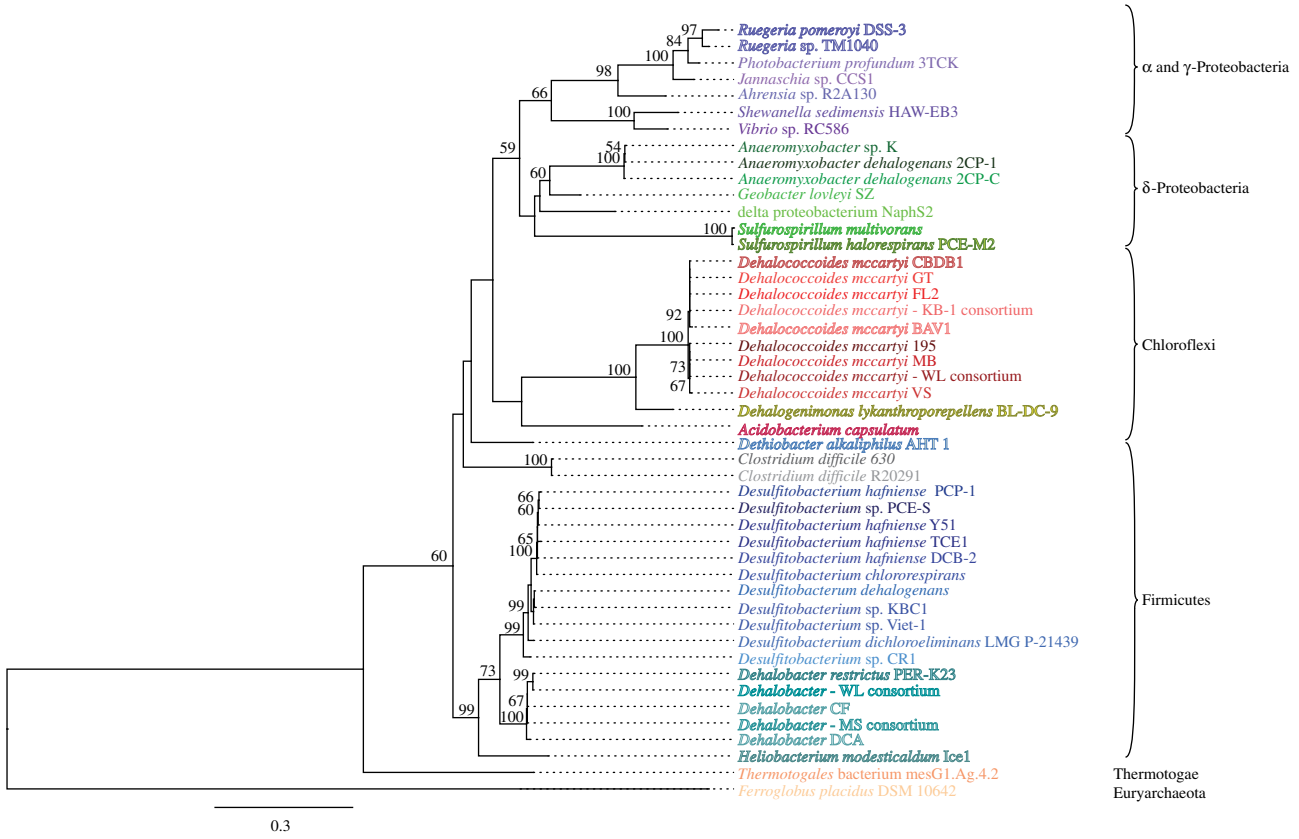

Figure 1. Maximum-likelihood (ML) tree of 16S rRNA gene sequences from all known organisms containing a reductive dehalogenase homologous (*rdhA*) gene. For organisms with multiple 16S rRNA genes, one representative gene sequence was chosen for clarity (see the electronic supplementary material, table S2 for 16S rRNA gene sequence accessions). 16S rRNA gene sequences were mined from IMG-M and NCBI. The sequences were aligned using the greengenes NAST alignment algorithm [55], and manually curated and masked in Geneious [56] to a final alignment of 1117 unambiguously aligned positions. Ten ML trees were calculated using RAxML HPC v. 7.2.8 [57] under the GTR + γ model of nucleotide evolution, and the tree with the highest likelihood chosen. Organism names are coloured to correspond to the colours on the RdhA tree (figure 2).

