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Minireview

Molecular techniques in the biotechnological fight against halogenated compounds in anoxic environments

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Summary

Microbial treatment of environmental contamination by anthropogenic halogenated organic compounds has become popular in recent decades, especially in the subsurface environments. Molecular techniques such as polymerase chain reaction-based fingerprinting methods have been extensively used to closely monitor the presence and activities of dehalogenating microbes, which also lead to the discovery of new dehalogenating bacteria and novel functional genes. Nowadays, traditional molecular techniques are being further developed and optimized for higher sensitivity, specificity, and accuracy to better fit the contexts of dehalogenation. On the other hand, newly developed high throughput techniques, such as microarray and next-generation sequencing, provide unsurpassed detection ability, which has enabled large-scale comparative genomic and whole-genome transcriptomic analysis. The aim of this review is to summarize applications of various molecular tools in the field of microbially mediated dehalogenation of various halogenated organic compounds. It is expected that traditional molecular techniques and nucleic-acid-based biomarkers will still be favoured in the foreseeable future because of relative low costs and high flexibility. Collective analyses of metagenomic sequencing data are still in need of information from individual dehalogenating strains and functional reductive dehalogenase genes in order to draw reliable conclusions.

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Introduction

Pollution caused by anthropogenic halogenated organic compounds has been a serious environmental problem since the middle of the 20th century. Halogenated compounds (including chlorinated and brominated) constitute more than 50% of the top hundred species in the 2007 CERCLA Priority List of Hazardous Compounds (http://www.atsdr.cdc.gov/cercla/07list.html). In order to remove halogenated compounds from the anoxic subsurface environments, bacteria that are capable of reductive dehalogenation are promising because they can replace chlorine/bromine with hydrogen and derive energy for growth, i.e. with chlorinated/brominated ethenes, polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs), chlorinated/brominated phenols, chlorobenzenes, and dioxins, through a process called dehalorespiration (Shelton and Tiedje, 1984; Maymó-Gatell *et al*., 1997; Boyle *et al*., 1999; Holliger *et al*., 1999; Adrian *et al*., 2000; 2007a; Bunge *et al*., 2003; May *et al*., 2008; Ye *et al*., 2010; L.K. Lee *et al*., 2011).

Molecular detection and characterization of dehalogenating bacteria have greatly facilitated application of dehalogenating bacteria in bioremediation. Gene expression studies by reverse-transcript quantitative polymerase chain reaction (RT-qPCR), RNA-sequencing (RNA-seq) and microarray have established links between genes and their dehalogenating activities. Advances in proteomics have initiated the discovery of numerous reductive dehalogenases (RDases) and elucidation of dehalogenation mechanisms. Previously, Northern blotting was used to monitor expression of only a limited number of genes, while microarray technology is able to measure thousands of genes in one chip. For small bacterial genomes, one microarray chip can cover the whole collection of protein-coding genes, allowing a thorough screening of transcript abundance and gene regulation (West *et al*., 2008). Therefore, the novel techniques such as microarrays and next-generation deep genome sequencing further enable the in-depth study of dehalogenating microbes, in aspects that used to require intensive labour

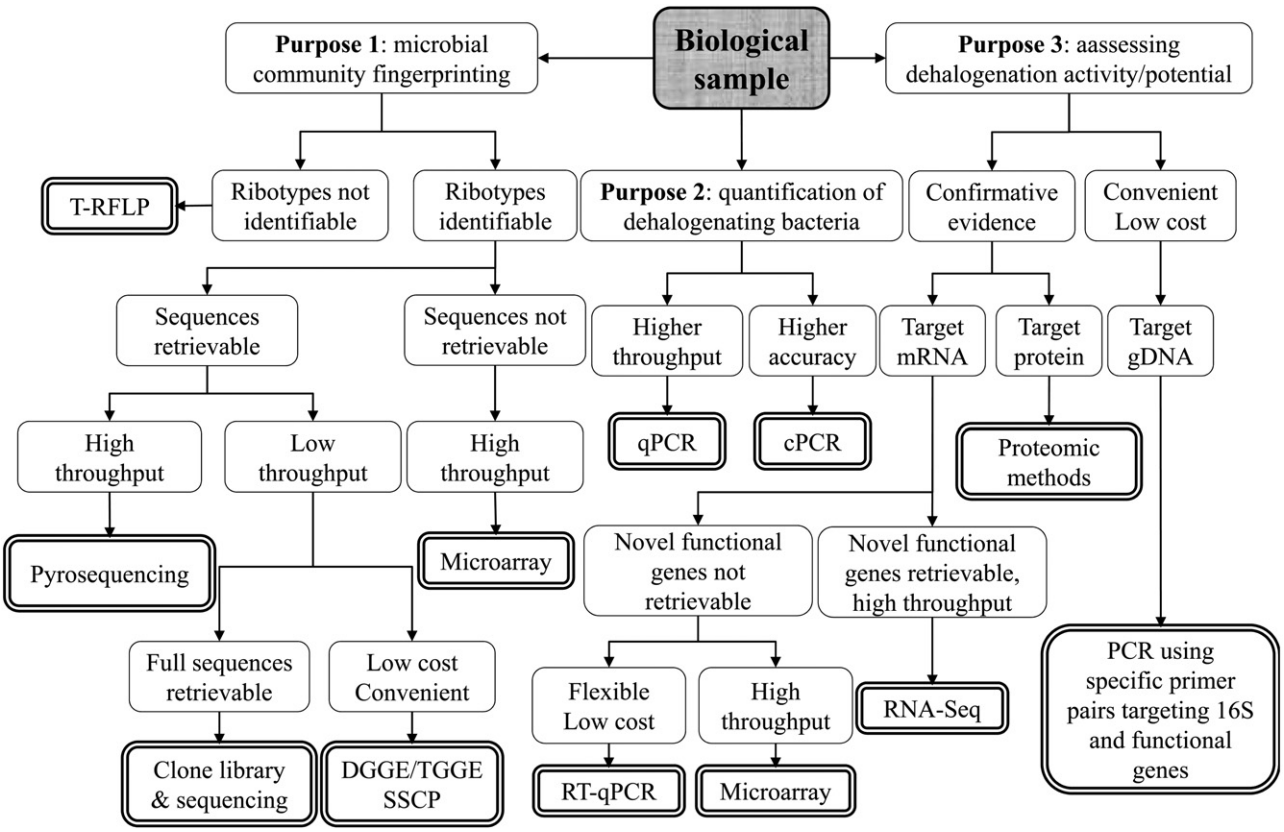

Fig. 1. An overview of molecular techniques utilized in the studies of reductive dehalogenation. Double line text boxes indicate various molecular techniques.

work. Figure 1 depicts various traditional and recently developed molecular techniques that are discussed in this review with their advantages/disadvantages indicated in different scenarios.

This review summarizes molecular techniques that have been utilized or will potentially be applied in studying dehalorespiration of halogenated compounds. It should be noted that there have also been studies on oxygenolytic/hydrolytic dehalogenation as well as co-metabolic reductive dehalogenation, which will not be covered in this review (Fetzner, 1998; Löffler *et al*., 2003; Mattes *et al*., 2010).

Detection and quantification of dehalogenating bacteria

Phylogenetic classification methods

The conventional 16S rRNA gene-based phylogenetic classification method is still widely used because of the large database available. Genus-specific primers targeting 16S rRNA genes are available for the detection of various dehalogenating genera, as summarized in Table 1. However, 16S rRNA gene-based techniques have their disadvantages due to some inherent drawbacks, such as low evolution rates of rRNA gene sequences, and existence of multiple 16S rRNA gene copies in bacterial genomes (Yamamoto and Harayama, 1995; Klappenbach *et al*., 2001; Acinas *et al*., 2004). Moreover, the information on 16S rRNA gene alone may not be enough to confirm the phylogeny of a species/strain. Sometimes, microbes sharing very similar 16S rRNA gene sequences (e.g. > 99.5% similarity) are actually different species based on DNA–DNA hybridization results (e.g. only 41 % similarity via DNA–DNA hybridization) (Fox *et al*., 1992). On the other hand, in the genus of *Desulfitobacterium*, strains previously thought to belong to different species based on 16S rRNA gene sequences were later found to be in the same species according to > 80% homology in DNA–DNA hybridization (Villemur *et al*., 2006).

