Terry J. McGenity
Kenneth N. Timmis
Balbina Nogales   *Editors*

Hydrocarbon and Lipid Microbiology Protocols

Primers

Springer

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Terry J. McGenity · Kenneth N. Timmis · Balbina Nogales
Editors

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Editors
Terry J. McGenity
School of Biological Sciences
University of Essex
Colchester, Essex, UK

Kenneth N. Timmis
Institute of Microbiology
Technical University Braunschweig
Braunschweig, Germany

Balbina Nogales
Department of Biology
University of the Balearic Islands
  and Mediterranean Institute for Advanced
  Studies (IMEDEA, UIB-CSIC)
Palma de Mallorca, Spain

Preface to Hydrocarbon and Lipid Microbiology Protocols[1]

All active cellular systems require water as the principal medium and solvent for their metabolic and ecophysiological activities. Hydrophobic compounds and structures, which tend to exclude water, although providing *inter alia* excellent sources of energy and a means of biological compartmentalization, present problems of cellular handling, poor bioavailability and, in some cases, toxicity. Microbes both synthesize and exploit a vast range of hydrophobic organics, which includes biogenic lipids, oils and volatile compounds, geochemically transformed organics of biological origin (i.e. petroleum and other fossil hydrocarbons) and manufactured industrial organics. The underlying interactions between microbes and hydrophobic compounds have major consequences not only for the lifestyles of the microbes involved but also for biogeochemistry, climate change, environmental pollution, human health and a range of biotechnological applications. The significance of this "greasy microbiology" is reflected in both the scale and breadth of research on the various aspects of the topic. Despite this, there was, as far as we know, no treatise available that covers the subject. In an attempt to capture the essence of greasy microbiology, the *Handbook of Hydrocarbon and Lipid Microbiology* (http://www.springer.com/life+sciences/microbiology/book/978-3-540-77584-3) was published by Springer in 2010 (Timmis 2010). This five-volume handbook is, we believe, unique and of considerable service to the community and its research endeavours, as evidenced by the large number of chapter downloads. Volume 5 of the handbook, unlike volumes 1–4 which summarize current knowledge on hydrocarbon microbiology, consists of a collection of experimental protocols and appendices pertinent to research on the topic.

A second edition of the handbook is now in preparation and a decision was taken to split off the methods section and publish it separately as part of the Springer Protocols program (http://www.springerprotocols.com/). The multi-volume work *Hydrocarbon and Lipid Microbiology Protocols*, while rooted in Volume 5 of the Handbook, has evolved significantly, in terms of range of topics, conceptual structure and protocol format. Research methods, as well as instrumentation and strategic approaches to problems and analyses, are evolving at an unprecedented pace, which can be bewildering for newcomers to the field and to experienced researchers desiring to take new approaches to problems. In attempting to be comprehensive – a one-stop source of protocols for research in greasy microbiology – the protocol volumes inevitably contain both subject-specific and more generic protocols, including sampling in the field, chemical analyses, detection of specific functional groups of microorganisms and community composition, isolation and cultivation of such organisms, biochemical analyses and activity measurements, ultrastructure and imaging methods, genetic and genomic analyses,

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[1] Adapted in part from the Preface to *Handbook of Hydrocarbon and Lipid Microbiology*.

systems and synthetic biology tool usage, diverse applications, and the exploitation of bioinformatic, statistical and modelling tools. Thus, while the work is aimed at researchers working on the microbiology of hydrocarbons, lipids and other hydrophobic organics, much of it will be equally applicable to research in environmental microbiology and, indeed, microbiology in general. This, we believe, is a significant strength of these volumes.

We are extremely grateful to the members of our Scientific Advisory Board, who have made invaluable suggestions of topics and authors, as well as contributing protocols themselves, and to generous *ad hoc* advisors like Wei Huang, Manfred Auer and Lars Blank. We also express our appreciation of Jutta Lindenborn of Springer who steered this work with professionalism, patience and good humour.

Colchester, Essex, UK   Terry J. McGenity
Braunschweig, Germany   Kenneth N. Timmis
Palma de Mallorca, Spain   Balbina Nogales

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About the Editors

Terry J. McGenity is a Reader at the University of Essex, UK. His Ph.D., investigating the microbial ecology of ancient salt deposits (University of Leicester), was followed by postdoctoral positions at the Japan Marine Science and Technology Centre (JAMSTEC, Yokosuka) and the Postgraduate Research Institute for Sedimentology (University of Reading). His overarching research interest is to understand how microbial communities function and interact to influence major biogeochemical processes. He worked as a postdoc with Ken Timmis at the University of Essex, where he was inspired to investigate microbial interactions with hydrocarbons at multiple scales, from communities to cells, and as both a source of food and stress. He has broad interests in microbial ecology and diversity, particularly with respect to carbon cycling (especially the second most abundantly produced hydrocarbon in the atmosphere, isoprene), and is driven to better understand how microbes cope with, or flourish in hypersaline, desiccated and poly-extreme environments.

Kenneth N. Timmis read microbiology and obtained his Ph.D. at Bristol University, where he became fascinated with the topics of environmental microbiology and microbial pathogenesis, and their interface pathogen ecology. He undertook postdoctoral training at the Ruhr-University Bochum with Uli Winkler, Yale with Don Marvin, and Stanford with Stan Cohen, at the latter two institutions as a Fellow of the Helen Hay Whitney Foundation, where he acquired the tools and strategies of genetic approaches to investigate mechanisms and causal relationships underlying microbial activities. He was subsequently appointed Head of an Independent Research Group at the Max Planck Institute for Molecular Genetics in Berlin, then Professor of Biochemistry in the University of Geneva Faculty of Medicine. Thereafter, he became Director of the Division of Microbiology at the National Research Centre for Biotechnology (GBF)/now the Helmholtz Centre for Infection Research (HZI) and Professor of Microbiology at the Technical University Braunschweig. His group has worked for many years, *inter alia*, on the biodegradation of oil hydrocarbons, especially the genetics and regulation of toluene degradation, pioneered the genetic design and experimental evolution of novel catabolic activities, discovered the new group of marine hydrocarbonoclastic bacteria, and conducted early genome sequencing of bacteria that

became paradigms of microbes that degrade organic compounds (*Pseudomonas putida* and *Alcanivorax borkumensis*). He has had the privilege and pleasure of working with and learning from some of the most talented young scientists in environmental microbiology, a considerable number of which are contributing authors to this series, and in particular Balbina and Terry. He is Fellow of the Royal Society, Member of the EMBO, Recipient of the Erwin Schrödinger Prize, and Fellow of the American Academy of Microbiology and the European Academy of Microbiology. He founded the journals *Environmental Microbiology, Environmental Microbiology Reports* and *Microbial Biotechnology*. Kenneth Timmis is currently Emeritus Professor in the Institute of Microbiology at the Technical University of Braunschweig.

Balbina Nogales is a Lecturer at the University of the Balearic Islands, Spain. Her Ph.D. at the Autonomous University of Barcelona (Spain) investigated antagonistic relationships in anoxygenic sulphur photosynthetic bacteria. This was followed by postdoctoral positions in the research groups of Ken Timmis at the German National Biotechnology Institute (GBF, Braunschweig, Germany) and the University of Essex, where she joined Terry McGenity as postdoctoral scientist. During that time, she worked in different research projects on community diversity analysis of polluted environments. After moving to her current position, her research is focused on understanding microbial communities in chronically hydrocarbon-polluted marine environments, and elucidating the role in the degradation of hydrocarbons of certain groups of marine bacteria not recognized as typical degraders.