halogenated electron acceptors used. Several molecular tools have been developed for examining a broad range of *rdhA* genes in a given sample (site, isolate or mixed culture), including both microarray-based methods and PCR-based protocols [60–63]. Identification of novel reductive dehalogenase genes has primarily come from PCR studies [32,33,35–37,39,40,42] and subsequently from genome and metagenome sequencing efforts [6,13,14,41,63], with each new organism or environment sampled increasing the known diversity of putative *rdhA* genes.

Analysis of genome sequences divides the OHRB into those whose genomes contain one or two *rdhA* genes and those containing several (more than two) different *rdhA* [5,64]. Genomes of *Desulfitobacterium*, *Dehalococcoides*, *Dehalogenimonas* and *Dehalobacter* strains contain multiple *rdhA* genes. The complete genome sequence of *Desulfitobacterium hafniense* strain DCB-2 contains seven putative *rdhA* genes [20]. From the *Dehalococcoides mccartyi* genomes, the current minimum number of *rdhA* genes per genome is 10 [15], with up to 36 *rdhA* genes present on a single genome [15]. The genome of *Dehalococcoides mccartyi* (formerly *ethenogenes*) strain 195 [65] harbours 17 different *rdhA* genes [14], while strain CBDB1's genome contains 32 *rdhA*s [13]. The genome of *Dehalogenimonas lykanthroporepellens* strain BL-DC-9, the only sequenced *Dehalogenimonas*, contains 19 *rdhA* genes [17,66]. The presence of multiple *rdhA* genes is also a feature in the *Dehalobacter* genus, with up to 24 complete putative *rdhA* genes within sequenced *Dehalobacter* genomes [21,22,67].

(a) Substrate specificity of reductive dehalogenases

Only few reductive dehalogenases have been biochemically characterized. The enzymes are inactivated by molecular oxygen, and a genetically tractable system for expressing a specific reductive dehalogenase is not yet available. Both these factors have impeded extensive biochemical experimentation in the past. In the case of *Dehalococcoides*, *Dehalogenimonas* and some *Dehalobacter*, detailed studies on the biochemistry of reductive dehalogenases have been hampered by difficulties in obtaining sufficient biomass. Substrates for RDases have been identified using culture-based methods as well as by enzymatic assays with pure RDases. Much of our understanding of reductive dehalogenases has come from well-studied organisms, including *Sulfurospirillum multivorans* and *Dehalobacter restrictus* PER-K23 which dechlorinate PCE to *cis*-dichloroethene (DCE) [7,68–70]. PceAs, active on the chlorinated ethenes PCE and TCE, have been identified from *Sulfurospirillum*, *Desulfitobacterium* and *Dehalobacter*. The four known *Desulfitobacterium* PCE/TCE reductive dehalogenases were isolated from *Desulfitobacterium hafniense* strains PCE-S, TCE1 and Y51, and *Desulfitobacterium* sp. strain PCE1. The *Sulfurospirillum* and *Desulfitobacterium* PCE/TCE reductive dehalogenases cannot dehalogenate isomers of DCE. The substrate spectrum dehalogenated by the reductive dehalogenase of *Desulfitobacterium hafniense* strain Y51 is similar to the PCE reductive dehalogenase of *Dehalobacter restrictus* [35,38].

CprA and CrdA dehalogenases from *Desulfitobacterium dehalogenans* and *Desulfitobacterium hafniense* dechlorinate a