According to *rrn*DB as of June 2011 (Z.M.P. Lee *et al*., 2009), among the 1074 entries of *Bacteria* and *Archaea*, only 20.2% genomes contain single-copy of 16S rRNA gene operons and the average number of 16S rRNA operons is 3.94 copies per genome (refer to Table 2 for 16S rRNA gene copies in genomes of common dehalogenating bacteria). Multiple and sometimes heteroge-

Table 1. List of genus-specific primer sets targeting 16S genes of dehalogenating bacteria.

Group	Primer	Sequence	T	Size	Specificity	Reference
Chloroflexi	Chl348F Dehal884R	GAG GCA GCA GCA AGG AA GGC GGG ACA CTT AAA GCG	60	470	*Chloroflexi*	Fagervold *et al.* (2005)
Acetobacterium	Aceto572f	GGC TCA ACC GGT GAC ATG CA	59	208	*Acetobacterium* in KB-1	Duhamel and Edwards (2006)
	Aceto784r	ACT GAG TCT CCC CAA CAC CT				
	Aceto572f	GGC TCA ACC GGT GAC ATG CA	63	219		Grostern and Edwards (2009)
	Aceto791r	CTG CGG CAC TGA GTC TCC CC				
Anaeromyxobacter	A60-86F A447-465R	(refer to the reference)				Dollhopf *et al.* (2005)
	60F 461R	CGA GAA AGC CCG CAA GGG ATT CGT CCC TCG CGA CAG T	56.5	401		Petrie *et al.* (2003)
	Ade399Fwd Ade466Rev	GCA ACG CCG CGT GTG T TCC CTC GCG ACA GTG CTT	60	67		Thomas *et al.* (2009)
	2CP444Fwd 2CP513Rev	TCG CGA GGG ACG AAT AAG G CGG TGC TTC CTC TCG AGG TA	60	69	2CP-like strains	Thomas *et al.* (2009)
	F112 R227	GTA ATC TGC CCT AGA GTC CGG A AGA GCG ATA GCT TGT GTA CAG AGG	60	115	*A. dehalogenans* strain 2CP-C	Sanford *et al.* (2007)
Clostridium	Chis150f	AAA GGR AGA TTA ATA CCG CAT AA	57	540	Majority of clusters I and II *Clostridia*	Hung *et al.* (2008)
	ClostIr	TTC TTC CTA ATC TCT ACG CA				
Dehalobacter	Deb179F Deb1007R	TGT ATT GTC CGA GAG GCA ACT CCC ATA TCT CTA CGG	53	828		Schlötelburg *et al.* (2002)
	Dre441F Dre645R	GTT AGG GAA GAA CGG CAT CTG T CCT CTC CTG TCC TCA AGC CAT A	58	225		Smits *et al.* (2004)
	Dre441F Dre1013R	GTT AGG GAA GAA CGG CAT CTG T CGA AGC ACT CCC ATA TCT	58	589		Smits *et al.* (2004)
	Dhb477f	GAT TGA CGG TAC CTA ACG AGG	63	~170		Grostern and Edwards (2006b)
	Dhb647r	TAC AGT TTC CAA TGC TTT ACG G				
Dehalobium	14F Dehal1265R	AGA GTT TGA TCC TGG CTC AG GCT ATT CCT ACC TGC TGT ACC	62	1215	o-17/DF-1-type *Chloroflexi*	Watts *et al.* (2005)
Dehalococcoides	DET730/Dhc730F DET1350/Dhc1350R	GCG GTT TTC TAG GTT GTC CAC CTT GCT GAT ATG CGG	58	620		Bunge *et al.* (2003)
	FL2F/Dhc728F/Dco728F FL2R/Dhc1164R	AAG GCG GTT TTC TAG GTT GTC AC CGT TTC GCG GGG CAG TCT	58	436	*Dehalococcoides* sp. strain FL2	Löffler *et al.* (2000)
	FpDHC1/Dhc1f RpDHC1377/1377R	GAT GAA CGC TAG CGG CG GGT TGG CAC ATC GAC TTC AA	55	1377		Hendrickson *et al.* (2002)[a]
	FpDHC1/Dhc1f Dhc264r	GAT GAA CGC TAG CGG CG CCT CTC AGA CCA GCT ACC GAT CGA A	59	~260		Grostern and Edwards (2009)
	DHE-for DHE-rev	AAG GCG GTT TTC TAG GTT CGT TTC GCG GGG CAG TCT	58	443		Dennis *et al.* (2003); Yan *et al.* (2009a)
	FpDHC1/Dhc1f 259r	GAT GAA CGC TAG CGG CG CAG ACC AGC TAC CGA TCG AA	59	258		Duhamel *et al.* (2004)
	FpDHC1/Dhc1f 1386r	GAT GAA CGC TAG CGG CG CCT CCT TGC GGT TGG CAC ATC	52	1380		Duhamel *et al.* (2004)
	DeF DeR	GCA ATT AAG ATA GTG GC ACT TCG TCC CAA TTA CC	55	1373		Cupples *et al.* (2003)
	FL2F/Dhc728F/Dco728F Dco944R	AAG GCG GTT TTC TAG GTT GTC AC CTT CAT GCA TGT CAA AT	58	216		Smits *et al.* (2004)
	Dhc193f Dhc1048r	GGT TCA YTA AAG CCG YAA GG CCT GTG CAA RYT CCT GAC T	53	855		Dowideit *et al.* (2010)
	567F RpDHC1377/1377R	CGG GAC GTG TCA TTC AAT AC GGT TGG CAC ATC GAC TTC AA	55	436		Fennell *et al.* (2001)
	DHC793f DHC946r	GGG AGT ATC GAC CCT CTC TG CGT TYC CCT TTC TGT TCA CT	60	153		Yoshida *et al.* (2005)
	DHC66f DHC180r	GGT CTT AAG CAA TTA AGA TAG TG CAC CAA GCR CCT TRC GGC	60	114		Yoshida *et al.* (2005)
	DhcForward DhcReverse	GGT AAT ACG TAG GAA GCA AGC G CCG GTT AAG CCG GGA AAT T	60	98	*'D. ethenogenes'* strain 195	Holmes *et al.* (2006)
Dehalogenimonas	(Thirteen primer sets)	(refer to the reference)	63			Yan *et al.* (2009a)