Introduction to Primer-Based Detection of Microbial Genes, Particularly Those Encoding Enzymes for Aromatic/Aliphatic Hydrocarbon Biodegradation

Howard Junca

Abstract

One major contribution to understand the microbial ecology of biodegradation of pollutants in the environment has been the retrieval of information by using primers targeting genes that code for enzymes acting on aromatic or aliphatic hydrocarbons, therefore helping to survey and understand the microbial catabolome related with pollutant biodegradation in contaminated environments. In this introduction the advantages and limitations of using oligonucleotides generally and for hydrocarbon catabolism specifically are presented, and some common technical and theoretical considerations about using primers are explained.

Keywords Primers, Oligonucleotides, Aromatic, Aliphatic, Biodegradation, Bacterial, Microbial Communities, Environmental, Catabolic Pathways, Gene Targets

1 The Versatility of Oligonucleotides

Few other simple yet versatile and highly used molecules in biology are as important as synthetic oligonucleotides, abbreviated depending on the experimental context as oligos, probes or primers. They are among the most powerful tools in molecular biology and, as a logical extension, to modern techniques in environmental microbiology and microbial ecology. The annealing of complementary DNA strands, with precision that can be easily modulated by chemical and physical means to be either quite strict or relatively relaxed regarding overall similarity of the annealing molecules, has been a great help to countless research interests and needs. This feature was observed and mainly developed by utilizing DNA fragments from genomes as a source for oligonucleotide probes in DNA hybridization assays. While the synthesis of oligonucleotides with a precise sequence of nucleotides was in constant development since the middle of the twentieth century, semi-automated

platforms were developed by the end of the 1980s for commercially producing such oligos with the nucleic acid sequence of interest [1]. Almost simultaneously, the community of researchers requiring the production and availability of oligos grew rapidly thanks to the invention of PCR [2]. There are many different, complementary or even combinatorial applications for which synthetic oligonucleotides have been used for a wide diversity of applications, such as for detection, quantification, differentiation, diagnostics, therapeutics, genomic sequencing, gene cloning, gene diversity analysis and protein engineering by gene mutagenesis [3, 4]. The possibilities to couple primers to another molecular biology technique are virtually endless. While there has been an explosion of genomic data production thanks to the emergence of the new sequencing technologies, it is important to note that despite looking at the utilization of primers for detecting or amplifying genes targeted in environmental DNA as a rather old and fading technique, its use is indeed steadily increasing precisely because of widespread use in the current state-of-the-art technologies or in synthetic genomics [5, 6]. It is not surprising that, after all, the evolutionary process resulted in such molecules to host the genetic information of all living forms and all its encoded complexity from very basic patterns and chemical building blocks. Thus, it is possible to assume that we are in the early stages of discovering the extensive applications of primers. In the beginning, primer applications were rather determinative, with important functions in all kinds of diagnostics, i.e. using known DNA sequence information, it was then possible to design a primer set to amplify a specific fragment to obtain a discrete presence/absence result about a gene fragment targeted in the template DNA under scrutiny.

Some basic categorizations on the use of oligos include: primers acting on dsDNA as a starting point for $5'$-$3'$ DNA polymerization, a feature used for amplifying a specific nucleic acid sequence, as in PCR and its numerous variants, or for Sanger sequencing using dideoxy nucleotides. Oligos can also be used as probes in hybridization assays on complementary strands of the sample tested, for instance, in macro- or microarrays or in fluorescence in situ hybridization. There are also possibilities of using probing and priming capabilities together. For example, they can be used as capturing probes coupled to its use as primers for amplification of the fragment rescued, or for specific enrichment of genomes targeted in metagenomes by adding specific probing primers on isothermal amplifications. Another complementary application of both features is for example when oligos are the overlapping building blocks joined and extended by ligation and polymerization for directed mutagenesis or for synthetic genomics and systems biology applications.

2 The Contribution of Primer-Based Surveys to the Improved Understanding of the Microbial Ecology of Environmental Pollutant Biodegradation

In this accompanying volume we have an outstanding collection of authoritative chapters showing many different examples of how to design and use primers, especially to retrieve functional gene fragments related to catabolism of hydrocarbons, including chlorinated hydrocarbons, as well as for investigating methanogens, methanotrophs, methylotrophs and other microbial processes of relevance to oil reservoir management. Each chapter thoughtfully explores: the function of interest, the main gene group or groups targeted (generally the gene families or subfamilies encoding proteins essential for the enzymatic activities of interest), and the different experiments detecting or quantifying gene copies or discerning gene complexities in the analysed samples, mostly from sites or treatments under the effect of the selecting compound. Basically there is a common experimental workflow on primer design and testing, such as the use of sequence alignments of the gene families and experimental testing after a careful primer design, especially when using primers to amplify from environmental samples where the presence of the gene family or subfamily targeted is unknown. In addition, an important limitation of PCR-based methods is potential variability at the conserved annealing site with priming-site sequences in the environmental sample differing from those in the databases. Another consideration is when the complexity of the gene family targeted and amplified requires further methods to discriminate the sequence diversity composition of the amplicon mixture, as is the case resulting from metagenomic DNA from an environmental microbial community. It was soon realized that PCR was a way to finally decipher whether the large proportion of non-cultured microorganism quantified and alive in environmental samples were similar or very divergent compared to our reference microbial type species. In that case the rationale was, and still is, to use one taxonomically informative and extremely conserved gene [7]. The results obtained revolutionized our understanding of the diversity of the microbial life in our planet. The use of 16S rRNA gene amplicons to describe the bacterial and archaeal community composition was adapted to detect functions only found in certain microbes in order to determine the distribution of those functions in the environment and how they were selected by different treatments and environmental conditions. The chapters in this volume highlight the approaches for designing primers targeting a given gene family, e.g. catabolic genes, which differ in many respects to the approaches applied to describe diversity of those non-protein coding and extremely conserved in microbial evolution such as coding rRNA genes. A common finding of all the chapters in this volume is that obtaining a "universal primer" is

close to unattainable or even undesirable to some extent in non-central metabolism protein-coding genes: the variability of silent mutations and the divergence inside a family imply that, in order to detect its major members by PCR-based approaches, it is more appropriate to use multiple primer sets able to cover as many members of each subfamily as possible. The subfamilies are defined and based on sequence distances; these distances are visualized as clusters of gene sequences composing branches in a gene phylogeny tree calculated from multiple sequence alignments of the family. For the case of bioremediation of contaminated environments, the purposes can be, for instance, detecting whether, in a bioaugmentation process, the functions are maintained over the threshold of PCR detection, indicating that the deployed microorganisms with the desired activities are maintained, possibly improving the bioremediation process. On the other hand, it could also be used to assess whether the same kind of genes from autochthonous microbes, even if not identical, are increasing in abundance due to selection when a sample is exposed to the contaminant or when it is subjected to biostimulation [8]. There are many other cases where the use of primers targeting a gene subfamily may not be the best way to find the gene responsible for the activity of interest. As detailed in the chapters, and almost for all the gene families mentioned, in the last 8 years there have been reports of the discovery, in isolates or in metagenomic libraries, of at least one new gene family having a functional convergence to the activity known to be encoded by gene members of completely different gene family or superfamily. Thus, as with virtually all methods, primers are a powerful *complementary* tool that greatly helped us comprehend the kind and composition of catabolic functions in contaminated environments, but it requires additional information to improve the precision of our understanding of biodegradation processes and to enrich our vision on the possibilities for a process in a given environment, e.g. in the microbial ecology of bioremediation.