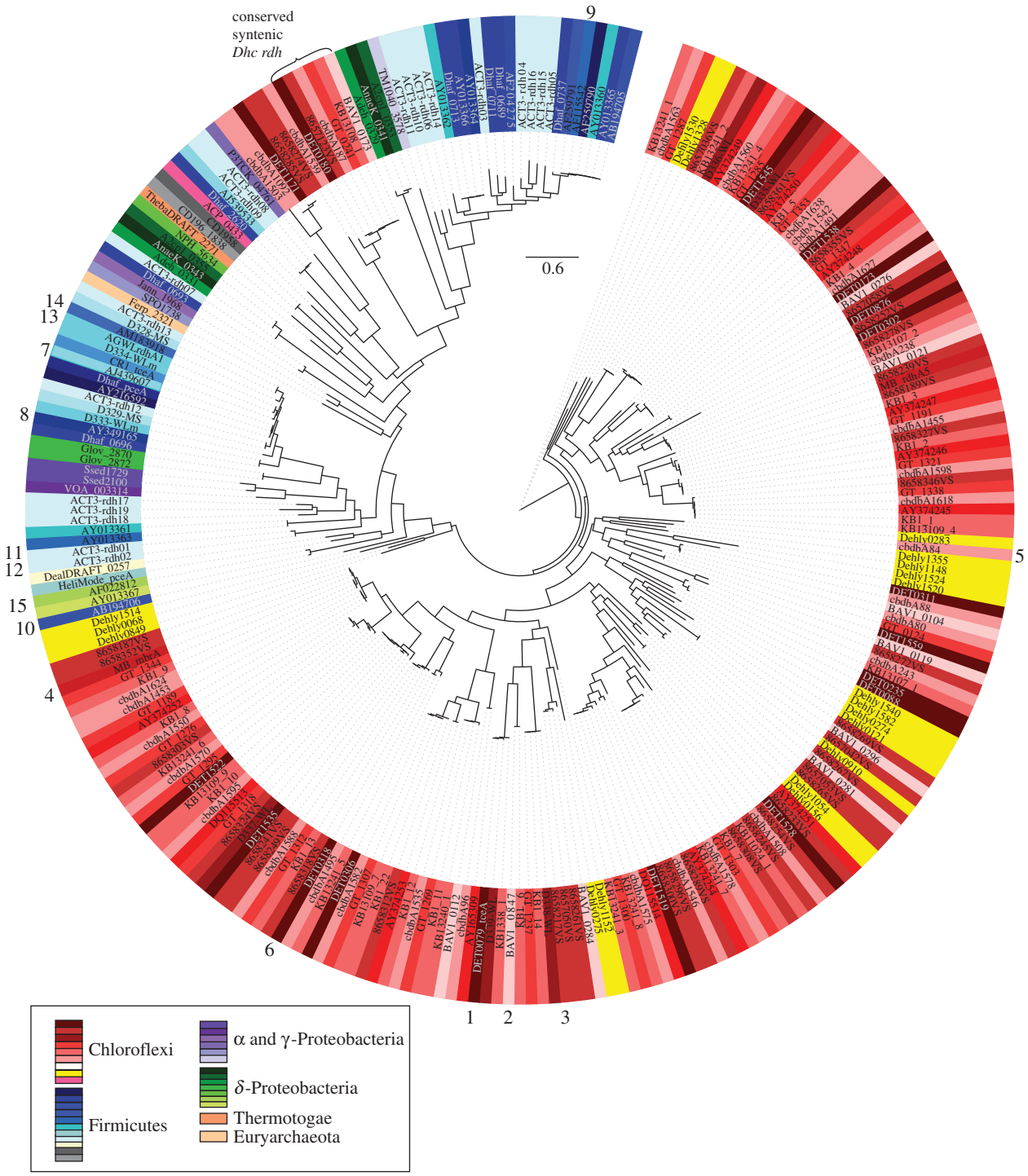

Figure 2. ML tree of 264 reductive dehalogenases and translated *rdhA* genes based on an amino acid alignment of the TIGRFAM domain and FeS-binding domains. The protein sequences were aligned using the Geneious muscle plugin [56,88] and the alignment iteratively refined using Hmmer v. 2.3.2 until the iterations converged on a single solution [89]. The alignment was manually masked to remove ambiguously aligned positions, leaving a final alignment with 831 positions. The best model of amino acid substitution was determined using ProtTest v. 3 [90,91]. ML trees were generated using RAxML HPC v. 7.2.8 [57] under the LG + γ+F model of amino acid substitution with 100 bootstrap resampling trees conducted. Bayesian inference trees were generated using Mr. Bayes [92], with aamodelpr set to 'mixed', Iset rates as 'invgamma', and default conditions otherwise. The analyses were continued until the standard deviation of split frequencies was below 0.1 (400 000 generations). Posterior probabilities were generated with a final burn-in of 150 000 and were mapped onto the nodes of the ML tree for display. Protein accessions are coloured by organism as in figure 1: red, *Dehalococcoides*; yellow, *Dehalogenimonas*; blue/grey, Firmicutes; purple, α and γ-Proteobacteria; green, δ-Proteobacteria; orange, Thermotogae and Archaea. See electronic supplementary material, figure S1 for a rectangular version of this phylogeny with scale bar and bootstrap support values for this tree. Reductive dehalogenases with known function on specific substrates, based on protein isolation, proteomics or transcriptomics are numbered as follows: (1) TceA (DET0079_tceA, *Dehalococcoides mccartyi* st. 195; TCE) [34]; (2) BvcA (BAV1_0847, *Dehalococcoides mccartyi* st. BAV1; VC, *cis*-DCE, 1,2-DCA) [40]; (3) VcrA (8658217VS, *Dehalococcoides mccartyi* st. VS; VC, TCE, *cis*-DCE) [74]; (4) MbrA (GU120391 (MB_mbrA on tree), *Dehalococcoides mccartyi* st. MB; TCE) [60]; (5) CbrA (cbdbA84, *Dehalococcoides mccartyi* st. CBDB1; 1,2,3,4-tetrachlorobenzene, 1,2,3-trichlorobenzene, pentachlorobenzene) [75]; (6) PceA (DET0318, *Dehalococcoides mccartyi* st. 195; PCE) [54]; (7) PceA (AJ439607, *Dehalobacter restrictus* PER-K23; PCE) [38]; (8) CprA (AY349165, *Desulfitobacterium hafniense* st. PCP-1; pentachlorophenol, 2,3,4,5-tetrachlorophenol, and 2,3,4-trichlorophenol) [71]; (9) CprA (AF259790, *Desulfitobacterium dehalogenans*; ortho-chlorophenols) [31]; 10-PrdA (AB194706, *Desulfitobacterium* sp. strain KBC1; PCE) [93]; (11) CfrA (ACT3_rdh02, *Dehalobacter* sp. strain CF; 1,1,1-trichloroethane, CF) [81]; (12) DcrA (ACT3_rdh01, *Dehalobacter* sp. strain DCA; 1,1-DCA) [81]; (13) DcrA (FJ010189 (AGWLrdh01 on tree), *Dehalobacter* sp. strain WL; 1,2-DCA) [94]; (14) DcaA (AM183918, *Desulfitobacterium dichloroeliminans*; 1,2-DCA) [73]; (15) PceA (AF022812; *Sulfurospirillum multivorans*; PCE) [51].

variety of chlorinated phenols [31,37,39,71]. Substrate specificities towards ortho or meta and para dechlorination are observed for the different CprAs. CrdA is a unique chlorophenol reductive dehalogenase that is capable of ortho dechlorination of several polychlorinated phenols but not of 3-chloro-4-hydroxyphenylacetate, in contrast to the other chlorophenol reductive dehalogenases [37]. CrdA was isolated from *Desulfitobacterium* strain PCP-1 and the *crdA* gene is also present in *Desulfitobacterium hafniense* strains TCP-A, DP7, DCB-2, Y51 and TCE1, *Desulfitobacterium dehalogenans*, *Desulfitobacterium chlororespirans* and *Desulfitobacterium* sp. strain PCE-1 [72]. Another unique chlorophenol reductive dehalogenase is CprA5 found in *Desulfitobacterium hafniense* strains PCP-1, TCP-A and DCB-2, which has dechlorination activity against several chlorophenols at all three substituent (ortho, meta and para) positions [39,72].

A 1,2-dichloroethane (1,2-DCA)-specific reductive dehalogenase was identified in *Desulfitobacterium dichloroeliminans* strain DCA1 and also in the metagenome of a 1,2-DCA organohalide-respiring enrichment culture from a contaminated aquifer [73]. Sequence analysis of the catalytic subunit of these DcaA proteins showed specific sequence differences and signature motifs compared with the other known reductive dehalogenases, suggesting that these enzymes may have specifically adapted to 1,2-DCA reductive dechlorination [73].