Table 1. *cont.*

Group	Primer	Sequence	T	Size	Specificity	Reference
Desulfitobacterium	Dd1/ Dsb174F	AAT ACC GNA TAA GCT TAT CCC	55	1199		El Fantroussi *et al*. (1997)
	Dd2/Dsb1373R	TAG CGA TTC CGA CTT CAT GTT C				
	Dd3/Dsb460F	TCT TCA GGG ACG AAC GGC AG	55	624		El Fantroussi *et al*. (1997)
	Dd4/Dsb1084R	CAT GCA CCA CCT GTC TCA T				
	Dsb406F	GTA CGA CGA AGG CCT TCG GGT	58	213		Smits *et al*. (2004)
	Dsb619R	CCC AGG GTT GAG CCC TAG GT				
	dsb434f	TAC TGT CTT CAG GGA CGA AC	60	865		Dowideit *et al*. (2010)
	dsb1299r	TGA GAC CAG CTT TCT CGG AT				
	Dsb406F	GTA CGA CGA AGG CCT TCG GGT	58	213		Smits *et al*. (2004)
	Dsb619R	CCC AGG GTT GAG CCC TAG GT				
	Dsb406F	GTA CGA CGA AGG CCT TCG GGT	58	967		Smits *et al*. (2004)
	Dd2/Dsb1373R	TAG CGA TTC CGA CTT CAT GTT C				
Desulfomonile	Dt1/Dsm59F	CAA GTC GTA CGA GAA ACA TAT C	55	995		El Fantroussi *et al*. (1997)
	Dt2/Dsm1054R	GAA GAG GAT CGT GTT TCC ACG A				
	Dt3/Dsm205F	GGG TCA AAG TCG GCC TCT CGA CG	55	423		El Fantroussi *et al*. (1997)
	Dt4/Dsm628R	GCT TTC ACA TTC GAC TTA TCG				
	DSMON85F	CGG GGT RTG GAG TAA AGT GG	62	1334		Loy *et al*. (2004)
	DSMON1419R	CGA CTT CTG GTG CAG TCA RC				
Desulfovibrio	DSV230	GRG YCY GCG TYY CAT TAG C	61	610	*Desulfovibrio*/ *Desulfomicrobium*	Daly *et al*. (2000)
	DSV838	SYC CGR CAY CTA GYR TYC ATC				
	DSB1180F	CCT AGG GCT ACA CAC GTA CTA A	61	225		Grostern and Edwards (2006a)
	DSB1405R	CCG GCT TCG GGT AAA ACC AG				
	DSV691-F	CCG TAG ATA TCT GGA GGA ACA TCA G	63	135		Fite *et al*. (2004)
	DSV826-R	ACA TCT AGC ATC CAT CGT TTA CAG C				
Desulfuromonas	BB1F/Dsf205F	AAC CTT CGG GTC CTA CTG TC	58	815		Löffler *et al*. (2000)
	BB1R/Dsf1020R	GCC GAA CTG ACC CCT ATG TT				
Geobacter	Geo564F	AAG CGT TGT TCG GAW TTA T	57	276	*Geobacteraceae* family	Cummings *et al*. (2003); Sanford *et al*. (2007)[b]
	Geo840R	GGC ACT GCA GGG GTC AAT A				
	Geo196F	GAA TAT GCT CCT GAT TC	53	820	*Geobacter* sp. strain SZ	Sung (2005)
	Geo999R	ACC CTC TAC TTT CAT AG				
	Geo73f	CTT GCT CTT TCA TTT AGT GG	59	412	*Geobacter* sp. strain SZ	Duhamel and Edwards (2006)
	Geo485r	AAG AAA ACC GGG TAT TAA CC				
	Geo196F	GAA TAT GCT CCT GAT TC	50	357	*Geobacter* sp. strain SZ	Amos *et al*. (2007)
	Geo535R	TAA ATC CGA ACA ACG CTT				
	Geo63F	CAG GCC TAA CAC ATG CAA GT	62	1443	*Geobacteraceae* family	Dennis *et al*. (2003)
	Geo418R	CCG ACC ATT CCT TAG GAC				
Sulfurospirillum	Sulfuro114f	GCT AAC CTG CCC TTT AGT GG	59	307	*Sulfurospirillum* in culture KB-1	Löffler *et al*. (2005); Duhamel and Edwards (2006)
	Sulfuro421r	GTT TAC ACA CCG AAA TGC GT				

This table contains most of the primer sets for dehalogenating bacteria, but should not be considered all inclusive. Primers are genus-specific unless specified according to statements in the references. For some primers, more than one primer names are listed, separated by '/'.
a. There are in total seven primer sets in the study by Hendrickson and colleagues (2002), among which three sets need to raise their annealing temperatures to ensure specificity on *Dehalococcoides* according to Yan and colleagues (2009a).
b. This primer set also amplifies *Anaeromyxobacter* 16S rRNA genes according to Bedard and colleagues (2007).

neous 16S rRNA gene copies in a single genome have notable inconvenience when analysing the phylogenetic sequence (Tourova, 2003) and also when querying the abundance of organisms (Fogel *et al*., 1999). For example, in order to query methanogen abundance in dechlorinating consortia, 16S gene copy number per genome in *Methanococcus maripaludis* has be to be estimated in order to convert measured gene copy numbers into actual cell numbers (Daprato *et al*., 2007).

To tackle these problems, the use of the *rpoB* gene (Dahllöf *et al*., 2000), the fast-evolving gene *gyrB* and the internal transcribed spacer (ITS) (Yamamoto and Harayama, 1995; Dauga, 2002; Brown and Fuhrman, 2005; Stingl *et al*., 2007) have been proposed to complement the 16S rRNA gene-based classification method (at least for some phylogenetic groups). The *gyrB*- and ITS-based phylogenetic analysis might serve as a promising way to determine culture's purity and suggest phyloge-

Table 2. Numbers of SSU rRNA gene copies per genome in common dehalogenating bacteria (Villemur *et al*., 2006; Z.M.P. Lee *et al*., 2009).

Genus	Species	Strain	16S	ITS	23S	5S	tRNA
Dehalococcoides	*ethenogenes*	195	1	0	1	1	46
Dehalococcoides	sp.	BAV1	1	0	1	1	46
Dehalococcoides	sp.	CBDB1	1	0	1	1	47
Desulfovibrio	*vulgaris*	DP4	5	5	5	6	68
Desulfovibrio	*desulfuricans*	G20	4	4	4	4	66
Desulfovibrio	*vulgaris*	Hildenborough	5	5	5	6	68
Desulfovibrio	*vulgaris*	Miyazaki F	4	4	4	4	64
Desulfitobacterium	*hafniense*	Y51	6	6	6	6	59
Desulfitobacterium	*hafniense*	DCB-2	6	–	–	–	–
Clostridium	*(Multiple)*	(Multiple)	9	8.72	9	8.81	79.16
Anaeromyxobacter	*dehalogenans*	2CP-C	2	2	2	2	49
Anaeromyxobacter	sp.	Fw109-5	2	2	2	2	49
Anaeromyxobacter	sp.	K	2	2	2	2	49
Enterobacter	sp.	638	7	7	7	8	84
Enterobacter	*sakazakii*	ATCC BAA-894	7	7	7	8	80

All data are presented as of 8Jun2011 from *rrn*DB. SSU rRNA gene copy numbers for *Clostridium* are average of 27 *Clostridium* strains.

netic distances among strains, especially for bacterial groups sharing very similar 16S gene sequences, e.g. '*Dehalococcoides*' (Cheng and He, 2009).

Biomarker based-stable isotope probing (SIP)

Dependence on bacteria's cultivability limits discovery of some difficult-to-cultivate bacterial species that degrade environmental pollutants. This challenge can be circumvented by biomarker based-SIP, which uses stable isotope as a tracer and analyses biomarkers after cells incorporate the isotope-containing substrate into biomass (Radajewski *et al*., 2000; Manefield *et al*., 2002). The available biomarkers include DNA, rRNA, and phospholipid-derived fatty acid (Neufeld *et al*., 2007) as well as mRNA and protein (Jehmlich *et al*., 2010; Dumont *et al*., 2011). SIP works well when the bacteria in query are able to break down targeted substrates and incorporate the labelled atoms into biomass, examples including benzene (Herrmann *et al*., 2010), phenol (Manefield *et al*., 2002), biphenyl (Leigh *et al*., 2007; Sul *et al*., 2009) and nitrotoluenes (Gallagher *et al*., 2010).

However, SIP encounters problems with reductive dehalogenation where assimilation of atoms in the halogenated substrates does not usually take place (Holliger *et al*., 1999). An alternate way is adding ^{13}C-labelled carbon source (usually ^{13}C-acetate) together with unlabelled halogenated compounds to the bacterial consortia, as proposed by Kittelmann and Friedrich (2008a) in a study of microbial community in pristine river sediment. The underlying principle is that acetate-utilizing bacteria should also be actively involved in the dehalogenation process when halogenated compounds are supplied as the major electron acceptors (Kittelmann and Friedrich, 2008a). Following this strategy, several novel bacteria were identified, which played important roles in tetrachloroethene (PCE) dechlorination, such as bacterial cluster LC from river sediments and *Dehalobium* from tidal flat sediments (Kittelmann and Friedrich, 2008a,b). Notably, all the dechlorinators identified in the study of Kittelmann and Friedrich belong to the *Chloroflexi* phylum. It is possible that some dehalogenating bacteria capable of fermentation were missed out by SIP since they may utilize other fatty acids instead of acetate as a carbon source. Therefore, the results of SIP in dehalogenation application are of importance, but may not be considered comprehensive in terms of its coverage of potential dehalogenating bacteria.