Many chapters in this volume describe the approaches to detecting a particular part of what can be called collectively the microbial catabolome, a group of catabolic gene families encoding enzymes crucial for aerobic/anaerobic aromatic/aliphatic (chlorinated) hydrocarbon degradation, and as such, for pollutant bioremediation in the environment. Thus, in this concept the variable part is the contaminant and consequently, the gene family involved in its degradation and the electron acceptor condition where it can take place. As soon as this is defined, then it is possible to proceed to establish the information available for the gene or gene families targeted, the feasibility of finding conserved regions with the widest coverage for a given subgroup (subfamily), the meaning in terms of substrate specificity that the presence of this gene type may represent, and the meaning of the sequencing variations regarding possible modification of structure and/or substrate specificity.

The authors of the chapters are some of the most renowned experts on each kind of gene group targeted. The chapters summarize the vast knowledge accumulated over the last 60 years on each gene family identified as important from isolated microorganisms and environmental samples. Apart from the technical details of protocol and primer design, the following are considered in most chapters: further possibilities for sequence diversity assessment; the meaning of this diversity regarding substrate activities and specificity; genomic and taxonomic associations of catabolic capabilities hosted and community catabolic gene flow, selection, horizontal gene transferring and natural protein evolution adapting existing enzymes to new analogous substrates.

While an enormous diversity of such catabolic functions is continuously discovered in isolates, enrichments and in environments, contaminated sites select for microbiomes that are enriched in gene variants related to members of known catabolic gene families, i.e. that are not completely different to what has been already reported. This is not just an issue of the bias of obtaining something similar to what is known as the most obvious limitation of a targeted PCR-based approach; it is also observed in results from functional screening of metagenomic libraries or detected in direct metagenomic sequencing results [9, 10]. Nevertheless, given the importance of primers as a means of obtaining a window into microbial diversity, it is important to consistently check primer validity based on new information, and refine them accordingly.

For almost all these activities and contaminant conditions, a few years ago it was still an open question whether the known genes and functions found in isolates were also present and important in contaminated environments, as it can be seen, in the last years there are many results applying primer surveys to environmental samples that support the idea of the environmental importance of such mechanisms. While there are various gene types that were not known and very different to what was described for the activity encoded, and possibly there are many more of this kind to be found, the catabolic gene families we know so far seem to encode the main kind of activities and bioremediation processes in the environment. In contaminated sites it is still hard to predict the selection and emergence in certain catabolic gene family members of the subfamily type mainly associated with the preferential substrate specificity against the pollutant at highest concentration in situ. For instance, it is sometimes possible to detect abundant members of other subfamilies associated with a different substrate preference and gene variants with few sequence changes that are producing enzymes that are able to degrade the contaminant, as we observed in a previous study [11].

In their corresponding chapters Lueders and von Netzer (anaerobic aromatics/aliphatics degradation) [12], Lu et al. (organohalide-respiring bacteria) [13], and Narihiro and Sekiguchi

(methanogens) [14] review the current understanding of the respective microbial processes and the primers available to detect the gene or genes identified as the most important in those activities under anaerobic conditions. Dumont [15] explores primers for detecting genes important for methylotrophs and methanotrophs, and Smith et al. [16] detail primers for key functions in nitrogen-cycling microbes in oil reservoirs. In general, the more halogenated an organic molecule is, the more recalcitrant it is to aerobic degradation, while being susceptible to anaerobic degradation. While Lu et al. [12] focus on organohalide respiration, Coleman [17] presents the functional gene families found in microorganisms involved in aerobic degradation of chlorinated hydrocarbons and the primers developed for its detection, explaining in a very detailed, entertaining and practical way how to do the whole process from scratch right through to fine assessment of the results. The chapters by Scoma et al. [18] and Hernandez-Sanabria et al. [19] provide a summary of our current understanding about the aerobic functions involved in aromatic and aliphatic biodegradation and primers designed and available for detection of selected subfamilies and its correlation with substrate specificity. Such information can help to generate tools to improve the annotation and inferences from catabolic gene sequences or genomic metabolic reconstructions [20].

I am convinced that the readers of this volume will find the information provided by the authors on each chapter valuable and insightful. They include updated information about genes to target depending of the function of interest, and give useful advice, strategies, tips and comments to successfully achieve the detection of the gene subfamily or microbial group of interest. In conclusion, the chapters provide a great resource for anyone interested in detecting or quantifying genes for catabolic functions, methanogenesis and nitrogen cycling in isolates or in environmental samples. I thank Prof. Dr. Dietmar H. Pieper for sharing with me, over the last 15 years, his knowledge and support in our studies about microbial biodegradation and ecology.

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Current Landscape of Biomolecular Approaches for Assessing Biodegradation of Aromatic Hydrocarbons

Emma Hernandez-Sanabria, Alberto Scoma, Tim Lacoere, Marcia Duarte, Nico Boon, Dietmar H. Pieper, and Ramiro Vilchez-Vargas

Abstract

The ability of bacteria to degrade hazardous pollutants is a valuable tool that can be employed for cleaning contaminated sites. As a result of the complex mixtures of organic compounds present in contaminated areas, the combined genetic information of more than one organism is necessary to enhance the degradation process. Aromatic compounds are believed to constitute approximately 25% of all biomass on earth. Community profiling and other molecular techniques, such as quantitative real-time PCR and fluorescence in situ hybridization, provide the phylogenetic context of the potential key genes associated with the degradation of aromatic compounds. The application of molecular techniques may help to identify potentially remediating organisms and to discover particular degradation abilities. Increased knowledge on the microbial diversity in environments contaminated with aromatic compounds may assist in the characterization of highly efficient and tolerant bacteria when exposed to a broad range of stresses. Ultimately, such knowledge may support the development of novel and effective bioremediation strategies.

Keywords Biomolecular approaches, Biodegradation, Aromatic hydrocarbons

1 Microbial Consortia and Their Role in Aromatic Hydrocarbon Degradation

A cost-effective biodegradation of organic pollutants can be achieved through diverse microbial metabolic processes. Indeed, microorganisms are capable of degrading environmental contaminants in diverse matrices and environments [1]. Thus, the ability of bacteria to degrade hazardous pollutants is a valuable tool that can be employed for cleaning contaminated sites [2]. A goal of bioremediation is to use organic pollutants as carbon and energy sources and ultimately mineralize the pollutants [3]. Many factors influence the efficient degradation of pollutants and their co-metabolism. Understanding the degradation mechanisms and responsible enzymes is fundamental to outline an efficient strategy for clean-up of pollutants. Aromatic compounds are believed to constitute approximately 25% of all biomass on earth [4] and may be of natural

as well as anthropogenic origin. There are three major groups of aromatic pollutants, which are polycyclic aromatic hydrocarbons (PAHs), heterocyclics, and substituted (e.g. chloro- or nitro-substituted) aromatics.