Within the *Dehalococcoides*, five proteins involved in respiring chlorinated ethenes, ethanes and chlorinated benzenes have been partially characterized. PceA has been shown to dechlorinate PCE to TCE [54]. TceA has been shown to dechlorinate TCE to ethene, though was most active on TCE [34], and VcrA catalyses the dechlorination of TCE, DCE isomers and vinyl chloride (VC) to ethene [40,74]. The VcrA protein is additionally capable of dihaloelimination of 1,2-DCA to ethene [74]. Using native polyacrylamide gel electrophoresis (PAGE), CbrA from *Dehalococcoides mccartyi* strain CBDB1 was found to dechlorinate 1,2,3,4-tetrachlorobenzene and 1,2,3-trichlorobenzene [75]. Recent studies using blue native PAGE have confirmed that BvcA also catalyses dechlorination of TCE, DCE isomers, VC and 1,2-DCA and further expands the known substrate ranges for the enzymes BvcA, VcrA and TceA [76], indicating that at least these RDases, and probably many others, can dechlorinate multiple substrates.

Beyond these partially characterized reductive dehalogenases, many RdhA proteins have been assigned a putative function through transcriptional analyses in the presence of specific substrates, or through peptide sequencing of partially purified proteins produced during specific stages of dechlorination [40,61,77,78]. While these proteins often cannot be assigned substrate specificity with certainty, these experiments provide evidence for function and can be used to better illustrate the sequence similarity/substrate affinity relationship within this group of enzymes. These methods have been somewhat confounded in the case of *Dehalococcoides*, where multiple reductive dehalogenase genes are typically expressed in the presence of a single halogenated substrate [79,80].

The oxygen sensitivity of reductive dehalogenases, their association with the cell membrane, and the tendency for multiple *rdhA* genes to be transcribed have impeded determination of substrate specificities or tertiary structures for these enzymes. Blue Native PAGE is a promising approach for examining *in vitro* reductive dehalogenase activity [76,81]. The current frontier of biochemical research on the reductive dehalogenases involves attempts to heterologously express reductive dehalogenase encoding genes and to engineer an OHRB with desirable substrate preferences. Reductive dehalogenase genes have been successfully cloned, but efforts to produce an active enzyme have failed so far [30,82,83].

(b) Sequence analysis of reductive dehalogenases

This themed issue is the result of a meeting at the KAVLI centre, UK, on 4–5 July 2011. At the meeting, a need for a classification system and a consistent nomenclature was identified. To address this need, the diversity of reductive dehalogenases is examined based on sequence analysis on a curated protein dataset of RDases and RdhA proteins. The ability to predict an organism's dechlorination potential based on sequence similarity and the known functional information for RDases is examined, and orthologous groups for transparent nomenclature of the gene family are defined. The orthologue groups are intended to serve as a reference point for all subsequently identified RdhA proteins, and to facilitate comparisons between sequenced organohalide respirers. Orthologues were defined based on pairwise sequence identities greater than 90 per cent at the amino acid level, and orthologue groups constrained to this level of sequence identity. Additionally, orthologue groups were confirmed as highly supported clades in protein tree analyses.

A basic search for 'reductive dehalogenase' in the NCBI nucleotide database results in over 600 sequences as of June 2012. For the curated, comprehensive dataset of RdhA proteins discussed below, a reductive dehalogenase homologue had to have been sequenced from a known organism for which a 16S rRNA gene sequence was available. Based on this filter, environmental sequences from contaminated sites, ocean surveys and uncharacterized enrichment cultures were excluded. It was not required that the organism had demonstrated reductive dehalogenation activity. In the majority of cases, *rdhA* genes were mined from finished genome sequences, or from known dechlorinating isolates whose genomes have not yet been sequenced (e.g. *Dehalococcoides mccartyi* strain MB [60,61]). *rdhA* genes from consortia (KB-1, ACT-3 and WL) were included in cases where metagenome sequences or quantitative PCR studies allow connection of *rdhA* sequences with specific OHRB genera [24,84–86]. Additionally, the *rdhA* genes were required to be full-length or close to full-length (greater than 300 amino acids), which excluded many partial *rdhA* genes in the database. Only the catalytically active subunit (RdhA) was included in this analysis; the RdhB proteins were not considered. The final curated set contained 264 RdhA proteins from 44 micro-organisms (see the electronic supplementary material, table S1 for sequence names, accessions and classification identifiers). Sequence comparisons were conducted both including and excluding the twin-arginine translocation (Tat) signal sequences (approx. 50 amino acids, composed of the characteristic Tat-motif and a hydrophobic stretch) at the N-termini of the proteins, which may be expected to evolve at a different rate from motifs involved in catalysis [87]. For further comparison, phylogenetic analyses on the aligned TIGRFAM reductive dehalogenase domain (removing Tat signal sequences as well as C-terminal FeS cluster-binding motifs from the original alignment) were conducted.