Quantification techniques

Quantitative real-time PCR (qPCR) and competitive PCR (cPCR) are two powerful PCR-based nucleic acid quantification techniques. Difference in their mechanisms is that qPCR quantifies fluorescence intensities during the amplification process while cPCR measures signals at the amplification endpoint. In addition, applying molecular fingerprinting techniques on serially diluted samples may also provide quantitative estimates of operational taxonomic units, a strategy called 'qfingerprinting' (Ramette, 2009).

qPCR finds its extremely versatile usage in quantification of dehalogenating species due to its huge merits in producing precise and fast results (Cupples, 2008) and offering high sensitivity compared with terminal restriction fragment length polymorphism (T-RFLP) and RFLP plus clone sequencing (Freeborn *et al*., 2005; Rahm *et al*., 2006a). A fast approach of synthesizing DNA standards and controls using long oligonucleotide hybridization has made the setup of qPCR even more convenient (David *et al*., 2008). The wide applications of qPCR in dehalogenation studies include: (i) establishing relationship between species and dehalogenating activities (Lendvay

et al., 2003; Yoshida *et al*., 2005; Taş *et al*., 2009; 2010a); (ii) examining interactions between dehalogenating bacteria and other species (Duhamel and Edwards, 2006; 2007; Cheng *et al*., 2010); (iii) demonstrating growth-linked dehalorespiration (He *et al*., 2003; Bedard *et al*., 2007; Grostern and Edwards, 2009; Yan *et al*., 2009b; L.K. Lee *et al*., 2011) and assessing culture purity (Sung *et al*., 2006); (iv) assessing spatial and temporal distributions of dehalogenating bacteria (Amos *et al*., 2009); and (v) analysing effects of growth factors on dehalogenating bacteria (He *et al*., 2007). As a standardized method, qPCR is sometimes used as a validation for other quantification methods (Adrian *et al*., 2007a). However, the accuracy and precision of qPCR is prone to interference such as PCR amplification inhibition and differences in PCR amplification/DNA extraction efficiency (Cupples, 2008). Holmes and colleagues (2006) successfully applied a four-gene plasmid standard to lower down the discrepancy between the 16S rRNA gene and RDase gene copy number. However, this methodology lacks flexibility, and can only increase precision of qPCR measurement but not accuracy.

cPCR is more accurate than qPCR in quantifying nucleic acids, showing good reproducibility when detecting very small variations of nucleic acid concentrations (Cupples *et al*., 2003; Zentilin and Giacca, 2007). Recent modification of cPCR (namely, alternately binding probe competitive PCR) allows good fitting standard curve ($R = 0.999$) and lower detection limit (10 copies μl^{-1} template DNA), specifically for *Dehalococcoides* (Miyata *et al*., 2010). cPCR has been used in enumeration of *Dehalococcoides* (Cupples *et al*., 2003; 2004), *Desulfitobacterium* (Lévesque *et al*., 1998), *Dehalobium* (May *et al*., 2008), and the *Chloroflexi* bacteria group (Fagervold *et al*., 2007). Although cPCR is both accurate and reliable, its limitations are obvious, which mainly lie in the construction of competitor standards that need to be as close as possible to the targeted template and in the cumbersome post-PCR electrophoresis-based detection and analysis step (Zentilin and Giacca, 2007). Its throughput is limited as multiple reactions are needed to quantify one single nucleic acid fragment.

Traditional fingerprinting techniques

Various molecular fingerprinting techniques are available aiming at retrieving microbial community structure information. Cloning and sequencing reveal nearly full-length sequences of 16S rRNA genes and thus allow discrimination based on subtle differences in the gene sequences. However, cloning and sequencing are performed at the expense of tedious work and high sequencing costs especially when a large number of clones are needed. Denaturing or temperature gradient gel electrophoresis (DGGE/TGGE) can separate DNA sequences differing only by one base pair (Myers *et al*., 1987; Muyzer and Smalla, 1998), which can be useful in the detection of *Dehalococcoides* strains with high identity of 16S rRNA genes (Hendrickson *et al*., 2002). Due to its low cost, fast results, high sensitivity (as low as 1% of total population), semi-quantitative ability, and good resolution, DGGE has been widely used in characterizing dehalogenating communities (Duhamel *et al*., 2002; 2004; May *et al*., 2008; Narihiro *et al*., 2010). However, DGGE/TGGE has multiple limitations (e.g. relatively short sequences of only 200–400 bp) as described by Muyzer and Smalla (1998). The choice of hypervariable regions (e.g. 'V1, V9 and V3 – the most variable regions' versus 'V1 and V4 – the most heterogeneous regions in terms of melting temperature') in 16S rRNA genes has significant impact on the resolving power of DGGE and thus the diversity implicated for the microbial community (Yu and Morrison, 2004). To further improve DGGE's separation resolution towards complex microbial communities, Wang and He (2011) developed a new method T-RFs-2D that separates terminal restriction fragments (T-RFs) of 16S rRNA genes on a two-dimensional gel electrophoresis. When characterizing a microbial community in a complex river-sediment that dechlorinates PCBs, T-RFs-2D separated 63 DNA fragments, while traditional DGGE detected only 41 DNA fragments in the same sample.

T-RFLP is a sensitive and high-throughput molecular fingerprinting method (Marsh, 1999). It was claimed that T-RFLP detected more 'ribotypes' and was considerably more sensitive than DGGE (Marsh *et al*., 1998). However, T-RFLP has often to be combined with clone library and sequencing to identify each fragment (Bunge *et al*., 2008; Kittelmann and Friedrich, 2008a,b) or with *in silico* analysis based on database sequences (Sung *et al*., 2006). Moreover, because of its non-confirmative results and the emergence of other new high-throughput fingerprinting techniques such as microarray and 16S-pyrosequencing, T-RFLP has become less frequently used except in initial tentative profiling of microbial community structures and changes.

There are other fingerprinting techniques such as single-strand conformation polymorphism (SSCP), amplified ribosomal DNA restriction analysis (ARDRA), and ribosomal intergenic spacer analysis (RISA), which were summarized and compared in a review by Justé and colleagues (2008). They were also occasionally applied in the studies of microbial reductive dehalogenation, albeit at a lower frequency. It should be noted that sometimes there is discrepancy observed in ribotype identities obtained using different 16S-rRNA-gene-based techniques, which may be caused by biases in sample preparations of different techniques. For example, in a study of microbial community in a biofilm sample that aerobically degraded

PCBs, although SSCP and clone library/sequencing detected bacteria species belonging to same genera, none of the sequences obtained by SSCP was identical to the sequences of clones obtained by PCR of 16S rRNA genes or RT-PCR of 16S rRNA (Tillmann *et al*., 2005).

High-throughput fingerprinting techniques

The next-generation sequencing and microarray techniques are developed that could overcome the limitations of traditional fingerprinting techniques, i.e. only limited number of DNA fragments can be displayed on the DGGE/TGGE gel or on the T-RFLP profile.

Next-generation sequencing techniques (Shendure and Ji, 2008) pushed forward genome sequencing by providing a low-cost and ultra-fast sequencing technique, which does not require cloning of sample DNA fragments. One of the sequencing techniques, pyrosequencing, was later applied in high-throughput sequencing of 16S rRNA gene fragments amplified from genomic DNA for microbial community analysis (Roesch *et al*., 2007). Multiplex barcoded pyrosequencing has further enhanced efficiency by pooling together primer barcoded DNA from multiple samples in a single run (Parameswaran *et al*., 2007; Smith *et al*., 2010). Zhang and colleagues (2010) successfully applied massively parallel pyrosequencing of a hypervariable region of the 16S rRNA genes on microbial samples from biofilm reactors with dechlorination activities. *Dehalococcoides* was found to thrive on the biofilm via dechlorinating trichloroethene (TCE), while a more diverse microbial community was observed in the biofilm fed with multiple chlorinated compounds, including sulfate-reducing bacteria (*Desulfovibrio*) and nitrate-reducing bacteria (*Geothrix* and *Pseudomonas*). J. Lee and colleagues (2011) retrieved over 10 000 sequences by using pyrosequencing on tidal flat microbial communities, and found *Desulfuromonas* and *Desulfovibrio* as potential PCE dechlorinators while *Dehalococcoides* was not detected. The pyrosequencing technique possesses a much higher resolution than conventional clone-library based approach.