The catabolic pathways for the degradation of aromatic compounds have been extensively studied and deeper insights have been reported [3, 5]. The activation of the aromatic ring is facilitated by either Rieske non-heme iron oxygenases, flavoprotein monooxygenases, or soluble diiron monooxygenases [6]. The further aerobic degradation of di- or trihydroxylated intermediates can be catalysed by either intradiol or extradiol dioxygenases [7]. The enzymes reported to be involved in the extradiol ring cleavage of hydroxylated aromatics can be categorized in three different super-families: type I extradiol dioxygenases (e.g. catechol 2,3-dioxygenases), which belong to the vicinal oxygen chelate super-family, type II or LigB superfamily extradiol dioxygenases, which comprise the protocatechuate 4,5-dioxygenases, among others, and type III enzymes such as gentisate dioxygenases, comprising enzymes of the cupin superfamily [6]. Alternatively, activation may be mediated by CoA ligases and the formed CoA derivatives are subjected to oxygenations that generate non-aromatic intermediates [8–15] which is extensively reviewed in Perez Pantoja et al. [16]. However, the limiting factor in aerobic degradation is the oxygen concentration, because heavily polluted ecosystems are often oxygen depleted [17]. Hence, facultative and strict anaerobes become essential to the bioremediation process [18]. For instance, studies focusing on the anaerobic degradation of BTEX compounds have indicated that these components are degraded anaerobically and over a longer timeframe when compared to aerobic processes [19, 20].

Our main knowledge on anaerobic degradation of pollutants is centred on biochemical studies involving both facultative and strict anaerobic microorganisms [18]. Strict anaerobes are challenging subjects, as a result of their sensitivity to sampling and storage and their fastidious growth requirements. Nevertheless, analysis of anaerobic naphthalene degraders and communities has been accomplished and new metabolic pathways [21], including novel ATP-independent benzoyl-CoA reductases or ring-reducing enzymes that act on substrates other than benzoyl-CoA, have been identified [18, 22, 23].

A detailed description of primers for detecting functional genes involved in anaerobic hydrocarbons degradation has been included in the current version of this volume [24].

Although many bacteria are able to metabolize organic pollutants (Fig. 1), a single bacterium does not possess the enzymatic capability to degrade all or even most of the organic compounds in a polluted soil. Mixed microbial communities have the most powerful biodegradation potential. Due to the complexity of the
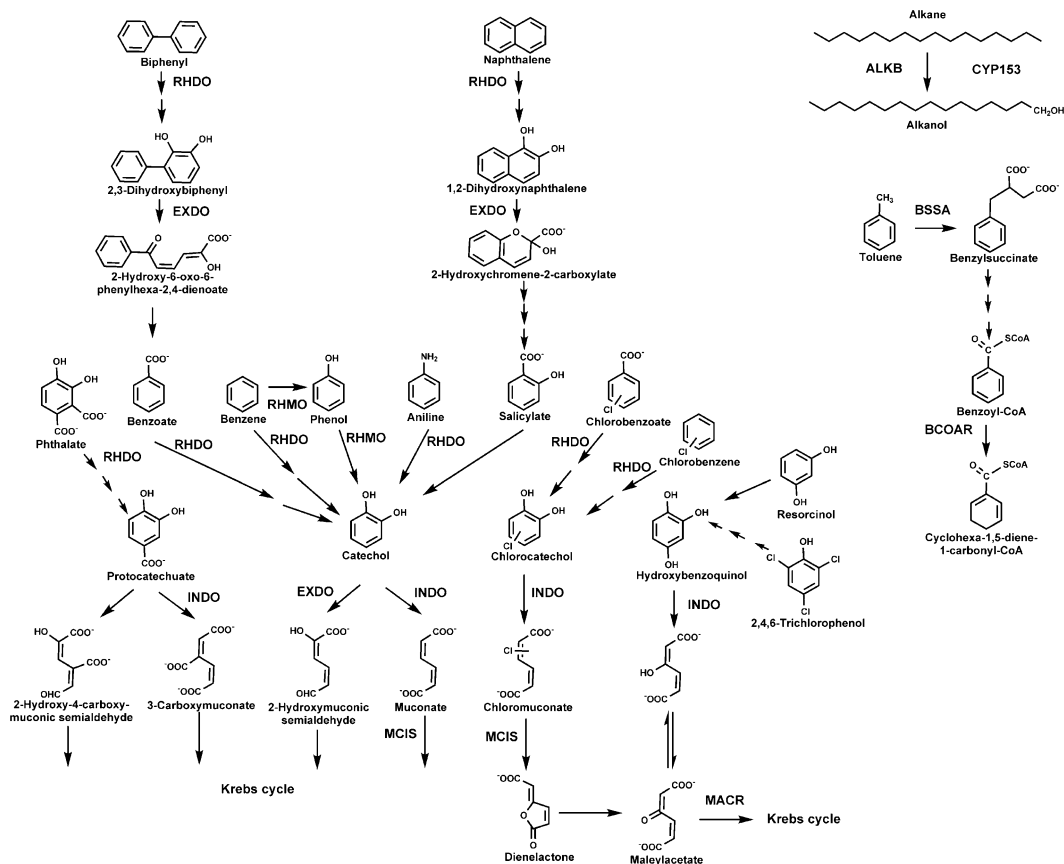
Fig. 1 Exemplary reactions catalysed by enzymes. For reactions catalysed by these enzymes, the respective family which the enzyme belongs to is indicated alongside the arrow with RHDO, ring hydroxylating dioxygenases; EXDO, extradiol dioxygenases of the vicinal chelate superfamily; INDO, intradiol dioxygenases; RHMO, ring hydroxylating monooxygenases; MCIS, muconate cycloisomerases processing catechol ring-cleavage products; MACR, maleylacetate reductases, involved in the degradation of chloroaromatics and nitroaromatics; ALKB, integral membrane bound alkane hydroxylases; CYP153, cytochrome P450 CYP153 alkane hydroxylases; BCOAR, benzoyl coenzyme A reductases and BSSA, benzylsuccinate synthases

mixtures of organic compounds present in contaminated areas, the combined genetic information of more than one organism is necessary to unravel the degradation process.

Community profiling and other molecular techniques, such as quantitative real-time PCR (qPCR) and fluorescence in situ hybridization (FISH), are typically used to analyse microbial community structures and thus the phylogenetic context in which biodegradation is occurring. On the other hand, the analysis of catabolic key genes associated with the degradation of aromatic compounds is crucial to understand biodegradation processes since there is often no direct correlation between taxonomy and catabolic potentials. As a result, the design of suitable primers targeting genes encoding

representative proteins involved in the above processes is a basic requirement for understanding biodegradation. To achieve the correct primer design it is crucial to generate broad, comprehensive, updated and manually curated databases that include catabolic key proteins involved in the degradation of aromatic pollutants.

2   Molecular Tools Employed for Describing the Functional Gene Diversity for Aromatic Degradation

Therefore, the targeted sequencing of functional genes may directly supply information to set a framework for classification as recently accomplished in Aromadeg [6]. A dedicated analysis of catabolic genes and genomes may also indicate to what extent catabolic genes may be used as a marker for the taxonomic identity of the organism harbouring such activity [16]. In summary, the application of molecular techniques may help to identify potential remediating organisms and to discover particular degradation abilities. Furthermore, increased knowledge on the microbial diversity in environments contaminated with aromatic compounds may assist in the characterization of highly efficient and tolerant bacteria exposed to a broad range of stresses. Ultimately, this may support the development of novel and effective bioremediation strategies.