The Tat signal sequence trimmed alignment gave the best-resolved tree in terms of strongest bootstrap support across the tree. In general, the relationships defined by the three trees (full-length proteins, Tat signal-trimmed and TIGRFAM

domain only) were consistent, with the same overall topology, and with all orthologue groups recovered with high bootstrap support and posterior probabilities (greater than 90%, greater than 0.9, respectively). Many of the differences between the three trees were in the branching order of divergent, long-branch sequences, which may be the result of long-branch attraction artefacts. Other topological differences were the result of rearrangements of the backbone of the tree, which was poorly supported in all three trees, and as such, the correct topology in the tree backbone cannot be ascertained. The Tat signal-trimmed tree is depicted in figure 2, while all three trees are presented with bootstrap support values in the electronic supplementary material, figures S1–S3.

The RdhA protein tree is dominated by sequences from *Dehalococcoides* (red), *Dehalogenimonas* (yellow), *Dehalobacter* (light blue) and *Desulfitobacterium* (dark blue) (figure 2). The preponderance of these organisms is partially due to the intense research efforts concentrated on these bacterial groups based on their environmental significance as dechlorinating organisms, but is also due to the fact that genomes from these organisms typically contain multiple *rdhA* genes [20,32,80,94].

The Chloroflexi RdhA proteins, predominantly from the *Dehalococcoides*, cluster within a large clade. Within this large Dehalococcoidales clade, several sub-clades of RdhA identified in multiple *Dehalococcoides mccartyi* strains share branching order with the 16S rRNA gene tree. In several instances, orthologous RdhAs (greater than 90% pairwise amino acid identity (PID) over the full-length of the sequences and bidirectional best BLASTp hits) are present in only two or three strains of the six sequenced strains. This patchy distribution of *rdhA* genes points either to rapid gene loss within the *Dehalococcoides*, or to lateral acquisition of novel *rdhA* genes within subsets of *Dehalococcoides mccartyi* strains. Lateral gene transfer has been invoked for *rdhA* gene acquisition in several scenarios [95–99] and is potentially a factor in the RdhA family diversification. Sequence information from new strains and complete genome sequences from strains for which some *rdhA* genes have been identified will help clarify orthologue and paralogue relationships.

The only core RdhA present in all sequenced *Dehalococcoides* genomes (11 o'clock in figure 2) falls outside of the main Dehalococcoidales RdhA clade. This core RdhA clade matches the 16S rRNA phylogeny in terms of strain relationships, and shares synteny across *Dehalococcoides* genomes [15], indicating it may have diverged within *Dehalococcoides* from a shared ancestral gene. A second smaller clade of *Dehalococcoides* sequences is also present in the non-*Dehalococcoides* region of the tree (next to marked conserved syntenic clade in figure 2), though only three sequenced strains are represented (CBDB1, 195 and VS). These clades may represent distant orthologues to non-*Dehalococcoides* RdhA proteins, though the current analysis is not conclusive.