Phylogenetic oligonucleotide arrays (POAs) (e.g. the PhyloChips) can detect the presence and abundance of *Bacteria* and *Archaea* by hybridization between matched DNA fragments and probes designed to target prokaryotic 16S rRNA genes (Brodie *et al*., 2006). A recent application of PhyloChip revealed a significantly altered community structure when monitoring microbial community prior to and after the oil spillage in the Gulf of Mexico (Hazen *et al*., 2010). In another TCE-contaminated site, PhyloChip measurement exhibited that TCE-respiring *Dehalococcoides* decreased, but methane-oxidizing organisms capable of TCE co-metabolism increased in wells distant from electron donor injection location (Conrad *et al*., 2010). The above observation indicates that electron donor addition that aimed at enhancing reductive dechlorination might also stimulate co-metabolism of TCE. Another POA designed by Sanguin and colleagues (2006a,b) showed that microbial community structure was significantly affected by as low as 1 p.p.m. TCE in soil and the most affected microorganisms from TCE treatment were identified (Nemir *et al*., 2010). However, unlike the PhyloChip which can distinguish bacterial phylogeny down to subfamily level, this POA has only 742 probes and thus possesses a much lower phylogenetic resolution.

The genome-probing microarray (GPM) is another type of microarray that spots bacterial genome DNAs instead of oligonucleotides onto glass slides to query sample DNA (Bae *et al*., 2005). Without the aid of PCR amplification, the detection limit of GPM was 2.5 ng of sample genomic DNA even in the presence of non-target DNA, which was added to test its effect on hybridization and detection sensitivity. The detection sensitivity of GPM was 0.25% of total microbial community. GPM avoided bias caused by PCR amplification and achieved a species-specific detection; however, genomic DNA needs to be prepared for each bacterial strain thus preventing large-scale production of arrays and application of this technique. Another limitation of GPM is that uncultured microorganisms cannot be used to establish genome probes on array chips. However, this was later solved by Chang and colleagues (2008a) using digital multiple displacement amplification to amply genomes from uncultured single bacterial cells. Despite these drawbacks, GPM is believed to have advantage over traditional DNA–DNA hybridization by having higher reproducibility, a lower background, and a less time-consuming procedure (Chang *et al*., 2008b).

Besides the above-mentioned POA and GPM, latter sections of this review will discuss other types of microarrays, i.e. functional gene arrays (FGAs) and whole-genome arrays (WGAs).

Assessing culture purity

Obtaining pure dehalogenating cultures is important for in-depth study of their physiological properties and dehalogenation mechanisms. Culture purity can be indicated by microscopic observation of uniform cell morphology, or by observing a single 16S rRNA fragment as detected by fingerprinting techniques (Yan *et al*., 2009b). In addition to molecular fingerprinting methods as mentioned above to assess culture purity, nested-PCR using genus-specific primer sets (Table 1) would be a recommended approach to detect other populations possibly existing in extreme minor populations. This is due to the fact that in some seemingly pure dehalogenating cultures, there might be another strain that is actually responsible for dehalogenating but could only grow to a very low cell density

caused by the rather low concentrations of halogenated compounds such as PCBs, PBDEs and dioxins. Colony picking from solid phase medium is an important isolation method but should not be relied on as evidence for purity of culture, since other possible taxa could be carried over during the colony picking process such as the case for coculture DPH-1 (Chang *et al*., 2000; Fletcher *et al*., 2008).

Even though all 16S rRNA gene-based techniques indicate a single 16S rRNA gene pattern in a culture, it may still be possible that multiple strains with the same 16S rRNA gene sequence exist, especially for *Dehalococcoides*. This is perfectly demonstrated in the isolation process of *Dehalococcoides* sp. strain GT (Sung *et al*., 2006). To cope with this challenge, RDases have been quantified together with 16S rRNA genes by qPCR based on the fact that common RDase genes such as *tceA* and *bvcA* are single copy genes in the *Dehalococcoides* genome (Krajmalnik-Brown *et al*., 2004; Sung *et al*., 2006). Pure culture identification must be performed carefully with appropriate molecular tools, or conclusions can be questionable. In a study of a PCE-to-ethene dechlorinating culture originated from Bitterfeld (Germany), one single *Dehalococcoides* strain in the culture was claimed to be responsible for all dechlorination steps from PCE to ethene, based on substrate specificity and 16S rRNA gene-based DGGE tests (Cichocka *et al*., 2010). However, the purity of *Dehalococcoides* in the culture was still questionable (unless a simultaneous quantification of RDase genes were performed), although the author attributed the random variations in 16S rRNA gene sequences of *Dehalococcoides* clones to method-introduced errors.

Investigation on dehalogenating activity

Identification of novel functional genes

Techniques available. Identification of novel functional genes responsible for dehalogenation is crucial in elucidating mechanisms of catalytic dehalogenation and in optimizing dehalogenation rates. In particular, dehalogenating bacteria possess RDases that catalyse the terminal electron transfer in the dehalorespiration process (Holliger *et al*., 1999). Ni and colleagues (1995) successfully identified the first RDase from *Desulfomonile tiedjei* strain DCB-1 by using chromatography separation. This RDase catalyses dechlorination of 3-chlorobenzoate to benzoate in an energy-yielding process. Up till now, more than 20 RDases have been linked to specific dehalogenation activities (Table 3), although PCB/PBDE/dioxin-related RDases remain largely undiscovered (Sakaki and Munetsuna, 2010) except for a few tentative cases (Zanaroli *et al*., 2010). Earlier identification of RDases was achieved by protein separation combined with *in vitro* activity test, and subsequent N-terminal sequencing of the enzyme. Later, after more homologue sequences were obtained, degenerate PCR primers based on conserved regions of RDases became popular in pulling out putative RDases. The rapid development of next-generation sequencing and microarray techniques also greatly aided novel RDase identification. Figure 2 depicts the common workflow of RDase gene identification.

Proteomic methods can identify RDases with no prior knowledge of the RDase gene sequences. Separation of whole cell proteins is achieved by either liquid chromatography (LC) (Magnuson *et al*., 1998) or polyacrylamide gel electrophoresis (PAGE) (Adrian *et al*., 2007b). Differential abundance of proteins in cultures with or without the targeted halogenated compounds may also give a hint of some possible candidates, since most RDase genes are inducible rather than constitutive (Cole *et al*., 1995; Lee *et al*., 2006). However, when using comparative proteomics technique to pick out potential RDases, one needs to bear in mind that differentially expressed genes might also result from the response of bacteria towards toxic substances and thus cannot guarantee a positive identification. After separation of proteins, native PAGE gel bands or LC effluent fractions containing RDase activities are collected for further analysis, e.g. SDS-PAGE (Ni *et al*., 1995; Magnuson *et al*., 1998; Maillard *et al*., 2003), N-terminal amino acid sequencing (Miller *et al*., 1998), or mass spectrometry (Thibodeau *et al*., 2004; Adrian *et al*., 2007b).

For certain bacterial species such as those within the *Chloroflexi* phylum, the commonly encountered problem in identification of RDases is extremely low biomass, which may be due to low energy yield and growth rate under anaerobic conditions, and may also be due to low solubility of some chloroaromatic compounds. Low biomass severely limits the application of proteomic techniques that usually require a large protein amount in order to ensure successful detection, either in gel or by chromatography (Müller *et al*., 2004; Adrian *et al*., 2007b). With accumulating RDase sequences identified (confirmed or putative) in recent years, using degenerate PCR primers to probe unknown cultures has become more popular (Table 4) (Krajmalnik-Brown *et al*., 2004; Regeard *et al*., 2004; Chow *et al*., 2010). To increase the chances of finding the most expressed RDases by degenerate primers, construction of clone library based on complementary DNA (cDNA) rather than on genomic DNA is helpful (Lee *et al*., 2008). It is impractical to cover all possible RDase sequences using one degenerate primer set as demonstrated by Wagner and colleagues (2009), who designed 13 primer sets to cover 32 RDases in *Dehalococcoides* sp. strain CBDB1. It is perceivable that clone libraries with multiple degenerate primer sets may retrieve more putative RDases, but work load is significantly higher.

Table 3. List of identified reductive dehalogenases and approaches employed.