2.1   Quantitative PCR (qPCR)

Real-time PCR (qPCR) has been widely employed in microbial ecology for profiling and bioprospecting of environmental samples [25]. The high sensitivity for quantifying specific genes in complex DNA mixtures also makes qPCR highly suitable for analysis of environmental samples [26, 27]. Furthermore, this technique can be employed to validate results of high-throughput methodologies at the genomic, metagenomic and metatranscriptomic levels.

The use of 16S rRNA gene qPCR in conjunction with functional gene analysis has been used successfully to acquire information regarding bacterial population size and dynamics [27]. However, the mere presence of a gene does not mean that it actually has a function under analysed conditions. Approximation of the importance of a particular function can be achieved through the use of reverse-transcriptase quantitative PCR (rt-qPCR), where mRNA is isolated from a sample and retro-transcribed to cDNA before being quantified using specific primers [28]. The use of rt-qPCR allows measuring both the in situ microbial activity in a particular environment [29] and the importance of targeted genes [30].

Multiplex real-time PCR is a methodology that utilizes multiple primer sets within a single PCR mix. Each primer set is labelled with distinct fluorescent dyes and therefore the excitation signals will not overlap. In this way, multiple genes are targeted at once and several amplicons are simultaneously produced. Careful design of primers and optimizing the annealing conditions are indispensable

to obtain accurate and reliable information. Internal controls (like house-keeping genes) allow for precise quantification of target genes. Multiplex PCR has been extensively employed in bioremediation studies, mainly to detect mono- or dioxygenase encoding genes involved in polycyclic aromatic hydrocarbons (PAH) metabolism [30–33]. Other studies using generic primers and real-time PCR focused on targeting the gene region encoding the Rieske iron sulfur center to track the population shifts of PAH-degrading microorganisms [34, 35]. However, care has to be taken to exclusively monitor genes/enzymes involved in PAH degradation, which are very different among, e.g., Proteobacteria and Actinobacteria [6, 36], and avoid amplification of genes that may be fortuitously enriched.

In summary, the use of rt-qPCR or multiplex qPCR using cDNA as a template allows for measuring both the in situ microbial activity in a particular environment and the involvement of targeted genes. In this way, the use of mRNA in rt-qPCR assays is a first approximation to obtain an overview of the key genes expressed within a community. Subsequently, new recently developed methods, such as proteomics, microarrays, or metatranscriptomics (see below), can provide more information on undiscovered genes and potential key players in the community.

2.2 Genetic Fingerprinting Methods

All techniques described below were initially developed for studies targeting 16S rRNA genes and, afterwards, they were applied in more or less extent to catabolic genes [37].

Denaturing (Temperature) Gradient Gel Electrophoresis (DGGE/TGGE) relies on the difference in melting behavior of different double-stranded DNA strands upon the application of heat (TGGE) or chemical denaturants (DGGE). If mixtures of homologous DNA fragments are subjected to electrophoresis on gels applying denaturing gradients, specific melting behaviors will be obtained depending on the sequence composition (G + C content). The so-called GC-clamp, a GC rich terminal region, is artificially introduced by means of PCR at one end of the amplification mixture, which ensures the connection of both strands thus preventing migration once the strands are partially melted.

DGGE/TGGE has been one of the methods of choice as a primary analysis in assessing the community composition of an environment [38], as it is accurate enough to determine the dominant members of microbial communities. DGGE has recently been applied to follow the presence of *bam*A genes in anaerobic environments. The gene product catalyses the hydrolysis of 6-oxocyclohex-1-ene-1-carbonyl-CoA formed after reduction of benzoyl-CoA as central intermediate in anaerobic aromatic degradation. The gene is considered as a good genetic marker for anaerobic aromatic metabolism since, in contrast to benzoyl-CoA reduction which in facultative and strict anaerobic bacteria is catalysed by different enzyme

classes, hydrolysis is catalysed by members of just one enzyme family in different bacteria. In accordance, DGGE-based analysis of *bamA* diversity has shown that the population structure of aromatic hydrocarbon degrading bacteria changes with depth [39].

Single Strand Conformation Polymorphism (SSCP) has been another powerful fingerprinting technique, which takes advantage of the fact that single stranded DNA (ssDNA), under nondenaturing conditions, acquires a secondary conformation of intramolecular loops and foldings due to complementarity of the bases on the same strand [37]. To a smaller extent, terminal-restriction fragment length polymorphism (T-RFLP) has also been applied for fingerprinting functional genes as mentioned in Junca and Pieper [37]. However, with the development of next generation sequencing methods such as barcode sequencing using the Illumina platform (see below), these methods are becoming more and more obsolete for the retrieval of sequence information from the environment.

2.3 RNA-SIP

Nucleic acid-based stable isotope probing (SIP) is another technique used for the identification of key microbes involved in degradation pathways [40]. SIP involves pulsing stable-isotope-labelled substrates into phylogenetic or functional marker molecules of microbial communities; for instance, through the incorporation of ^{13}C-labelled substrates into nucleic acids. Density gradient separation of labelled nucleic acids allows for the molecular identification of the microorganisms responsible for degrading the substrates [41]. In this way, SIP provides access to the relationship between environmental functions and the specific microbial community members involved in community performance [42]. SIP has been utilized to identify key microbes utilizing various aromatic and chlorinated hydrocarbons [42–46], methanotroph populations in soda lakes [47], microbial communities in activated sludge [48], in PAH contaminated soil [49], for the molecular analysis of arsenic reducing bacteria in groundwater sources [50] and to identify the key iron-reducing microorganisms involved in anaerobic benzene degradation [40]. Currently, a novel technique using high-throughput sequencing of labelled DNA obtained following isopycnic centrifugation [51] has been applied to monitor microbial communities consuming labelled toluene as a substrate. SIP, together with high-throughput sequencing, has enabled the identification of key bacteria involved in degrading specific compounds of interest, for example, the bacterial taxa involved in the degradation of polycyclic aromatic hydrocarbons in oil-contaminated waters [52].

2.4 Fluorescence In Situ Hybridization

FISH is a method used to quantify microbial subpopulations in a community sample. In this technique microbial cells are treated with fixative, hybridized with specific probes (usually 15–25 bp fluorescent dye-labelled oligonucleotide probes) on a glass slide

or on membrane filters, and then visualized with either epifluorescence or confocal laser microscopy, or quantified by flow cytometry. However, FISH alone does not provide insights in the metabolic functions of microorganisms. Moreover, the sensitivity of the methodology can diminish if microbial cells are small, slow-growing, starving or when they contain low amounts of cellular rRNA. Multiple group-specific rRNA probes can be used simultaneously for the quantification of physiologically active microbial populations in an environmental sample [53]. Population dynamics and interspecies relationships at the single cell level have been widely monitored using catalysed reporter deposition (CARD)-FISH in petroleum-contaminated sites [54]. Co-localization studies combining FISH and digital image analysis are providing comparative analysis of temporal or spatial information in structured ecosystems for metagenome analysis [53]. FISH can also be associated with other methods, such as microautoradiography, mRNA-FISH, and nanoSIMS, to simultaneously reveal the physiological and functional traits of selected microbial populations in complex environments [54].

One of the limitations of FISH is that the results depend on previously available sequence information to design probes. This issue may be of minor importance if a taxonomical overview of the community should be achieved. However, the limitation becomes important if the probe targets functional genes. In that case, probes need to be designed using updated and manually curated databases.