There are 15 RdhA proteins on the tree (see numbers in figure 2), for which functional information is available in the form of transcriptional data or biochemical characterization of purified proteins. These RDases are distributed across the RdhA tree, and are known to catalyse the dehalogenation of a broad range of substrates, including chlorinated ethenes (VcrA, BvcA, TceA, MbrA, PceA, PrdA [38,40,54,60,74,93,100]), chlorinated ethanes (CfrA, DcrA, DcaA [73,81,94]), and chlorinated aromatics (CbrA, CprA [31,71,75,101]). For most characterized reductive dehalogenases, the full substrate range is not known; instead activity on a specific substrate has been verified. It is clear that reductive dehalogenases that share a specific function do not necessarily share high sequence similarity: the four PCE-dehalogenating enzymes (nos. 6, 7, 10 and 15) are located at three distant positions on the tree. Similarly, the two chlorophenol reductive dehalogenases (nos. 8 and 9) are at opposite edges of the non-*Dehalococcoides* RdhA portion of the tree and have relatively low sequence similarity (28% PID**)**. One note from this is that neither the structure nor the identities of the catalytic amino acid residues in the active centre of the reductive dehalogenase enzymes are known. It is possible that the dehalogenation of similar targets is conducted using different catalytic mechanisms, but it is also possible that shared residues and motifs between these divergent sequences with shared function will provide clues as to the critical residues for RdhA folding and function. When genetic manipulation of these targets becomes possible, those shared residues would be primary targets of interest for mutation experiments.

In a single example of sequence identity hinting at function, the chlorinated ethene reductive dehalogenases TceA (DET0079_tceA), BvcA (BAV1_0847) and VcrA (DhcVS_1291) in *Dehalococcoides* form a monophyletic group on the tree (with approx. 48% PID), and proteins in this group are associated with enrichment cultures capable of dechlorinating chlorinated ethenes and 1,2-DCA. In general, the RdhA phylogeny does not indicate that similarity analysis of reductive dehalogenases will allow confident prediction of substrate specificity for novel RdhA proteins identified. An example of this concerns the DcrA (ACT3_rdh01) and CfrA (ACT3_rdh02) RDases from *Dehalobacter* strains DCA and CF, respectively, from the ACT-3 culture, which share a high level of sequence similarity (95% PID), group closely together (nos. 11 and 12, at 8.30 in figure 2), but do not share overlapping substrate specificities [81]. Similarly, the *Dehalobacter/Desulfitobacterium* PceAs share 88–89% PID with the *Desulfitobacterium dichloroeliminans* DcaA but do not share overlapping substrate specificities [73]. These two examples present the reverse scenario to the four PceA proteins, where amino acid residues that differ between the two proteins are potentially important in substrate specificity.

(c) Classification system for reductive dehalogenases

The need for a classification system for the reductive dehalogenases is tied to the rapid expansion of the family through genome and metagenome sequencing. These enzymes and genes are being used extensively as biomarkers for monitoring active and passive bioremediation at contaminated sites. Greater clarity in nomenclature would facilitate this transfer of science to practice. Typically, comparisons of proteins encoded by novel *rdhA* genes focus on the characterized reductive dehalogenases. As discussed above, these provide only a partial picture of the sequence diversity of the protein family. In defining a classification system for the reductive dehalogenases, we hope to clarify the relationships within the family as well as provide a platform for more robust comparisons of new sequences to the existing family. Furthermore, such classification can guide the selection of representative genes and proteins for more detailed functional characterization.

Reductive dehalogenases are included in the TIGRFAM and PFAM databases of homologous proteins (TIGR02486 and PF13486), but are not recognized as a protein family by other major databases (PROSITE and SMART). In the absence of a crystal structure and information as to key residues for folding or the active site, it is difficult to implement many of the standard

methods for classifying protein families. Instead, we have implemented a sequence identity-based classification of orthologues into groups, anchored by the tree of curated reductive dehalogenase protein sequences.

We defined orthologue groups based on a threshold of 90% PID in amino acid alignments between all members of a group that are additionally supported on the tree with bootstrap support values more than 90%. PID and similarity scores for all RdhAs within this curated amino acid dataset were generated using CLUSTALW [102,103]. The orthologue groups allow direct comparisons of RdhAs between current sequenced strains and facilitate placement of newly sequenced RdhAs in the protein family. Based on these criteria, we have identified 46 orthologue groups containing in total 176 RdhA proteins. The minimum number of proteins in a group is 2, while the current maximum is 10. A complete description of the defined groups, including organisms and gene names within each group, number of encoded proteins per group, and the respective PID ranges within the groups is presented in the electronic supplementary material, table S3, and the groups are highlighted in colour in the electronic supplementary material, figure S1.