RDase	Microorganism	Main substrate	Identification technique	Reference
3-ClBA–RD	*Desulfomonile tiedjei* strain DCB-1	3-chlorobenzoate	LC + *in vitro*	Ni *et al*. (1995)
CprA	*Desulfitobacterium chlororespirans* strain Co23	3-chloro-4-hydroxybenzoate, chlorinated phenols	LC + *in vitro* + Amino	Löffler *et al*. (1996); Krasotkina *et al*. (2001)
PceA	*Sulfurospirillum multivorans*	PCE, TCE, *cis*-/*trans*-DBE	LC + *in vitro* + Amino	Neumann *et al*. (1996; 1998); Ye *et al*. (2010)
PceA	*Dehalobacter restrictus*	PCE, TCE	LC + *in vitro* + Amino	Schumacher *et al*. (1997); Maillard *et al*. (2003)
PceA	*Dehalococcoides ethenogenes* strain 195	PCE	LC + *in vitro* + PAGE	Magnuson *et al*. (1998)
TceA	*Dehalococcoides ethenogenes* strain 195	TCE	LC + *in vitro* + PAGE	Magnuson *et al*. (1998; 2000)
PceA	*Desulfitobacterium hafniense* strain PCE-S	PCE, TCE, *cis*-/*trans*-DBE, VB	LC + *in vitro* + Amino	Miller *et al*. (1998); Ye *et al*. (2010)
CprA	*Desulfitobacterium hafniense* strain DCB-2	3-chloro-4-hydroxyphenylacetate	LC + *in vitro* + Amino	Christiansen *et al*. (1998)
CprA	*Desulfitobacterium dehalogenans*	A number of *ortho*-chlorinated phenols	LC + *in vitro* + Amino	van de Pas *et al*. (1999)
PceA	*Desulfitobacterium* sp. strain PCE1	PCE	LC + *in vitro* + Amino	van de Pas *et al*. (2001)
CprA	*Desulfitobacterium* sp. strain PCE1	Cl-OH-phenylacetate	LC + *in vitro* + Amino	van de Pas *et al*. (2001)
PceA	*Desulfitobacterium hafniense* strain TCE1	PCE, TCE	LC + *in vitro* + Amino	van de Pas *et al*. (2001)
PceC	Coculture DPH-1 (containing *Desulfitobacterium hafniense* strain JH1)	PCE, TCE	LC + *in vitro* + Amino	Okeke *et al*. (2001)
PceA	*Desulfitobacterium* sp. strain Y51	PCE, TCE	LC + *in vitro* + Amino	Suyama *et al*. (2002)
CrdA	*Desulfitobacterium hafniense* strain PCP-1	2,4,6-TCP, PCP	LC + *in vitro* + Amino + Genome	Boyer *et al*. (2003)
CprA5	*Desulfitobacterium hafniense* strain PCP-1	3,5-DCP	LC + *in vitro* + MS + Genome	Thibodeau *et al*. (2004)
VcrA	*Dehalococcoides* sp. strain VS	VC, *cis*-/*trans*-/1,1-DCE	LC + *in vitro* + Amino	Müller *et al*. (2004)
BvcA	*Dehalococcoides* sp. strain BAV1	VC	Dege + qPCR	Krajmalnik-Brown *et al*. (2004)
CbrA	*Dehalococcoides* sp. strain CBDB1	Chlorinated benzenes	PAGE + *in vitro* + MS + Dege + T-RFLP + Genome	Adrian *et al*. (2007b; Wagner *et al*. (2009)
DcaA	*Desulfitobacterium dichloroeliminans* strain DCA1	1,2-DCA	Dege + qPCR	Marzorati *et al*. (2007)
CBDBA1453	*Dehalococcoides* sp. strain CBDB1	1,2,3-TCB	Dege + T-RFLP + Genome	Wagner *et al*. (2009)
CBDBA187	*Dehalococcoides* sp. strain CBDB1	1,2,3-TCB	Dege + T-RFLP + Genome	Wagner *et al*. (2009)
CBDBA1624	*Dehalococcoides* sp. strain CBDB1	1,2,4-TCB	Dege + T-RFLP + Genome	Wagner *et al*. (2009)
WL RdhA1	*Dehalobacter* sp.	1,2-DCA	Dege + qPCR	Grostern and Edwards (2009)
(eight RdhAs)	*Dehalococcoides* culture TUT2264	Chloroethenes	Dege + qPCR	Futamata *et al*. (2009)
MbrA	*Dehalococcoides* sp. strain MB	TCE	Dege + qPCR	Chow *et al*. (2010)
CprA3	*Desulfitobacterium hafniense* strain PCP-1	PCP, TeCP, TCP	LC + *in vitro* + MS + Genome	Bisaillon *et al*. (2010)

Techniques: LC, chromatography separation; Amino, amino acid sequencing; PAGE, PAGE gel separation; Dege, degenerate primer detection; *in vitro, in vitro* activity test of RDases; Genome, sequenced genome of the targeted strain; MS, mass spectrometry detection of peptides; qPCR, transcriptional analysis by qPCR; T-RFLP, transcriptional analysis by T-RFLP.
Compounds: PCP, pentachlorophenol; TeCP, tetrachlorophenol; TCP, trichlorophenol; DCP, dichlorophenol; PCE, tetrachloroethene; TCE, trichloroethene; DCE, dichloroethene VC, vinyl chloride; DCA: dichloroethane; TCB, trichlorobenzene; DBE, dibromoethene; VB, vinyl bromide.

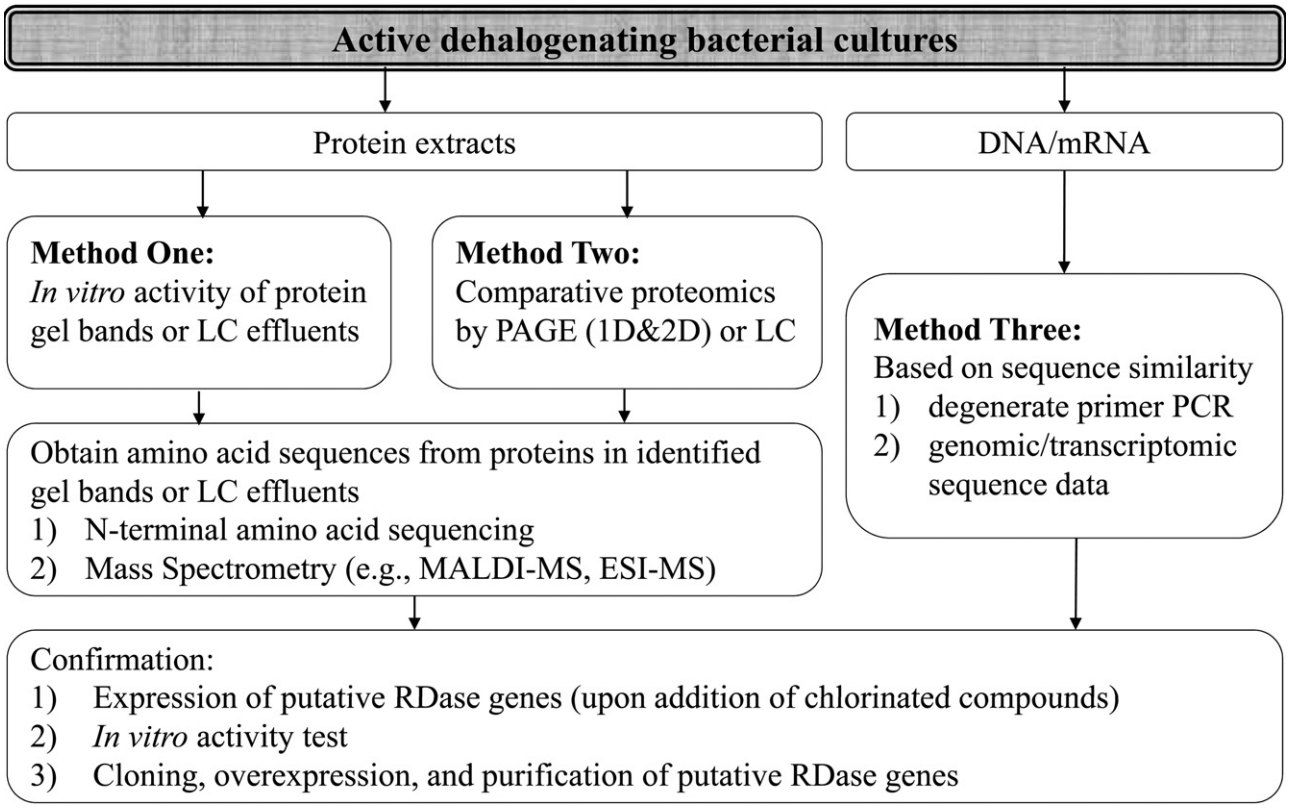

Fig. 2. Common work flow of reductive dehalogenase gene identification.