2.5 Microarrays

It should be noted that applying microarrays for the survey of functional genes in environmental samples face some obstacles in comparison with those microarrays applied to analyse gene expression in pure cultures. Expression analysis typically compares two different controlled experimental conditions, where due to the relative quantification approach the majority of signals arising from unspecific hybridization are eliminated during the normalization procedure and only signals exhibiting a defined fold change are considered. However, if microarray analyses are applied to environmental samples, the goal is usually gene detection rather than determination of expression levels and normalization across two different conditions is not possible. Thus, another experimental design should be taken into consideration. As normalization across two different conditions cannot be done, the proper approach to determine the reliability of microarray results requires the use of internal positive controls (an internal calibration in each single microarray) for setting the correct threshold according to the desired precision of the experiment, rather than artificial signal to noise ratios. Recently, a microarray to survey the metabolic potential of microbial communities for the degradation of aromatic but also of aliphatic environmental pollutants was reported [55]. This microarray was designed using curated databases of catabolic key

proteins. The use of internal controls allows systematic validation, optimization, normalization and cross-experiment comparisons. The newly developed protocol and engineered internal control also allowed for the analysis of catabolic gene expression levels and facilitates more accurate comparisons across environmental samples.

It should be noticed that the microarray approach is also a sequence-dependent technique as a specific probe is required with a threshold of homology between the probe and the target gene to allow its detection. A continuous update of the set of probes will increase the overview of the catabolic landscape in one environmental sample. As a matter of fact, new sequences with catabolic potentials that are continuously discovered using techniques such as clone libraries [56], metatranscriptomics [57], or genomics [58], among others, can be used for generating the corresponding probes and be implemented on microarrays in future updates.

Taking into account all the considerations mentioned above, the microarray may be considered a technique that produces the results of multiple PCRs (as many as probes are represented on the microarray) in a single experiment.

2.6 OMICS Approaches

Most culture-independent surveys of catabolic gene diversity in contaminated environments rely on conserved nucleotide sequences used for designing primers to examine the presence, abundance and diversity of catabolic genes encoding a defined group of enzymes which are assumed to be critical in the target environment. Designing primers requires prior knowledge of gene sequences, which have been identified from pure cultures, and therefore restrict the overview of the potential community functions. During the last years, new techniques for gene sequencing have been developed, opening a new frame in the research that allows for detecting new genes and their products without the need for pre-existing sequencing knowledge [58, 59].

Nevertheless, these techniques can be purely descriptive if they are applied without any specific questions. For instance, metagenomic approaches, i.e., the sequencing of the genetic content of a target environment, if applied arbitrarily, will lead to an accumulation of sequence information defined as "unknown," recently described as "the know unknown" [60], with no ecological meaning. On the other hand, if the aim is to detect new unknown genes involved in the degradation of pollutants in situ, metatranscriptomics studies based on the gene transcription levels by comparing two environmental samples (control and contaminated) may allow for the discovery of key-catabolic genes transcripts, which cannot be detected using sequence-based techniques as the ones described above. By means of comparative metatranscriptomics, De Menezes and collaborators recently identified an increase in transcripts associated with the metabolism of aromatic compounds, respiration and

stress response upon PAH amendment [1]. Moreover, they detected an up-regulation of heavy metal P-type ATPases and thioredoxin previously not associated with polycyclic aromatic hydrocarbon stress in microorganisms. Luo and collaborators investigated the genes involved in the anaerobic benzene and benzoate degradation in a nitrate-reducing enrichment culture applying comparative metatranscriptomics between the enrichment with and without the substrate. Outstandingly, the enrichment culture used for this experiment was the result of 16 years of maintenance, adding benzene and nitrate monthly and executing periodic transfers. As a result, the syntrophic association between a benzene-degrading *Peptococcaceae* strain and a benzoate-degrading denitrifying *Azoarcus* sp. was confirmed for the complete catabolism of benzene with nitrate as the terminal electron acceptor [57].

In addition, applying a combination of omics approaches allows discovering new pathways and enzymes, which could not be found using sequence-based techniques. Wu and collaborators studied the anaerobic degradation of terephthalate in methanogenesis consortia combining microbiomic and proteomic approaches. The community acts by degrading the terephthalate due to *Pelotomaculum* spp. transforming the substrate into acetate, carbon dioxide, and hydrogen. Afterwards, acetate is utilized by the acetoclastic *Methanosaeta* spp. producing methane and carbon dioxide, while hydrogen is utilized by the hydrogenotrophic *Methanolinea* spp. producing methane [61].

Finally, metabolomics investigations may be a powerful tool in finding molecular biomarkers in aromatic degradation as, although aerobic pathways have a high degree of complexity, the technique was successfully applied so far to anaerobic environments [62].

3   Databases

Nowadays, due to the rapid development of DNA sequencing technologies, massive amount of sequence information is continuously generated, which needs to be properly annotated. Databases play a fundamental role in understanding and increasing our knowledge of the sequences retrieved from the environment. Limited efforts have been invested in the collection and curation of sequences for being deposited into databases and to carry out the annotation in an accurate manner. Certainly, databases have to be continuously updated by including key-catabolic genes that are continuously being discovered, such as the new phenylacetyl-CoA, benzylsuccinate synthases, or benzoyl-CoA reductases genes.

A novel database termed AromaDeg (http://aromadeg.siona. helmholtz-hzi.de), an open source database with a total of 3,605 protein sequences of key-catabolic enzymes for aromatic degradation, allows query, and data mining of novel genomic,

metagenomic, or metatranscriptomic data sets [6]. Moreover, HyDeg (http://www.hydeg.ugent.be) assists in targeting subfamilies of genes involved in the catabolism of aromatic/aliphatic hydrocarbons [55] and provides practical examples of primer design [63].

Acknowledgements

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Primers: Bacterial Genes Encoding Enzymes for Aerobic Hydrocarbon Degradation

Alberto Scoma, Emma Hernandez-Sanabria, Tim Lacoere, Howard Junca, Nico Boon, Dietmar H. Pieper, and Ramiro Vilchez-Vargas

Abstract

Alkanes are saturated hydrocarbons that are ubiquitous in the environment. Microbial degradation pathways evolved to activate and catabolise these compounds in order to gain energy and building blocks for cell growth. These pathways involve a number of hydroxylases, which primarily differ according to the nature of the hydrocarbon itself (e.g. aromatic or aliphatic). Given the widespread distribution of alkanes in the environment, a number of variants of such enzymes are present among microbes. Hence, primers designed to detect such environmental variants would require a database with a sufficiently large number of sequences. In the present chapter, we selected the integral-membrane alkane hydroxylases (AlkB) and cytochrome P450 alkane hydroxylases (CYP153) superfamilies for sketching a general proposal of a design pipeline to target bacterial genes involved in aerobic alkane degradation. Further, we introduce *HyDeg*, a web-based tool that targets multiple subfamilies of enzymes involved in the microbial degradation of aromatic/aliphatic hydrocarbons. The website allows to retrieve amino acid and nucleotide sequences of the target family and proposes an evolutionary relationship for the selected enzyme.