In defining such a classification system for the reductive dehalogenase family, we endeavoured to create a flexible format that was informative, and which would not require constant revision as new RdhA proteins are described. To that end, all orthologue groups have been named sequentially based on the date of first publication of the earliest described sequence in the group. For example, the first sequenced RdhA was the tetrachloroethene reductive dehalogenase PceA from *Sulfurospirillum multivorans* in August 1998 [30], and as a result, the orthologue group containing this protein has been designated Reductive Dehalogenase Orthologue Group 1 (RD_OG1). In the case where multiple RdhA proteins were reported in the same article [13,14,32,40], group numbers were assigned based on locus tag or accession numbers, in ascending order. In one case, two RdhAs were presented in the same journal issue [39,74]; in this case, the lower group number was assigned based on alphabetical order of first author names.

An advantage to this classification method compared with large clade-based classification methods [9,15] is that the orthologue groups are not expected to change with identification of divergent sequences, while larger order relationships may shift with additional sequences. Given the lack of support for the backbone of the protein tree, the proposed classification system represents a conservative solution. The intention for this classification system moving forward is to allow the addition of new groups as novel RdhA proteins are discovered. A novel group would be created when a full-length or near full-length (greater than 75%) RdhA from a known organism was identified that had 90% or more PID with an RdhA not currently placed in an orthologue group. The group would be defined as containing that pair of sequences, and it would receive the next available group number. Such a classification system allows flexibility for adding novel sequences and provides a level of intuitive interpretation, where the lower the group number, the longer that particular RdhA group has been known or studied in multiple organisms.

(i) An example: the WBC-2 consortium

WBC-2 is a microbial consortium capable of dechlorinating chlorinated ethenes and ethanes [104]. This consortium contains *Dehalobacter*, *Dehalococcoides* and *Dehalogenimonas* strains [104]. Previous sequence data from this consortium have been limited to 16S rRNA gene clones, leaving the reductive dehalogenase complement unknown. A metagenome from a 1,1,2,2-tetrachloroethane-degrading WBC-2 subculture was sequenced and partially assembled, resulting in 144 846 contigs with total scaffold length of 68 536 230 bp, a maximum contig length of 334 963 and an N50 of 2192 bp. A total of 16 reductive dehalogenase genes were identified from the WBC-2 metagenome using tblastx with the curated gene set as queries. Of these, 13 were readily classified into existing orthologue groups as described above, while the remaining three shared greater than 90% PID with a single existing sequence. A protein tree placing the WBC-2 RdhA within the orthologue groups can be found in the electronic supplementary material, figure S4. As the reductive dehalogenases from the WBC-2 consortium cannot be ascribed to a specific OHRB, this example illustrates how novel sequences can be described in the context of the RD_OGs rather than how a new RD_OG could be generated and numbered.

The curated reductive dehalogenase dataset, a detailed method for incorporating novel reductive dehalogenase sequences into the RD_OG framework and for creating new RD_OGs, and a spreadsheet detailing the current RD_OGs are all available for public access and editing at the following link: docs.google.com/folder/d/0BwCzK8wzlz8ON1o2Z3FTbHFPYXc/edit. It is the hope of the authors that this allows simple adoption of this naming system for newly discovered reductive dehalogenase sequences, with iterations of the RD_OG framework easily generated and maintained.

3. Summary

Organohalide respiration is a unique metabolic process that is implicated in the global halogen cycle as well as being of environmental and societal significance for remediation efforts. The reductive dehalogenase family is diverse and growing: newly available genome sequences of organohalide-respiring organisms or gene surveys of natural and anthropogenic environments provide novel RdhA sequences, suggesting the diversity of the protein family is nowhere near exhausted. In this contribution, a curated database of RdhA proteins was generated and a phylogenetic comparison of the RdhA-encoding organisms and their respective RdhA proteins conducted. From this, a classification system for the RdhAs was proposed based on sequence identity. The intention for this classification is to facilitate placement of novel RdhAs within the context of the gene family, and to aid communication about and comparison of the multiple rdhA genes and their encoded proteins in different strains of organohalide-respiring bacteria.

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