Metagenomic sequencing and whole-genome sequencing extract huge amounts of sequence information from bacterial genomes, and thus pave the way for rapid identification of novel putative RDases. For example, the complete genome sequence of *Dehalococcoides* sp. strain CBDB1 revealed 32 putative RDases, implying the enormous dehalogenating potential of this microbe (Kube *et al*., 2005). Recently released complete genome sequence of the novel *Chloroflexi* microbe *Dehalobium chlorocoercia* strain DF-1 by J. Craig Venter Institute revealed at least 35 putative RDases, which may be responsible for DF-1's ability to dechlorinate PCB congeners as well as chlorinated ethenes (http://www.jcvi.org). Chan and colleagues (2010) verified activities of putative hydrolytic dehalogenases identified from five sequenced microbial genomes by expressing them in *E. coli*. The strategy of cloning, overexpression and purification of selected proteins as adopted in this study proved to be effective in screening potential functional genes from genome sequencing data.

Similar to the degenerate primers method, putative RDase genes in genome sequences can only be identified if they share a significant sequence similarity with identified RDases, thus certain novel RDases may be missed out if they are only distantly related with existing RDases. To tackle this, sequencing of bacterial transcriptome is a promising way to select possible candidates among the most abundant transcripts (Mao *et al*., 2008; Ansorge, 2009). In such scenarios, there will be more positive identifications because transcripts with either small sizes or low BLAST scores in public databases will still be identified as long as they are highly expressed upon the addition of halogenated compounds.

Understanding RDase structures. So far, several consensus sequences in RDases have been identified to be related to reductive dehalogenation, such as the iron–sulfur cluster binding motifs, cobalamin binding motifs, and twin-arginine signal sequence (Hölscher *et al*., 2004), as well as some conserved amino acid residues (e.g. tryptophan and histidine) that are potentially involved in catalysis of chloroethenes (Smidt *et al*., 2000). It is known that critical changes of amino acids in active sites may cause significant shift in catalytic activities as demonstrated in studies on hydrolytic dehalogenases (Pavlova *et al*., 2009; Beloqui *et al*., 2010). Obtaining such information with RDases will help in modification of RDases to achieve higher catalytic rates and broader substrate ranges.

Up to now, what we know about RDase catalysing mechanisms is still limited to the above-mentioned conserved regions. While next-generation gene sequencing

Table 4. List of degenerate primer sets for reductive dehalogenase gene identification.

Primer pair	Primer	Targeted region	Size	Detected RDase genes	Reference
1	RRF2	Twin arginine motif in strain 195	1500 ~ 1700	7 RDase genes in BAV1 including *bvcA*	Krajmalnik-Brown *et al*. (2004)
				7 RDases genes in MB including *mbrA* (together with RDH F1C/R1C)	Chow *et al*. (2010)
	B1R	'WYEW' motif in B genes in strain 195		13 RDase genes in CBDB1, 14 RDases in FL2	Hölscher *et al*. (2004)
				8 RDase genes in culture TUT2264	Futamata *et al*. (2009)
				4 RDase genes in environmental samples, including two novel RDases	Lee *et al*. (2008)
2	RDH F1C RDH R1C	Twin arginine motif 'PIDD' motif	1200	7RDases genes in MB including *mbrA* (together with RRF1 and B1R)	Chow *et al*. (2010)
3	mern2 mern5	Upstream of ISB region of orfA in strain CBDB1	1000	One RDase gene in CBDB1	Hölscher *et al*. (2004)
4	fdehal rdehal	Upstream of ISB region of orfA in strain CBDB1	500	Two RDase genes in CBDB1	Hölscher *et al*. (2004)
5	ceRD2L/ ceRD2S	Conserved sequence: 'AARLFGA(D/S)(L/S)VG'	750 ~ 900	Two known *pceA*, one new RDase gene in *Desulfitobacterium* sp.	Regeard *et al*. (2004)
				dcaA in *Desulfitobacterium dichloroeliminans* strain DCA1	Marzorati *et al*. (2006; 2007)
	RD7r	Conserved sequence: 'C(V/E)AVCP'		7 RDase genes (together with RRF2/RD7r)	Grostern and Edwards (2009)
6	ceRD2L/ ceRD2S RD5r	Conserved sequence: 'AARLFGA(D/S)(L/S)VG' Conserved sequence: 'P(D/T)KPI(D/K)(A/F)G'	550	Two new RDase genes in *D. restrictus*, one new RDase in *S. multivorans*	Regeard *et al*. (2004)
7	RD4f/RD4r/ RD5f	(refer to the reference)		No amplicons	Regeard *et al*. (2004)
8	RRF2 RD7r	Twin arginine motif in strain 195 Conserved sequence: 'C(V/E)AVCP'	1000	7RDase genes (together with ceRD2L/S&RD7r)	Grostern and Edwards (2009)
9	Dhu1080f Dhu1350r	highly conserved ISB region	450	Two RDase genes in a 2-bromophenol-degrading consortium	Rhee *et al*. (2003)
10	Dhar1000f	highly conserved iron–sulfur cluster binding motifs	350	One RDase gene in a 2-bromophenol-degrading consortium	Rhee *et al*. (2003)
	Dhu1350r			No amplicons	Kittelmann and Friedrich (2008b)
11	(unnamed primer sets)	Conserved regions in several known *pceA* genes	330	Two *pceA* genes	Kimoto *et al*. (2010)

has yielded billions of base pairs of gene sequences from either isolates or environmental samples, interestingly, the boosting RDase gene pools have not brought in revolutionary insights into structure–function analysis of the RDases. One reason is that expressing RDase genes in host cells is difficult due to their instability after purification and absence of activity after overexpression (Neumann *et al*., 1998; Sakaki and Munetsuna, 2010). More needs to be done to overcome such difficulties when trying to produce active RDases since this is the prerequisite for site-specific mutagenesis and further identification of RDase active sites. One possible solution is to create genetically modified strains using natural dehalogenating bacteria, either by modifying genes on chromosomes or introducing expression vectors containing RDase gene sequences. This strategy takes advantage of the natural transcription/translation in these bacteria and may circumvent the difficulties in constructing a suitable expression system in the commonly used *Escherichia coli* host strains.

On the other hand, deficiency in analysis of metagenomic data hinders novel enzyme identification (Fernández-Arrojo *et al*., 2010). It should be noted that sequencing data only provide an inventory of genes rather than proofs in functionality. A significant portion (5%) of open reading frames in the newly sequenced genomic data have little homology with genes of known functions, implying for many previously undescribed genes (Harrington *et al*., 2007). Also, miss-annotation exists in gene databases, especially in those without manual curation (Schnoes *et al*., 2009). To facilitate protein identification, semi-rational protein design that utilizes computational tools has become popular recently (Beloqui *et al*., 2010; Lutz, 2010). By preselecting promising target sites and limiting amino acid diversity, semi-rational protein design greatly reduces library sizes, which are usually large in

directed evolution of proteins. Thus, it holds promise for identification and modification of novel RDases in future.

Monitoring dehalogenating activities

Biomarkers indicating dehalogenating activities. Traditionally, the most evident sign for dehalogenation activity is direct monitoring of microbial degradation of substrates *in situ* (Kjellerup *et al*., 2008). However, biomarker-based techniques (e.g. DNA, mRNA, protein and phospholipid) are mainstream detection methods due to their high sensitivity (White *et al*., 2005; Lee *et al*., 2008; Futamata *et al*., 2009; Lu *et al*., 2009; Werner *et al*., 2009). DNA fragments such as 16S rRNA genes (Lu *et al*., 2009) or functional genes of dehalogenating bacteria indicate dehalogenating potential but are only indirectly related to dehalogenating activity because: (i) quantification of cell numbers (16S rRNA gene copies) often does not reflect the actual physiological state of the microbial community (Röling, 2007), as shown by the discrepancy between dehalogenating bacteria cell counts and *in situ* activity (Freeborn *et al*., 2005; Ritalahti *et al*., 2010); and (ii) functional genes may be present but not expressed at all, or targeted functional genes do not cover the entire group of genes with similar functions, since the current RDase database is far from complete (Ritalahti *et al*., 2010).