Keywords: Biodegradation pollutants compounds, Hydeg, Primer design

1 Targeting the Key Bacterial Genes Involved in Aerobic Alkane Degradation

Alkanes are saturated hydrocarbons with low chemical reactivity, which can be found arranged in different structures (linear, cyclic or branched, named *n*-, *cyclo-* or *iso*-alkanes, respectively). Besides being components of crude oil, several different prokaryotes and eukaryotes produce them, contributing to the widespread distribution of alkanes in both soil and wet environments. As a consequence, prokaryotes and eukaryotes possess enzymatic systems to oxidise hydrocarbons and to complete the biodegradation of alkanes [1].

Currently, the enzymes involved in aerobic alkane activation can be clustered in soluble di-iron methane monooxygenases (sMMO) and membrane-bound copper-containing methane

monooxygenases (pMMO) [2]; cytochrome P450 class I P450 (CYP153) and cytochrome P450 class II P450 (CYP52, CYP2E, and CYP4B) [3]; integral-membrane di-iron alkane hydroxylases (AlkB) [2]; flavin-binding monooxygenases (AlmA), firstly found in *Acinetobacter* strain DSM 17874 [4]; or long-chain alkane monooxygenases (LadA), initially found in *Geobacillus thermodenitrificans* NG80-2 [5] superfamilies. These discoveries indicate that new superfamilies of enzymes with alkane hydroxylase activity are still waiting to be recovered and characterised in the near future.

Concerning the chain length of the substrate, sMMO and pMMO oxidise C_1–C_4 hydrocarbons. CYP52, CYP2E and CYP4B, which are found in fungi and humans, oxidise alkanes in the C_6–C_{16} range; CYP153, which is typically found in *Eubacteria*, is able to oxidise C_4–C_{16} alkanes, whereas AlkB oxidises C_5–C_{20} and exceptionally up to C_{26} [6] alkanes. AlmA oxidises alkanes up to C_{40} and LadA oxidises alkanes between C_{15}–C_{36} [7]. Thus, it is possible that enzymes belonging to completely distinct superfamilies can hydroxylate similar alkane lengths.

Generating a database with large enough number of representative sequences is crucial for designing primers in a systematic manner, aiming to detect environmental variants. AlkB and CYP153 superfamilies have been extensively studied in environmental niches (Tables 1 and 2). Thus, massive sequence information is available. In the present chapter, we selected the AlkB and CYP153 superfamilies to outline a general pipeline for primer design to target bacterial genes involved in aerobic alkane degradation.

Based on the information for AlkB and CYP153 superfamilies previously reported [17], we created *HyDeg* (http://www.hydeg.ugent.be), a user-friendly interface with practical examples of primers designed to target subfamily groups of gene families included in the microbial catabolome for aromatic/aliphatic hydrocarbon biodegradation.

1.1 The HyDeg Website

HyDeg (http://www.hydeg.ugent.be) contains several superfamilies of functional genes known to be involved in the aerobic degradation of hydrocarbons, namely, alkane monooxygenases (AlkB in the *HyDeg* website) and cytochrome alkane hydroxylases P450 (CYP153), for aliphatic hydrocarbons, and alpha subunit of Rieske nonheme iron oxygenases (RHDO), extradiol oxygenases (EXDO), alpha subunit of soluble di-iron monooxygenases (RHMO), intradiol dioxygenases (INDO), muconate/chloromuconate cycloisomerases (MCIS) and maleylacetate reductases (MACR), for aromatic hydrocarbons. The website allows the user to retrieve both the amino acid and nucleotide sequences of each subfamily and to observe the evolutionary relationship of such enzymes within the microbial domain. Moreover, the website allows the user to select the desired node and to download all the nucleotide sequences employed for the design of a selected pair or primers.

Table 1
List of primers used in literature targeting alkane hydroxylases (AlkB)

Primer name	F/R[a]	Set of primer (5′–3′)	Targeted AlkB from	Designed for	Reference
alkBwf	F	AAYACNGCNCAYGARCTNGGVCAYAA	*Alcanivorax, Bacillus, Brachybacterium, Erythrobacter, Gordonia, Halomonas, Idiomarina, Kordiimonas, Leifsonia, Marinobacter, Martelella, Mesorhizobium, Microbacterium, Novosphingobium, Ochrobactrum, Parvibaculum, Salinisphaera, Solimonas, Sphingobium, Sphingopyxis, Tetrathiobacter, Tistrella*	PCR	[8]
alkBwr	R	GCRTGRTGRTCHGARTGNCGYTG			
alkB-1f	F	AAYACNGCNCAYGARCTNGGNCAYAA	*Acinetobacter* ADP1 (DSMZ 586), *Pseudomonas putida* GPo1 (ATCC 29347), *Bacillus subtilis* strain Marburg (DSMZ 10), *Escherichia coli* TG1	PCR	[9]
alkB-1r	R	GCRTGRTGRTCNGARTGNCGYTG			
TS2S	F	AAYAGAGCTCAYGARYTRGGTCAYAAG	*Pseudomonas, Burkholderia, Comamonas, Stenotrophomonas, Ralstonia, Acinetobacter, Rhodococcus, Amycolatopsis*	PCR	[10]
TS2Smod	F	AAYAGAGCTCAYGARITIGGICAYAAR			
TS2Smod2	F	AAYAGAGCTCAYGARITITCICAYAAR			
deg1RE	R	GTGGAATTCGCRTGRTGRTCIGARTG			
deg1RE2	R	GTGGAATTCGCRTGRTGRTCRCTRTG			
AF1	F	TCTACGGSCAYTTCTACRTCGA	*Geobacillus thermoleovorans* strain T70	PCR	[11]
AR1	R	CGGRTTCGCGGTGGRTGRT			
AH+ for	F	YRCSGCVCACGARYTSGGBCACAAG	*Nocardia, Rhodococcus, Gordonia*	PCR	[12]
AH+ rev	R	SGGATTCGCRTGRTGRTCRCTGTG			
alkBF	F	ATCAAYRCVGCVCAYGARYTVGGBCACAAG	*Gordonia*	PCR	[13]
alkBR	R	SGGRTTCGCRTGRTGRTCRCTGTGNSGYTG			
ALK-1F	F	CATAATAAAGGGCATCACCGT	*Pseudomonas, Burkholderia, Stenotrophomonas, Acinetobacter, Rhodococcus, Amycolatopsis*	PCR and Southern hybridisation	[14]
ALK-1R	R	GATTTCATTCTCGAAACTCCAAAC			
ALK-2F	F	GAGACAAATCGTCTAAAACGTAA			
ALK-2R	R	TTGTTATTATTCCAACTATGCTC			
ALK-3F	F	TCGAGCACATCCGCGGCCACCA			
ALK-3R	R	CCGTAGTGCTCGACGTAGTT			

[a]Forward (F) and reverse (R) is indicated in the second column

Table 2
List of primers described in literature targeting cytochrome P450 CYP153 alkane hydroxylases (CYP153)

Primer name	F/R[a]	Set of primer (5′–3′)	Targeted CYP153 from	Designed for	Reference
P450fw1	F	GTSGGCGGCAACGACACSAC	*Pseudomonas putida* GPo12(pGEc47B)	PCR	[15]
P450rv3	R	GCASCGGTGGATGCCGAAGCCRAA			
P450F	F	TGTCGGTTGAAATGTTCATYGCNMTGGAYCC	*Alcanivorax, Bacillus, Brachybacterium, Erythrobacter, Gordonia, Halomonas, Idiomarina, Kordiimonas, Leifsonia, Marinovarina, Martelella, Mesorhizobium, Microbacterium, Novosphingobium, Ochrobactrum, Parvibaculum, Salinisphaera, Solimonas, Sphingobium, Sphingopyxis, Tetrathiobacter, Tistrella*	PCR	[8, 16]
P450R	R	TGCAGTTCGGCAAGGCGGTTDCCSRYRCAVCKRTG			

[a]Forward (F) and reverse (R) is indicated in the second column

2 Preceding Considerations for Primer Design Approaches

2.1 Microbial Community Composition Versus Catabolic Gene Diversity

The 16S rRNA gene sequence has been recognised as the most reliable target for bacterial phylogenetic identification and classification and for biodiversity composition studies in complex communities [18]. It contains nine hypervariable regions which are flanked by highly conserved regions [19]. This particular feature allows the estimation of bacterial community composition and diversity in a single step. In this way, a single primer pair can be annealed to the highly conserved regions and the hypervariable regions between will be retrieved.