In view of the limitation of gene copy numbers, it is recommended to monitor gene expression (mRNA abundance) in order to assess *in situ* dehalogenation activity, using techniques such as RT-qPCR and microarrays. To account for mRNA loss during sample preparation, the addition of exogenous internal reference mRNA substantially improved the quantification accuracy for laboratory cultures (Johnson *et al*., 2005; Futamata *et al*., 2009). Transcripts of key functional genes such as RDase genes were found to correlate with active dechlorination of chlorinated ethenes (Lee *et al*., 2006; Futamata *et al*., 2009). For example, Wagner and colleagues (2009) adopted an innovative T-RFLP method to monitor the expression of all 32 RDases in strain CBDB1 genome, which is less labour-intensive and more cost-effective. However, because of primer degeneracy, certain low level transcripts were not successfully amplified. Moreover, primer degeneracy also leads to biased PCR amplification among different RDase transcripts, making this method only semi-quantitative. Nevertheless, T-RFLP seems promising for simultaneous monitoring of gene homologues other than 16S rRNA genes as long as suitable primer sets are available. Besides tracking the RDase transcripts, some other key genes (e.g. hydrogenase genes) in the respiratory chain may also be monitored for assessing microbial activities (Rahm *et al*., 2006b; Rahm and Richardson, 2008a,b; Rowe *et al*., 2008). It is noteworthy to point out that under certain stress conditions (e.g. elevated temperature and presence of oxygen), expression of functional genes may be upregulated but the corresponding microbial activity does not elevate simultaneously (Amos *et al*., 2008; Fletcher *et al*., 2011).

Proteins translated from mRNA are more confirmative evidence for dehalogenating activities because specific RDases directly catalyse the transformation of halogenated compounds. However, protein biomarkers are less utilized compared with nucleic acids because of lack of convenient and sensitive method for their detection and identification. Based on available *Dehalococcoides* genome annotation, mass spectrometry can identify specific peptides matching several respiratory enzymes (e.g. hydrogenases, formate dehydrogenase and several strain-specific RDases) present in active dechlorinating cultures, which may be used as biomarkers in environmental samples (Morris *et al*., 2006; 2007; Fung *et al*., 2007). However, conventional mass-spectrometry-based proteomic analyses are susceptible to contaminating proteins, and can only be carried out in less complex systems, or in membrane-associated cell fractions (Morris *et al*., 2007). Werner and colleagues (2009) introduced a highly selective and sensitive protein identification method in the detection of specific proteins in a complex environment. This method, referred to as multiple-reaction monitoring mass spectrometry, is able to quantify as low as 5 fmol peptide and requires protein from merely 1.4×10^6 *Dehalococcoides* cells for analysis.

Transcriptomic analysis by microarray and next-generation sequencing. The construction of microarray and next-generation sequencing techniques has turned high-throughput transcriptomic analysis into reality (Schena *et al*., 1995; Wang *et al*., 2009). Metabolism of key dechlorinators such as '*Dehalococcoides ethenogenes*' strain 195 is of constant interest to researchers. A WGA was designed to cover > 99% of the predicted protein-coding sequences for strain 195, based on which a series of studies were performed to query its metabolic pathways (Johnson *et al*., 2008; 2009; West *et al*., 2008; Tang *et al*., 2009). Using this array, changes in the strain 195 transcriptome were captured and linked to availability of growth factors such as corrinoid cofactor, electron acceptor, electron donor, carbon source and nitrogen source (Johnson *et al*., 2008; 2009; P.K.H. Lee *et al*., 2009). When targeting on genomic DNA rather than mRNA, comparative genomics by using WGAs have yielded interesting results in analysing intraspecies genome mutations among *Dehalococcoides* (West *et al*., 2008; P.K.H. Lee *et al*., 2011). The above studies show that although *Dehalococcoides* strains are similar to each other in genomes, they differ in genes located in integrated elements or high-plasticity regions where RDase genes usually locate.

Functional gene arrays target genes involved in key metabolic processes and are used to study microbially mediated geochemical, ecological and environmental processes, such as E-FGA (McGrath *et al*., 2010) and GeoChip (He *et al*., 2010). In the GeoChip 3.0, the number of probes was increased to 27 812, covering 56 990 functional genes for carbon, nitrogen, phosphorus and sulfur cycles, energy metabolism, and notably, degradation of organic contaminants including chlorinated compounds (He *et al*., 2010). Both genomic DNA and cDNA from reverse transcribed RNA can be detected by GeoChip, since its probes were designed based on protein-coding gene sequences (He *et al*., 2010). GeoChip found its versatile usage in tracking functional microbial communities in bioremediation sites (Leigh *et al*., 2007; Taş *et al*., 2009; Van Nostrand *et al*., 2009). An example was the study of dechlorinating community in soil samples from Ebro River (Taş *et al*., 2009), in which new probes were designed and added to the array in order to cover all RDases in public databases. Results showed that *Dehalococcoides* activity varied significantly at different locations.

In recent years, sequencing of cDNA library using next-generation sequencing techniques, termed as RNA-Seq, has gained enormous attention in the study of transcriptomics (Wang *et al*., 2009). Comparisons of microarray and RNA-Seq are frequently made, usually in favour of RNA-Seq in view of inherent limitations of microarray techniques (Shendure, 2008; Wang *et al*., 2009). Nevertheless, microarray is still frequently used because of shorter time to retrieve results and reasonable cost (Agarwal *et al*., 2010). It is expected that with reducing sequencing costs and further improvement of protocols, RNA-Seq will gradually replace microarray in most bacterial genome-wide transcriptomic analyses (Croucher and Thomson, 2010).

Future perspective

Rapid development of molecular techniques has revolutionized the study of dehalogenation in many ways, and some basic issues need to be re-evaluated, such as choices of biomarkers, evolution of mainstream techniques and overall analysis strategies.

From nucleic acids to peptide fragments as targeted biomarkers?

It is interesting to know whether characteristic peptides will surpass DNA/RNA and become the most frequent bioremediation biomarkers in the future. There are predictions of expecting the rise of proteomics in functional microbial ecology, judging from recent research trends that incorporate more data from shotgun proteomics (Maron *et al*., 2007; Desai *et al*., 2010). However, unlike nucleic acids which can be conveniently amplified and targeted, protein detection lacks suitable amplification methods, and is limited by sophisticated instrumentations. Therefore, before fast and high-throughput protein sequencing techniques become available, genomics/transcriptomics are still the most welcome approaches we can rely on to obtain an overall picture of microbial status *in situ*.

From traditional low-throughput techniques to novel high-throughput techniques?

Current PCR-based quantification/fingerprinting techniques are continuously being optimized to better suit to characterize dehalogenating microbial communities, to improve coverage, specificity and sensitivity in the detection of dehalogenating bacteria. On the other hand, emerging sequencing and microarray techniques allow analysis of very complex microbial community and thorough screening of gene expression in the microbial genome, which greatly facilitate the identification of functional genes and their regulation mechanisms with decreasing costs of genome/metagenome sequencing and tailor-made microarrays. Nevertheless, relative low costs and high flexibility will still keep traditional low-throughput molecular tools important in monitoring key dehalogenating bacteria and functional genes. The two sets of techniques are perfectly complementary to each other rather than replacing one for another.

From specific microbes and genes to an integrated network?

Taş and colleagues (2010b) argued an end of the so-called reductionist approaches in the studies of *Dehalococcoides*, which are confined to only a few selective biomarkers. Instead, they contended for a switch to a strategy aiming at the entire bioremediation system. Similar points were also raised out by Vieites and colleagues (2009) and Frias-Lopez and colleagues (2008). Although it is absolutely necessary to view the behaviour of microbes and functional genes collectively, it is still too early to move our focus from key dehalogenating bacteria and biomarkers to the holistic approaches. For example, many uncertainties and contributing factors exist in the analysis of metagenomic/metatranscriptomic sequencing data, such as miss-annotation of genes and lack of information on protein function analyses. More information is needed on functional genes and their regulation mechanisms before we can expect reliable inferences of relationship between genes and activities from next-generation sequencing data.

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