In contrast, this approach is not feasible with the majority of the catabolic genes. Because of the higher mutation rate within the protein-encoding families, the sequence variation is higher [20]. As illustrated in Fig. 1, the cumulative alignment of DNA sequences encoding AlkB from cluster 1 to cluster 9 shows the lack of a fully conserved region across the whole alignment (Fig. 1b). Instead, conserved sequences along highly similar subclusters are present (http://www.hydeg.ugent.be).

Some studies use the most conserved positions in the global alignments to design primers aiming to detect AlkB and CYP153. For example, the well-known conserved domains in alkane hydroxylases and in cytochrome P450 CYP153 alkane hydroxylases [16]. In the same way as the universal amplification of 16S rRNA gene fragments, such approaches are limited when applied to families with higher variation. For instance, a highly conserved motif "NTAHELGHK" [9] is absent in the alkane hydroxylases of *Flavobacteria* bacterium BBFL7 (ZP01201250), *Alcanivorax borkumensis* SK2 (BAC98365 or YP691842), *Rhodococcus* Q15 (AAK97446), *Pseudomonas putida* P1 (CAB51047), *Pseudomonas frederiksbergensis* (AAR13803), *Tetrahymena thermophila* (XP001020064), *Burkholderia lata* 383 (YP371980) and *Polaromonas* JS666 (YP552229) among others. In addition, motif "MFIAMDPP" [21] is absent in the cytochrome P450 CYP153 alkane hydroxylases of *Caulobacter crescentus* CB15 (NP418882), *Sphingopyxis macrogoltabida* HXN-200 (CAH61448 or CAH61454), *Bradyrhizobium diazoefficiens* USDA 110 (NP768493), *Rhodopseudomonas palustris* CGA009 (NP946959), *Erythrobacter litoralis* HTCC2594 (YP458852), *Novosphingobium aromaticivorans* DSM 12444 (YP495502) and *Oceanicola batsensis* HTCC2597 (ZP00997728) among others. Therefore, these domains cannot be considered universally conserved.

Consequently, designing primers based on such conserved motifs or nonexistent conserved regions may bias the aim of detecting all the key players involved in alkane degradation. Hence, primer design based on consensus regions of a subset of sequences will provide comprehensive coverage and detection range. Therefore, all the related family members that may be present in a given environment or isolate can be revealed.
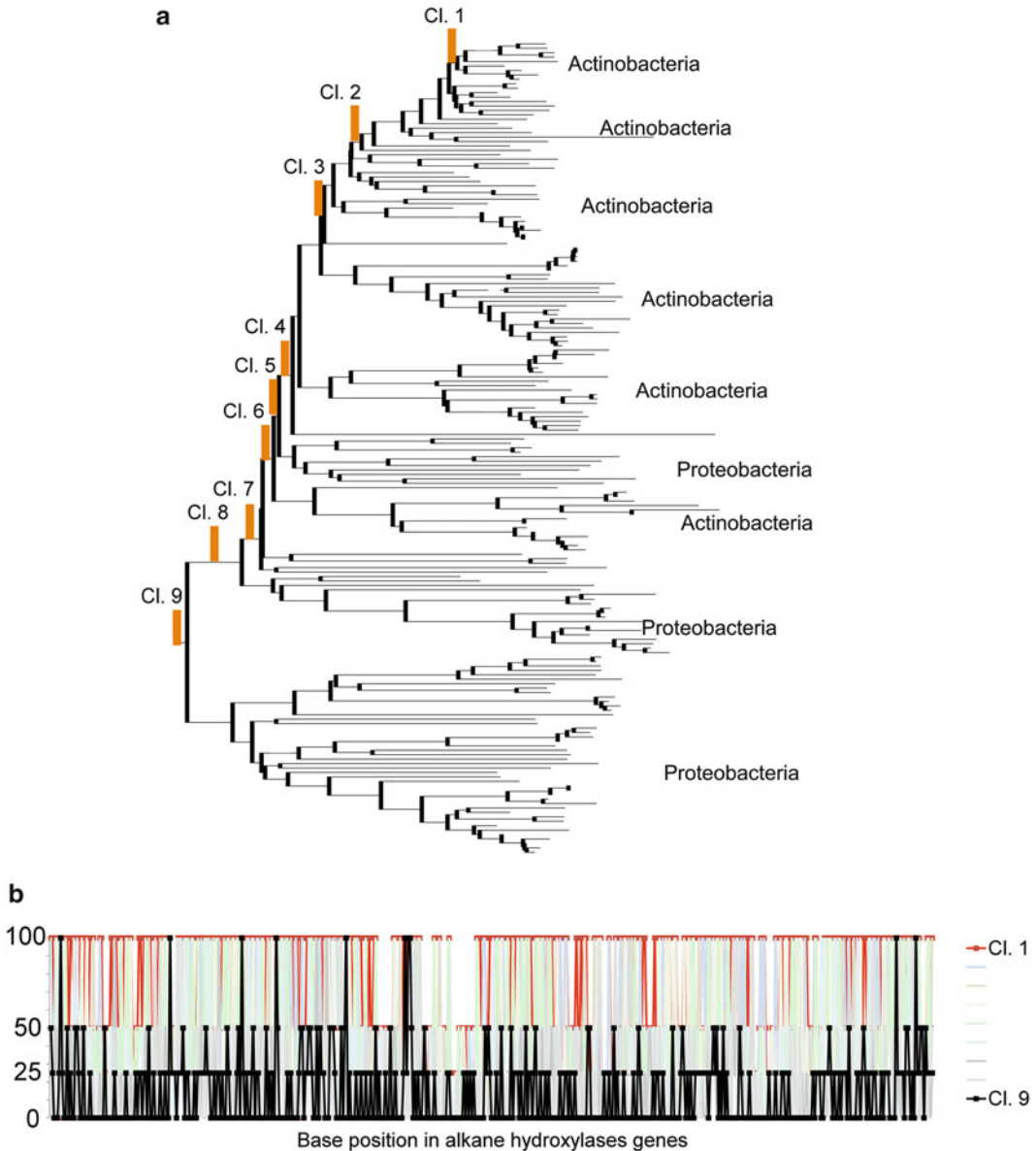
Fig. 1 (**a**) Evolutionary relationships of the alkane hydroxylases [17]. Nodes were defined considering clusters with an increasing number of representatives (cluster 1 to cluster 9). (**b**) Level of degenerate bases per base position in alkane hydroxylase genes (gaps were not considered) for each cluster showed in (**a**), cluster 1 and cluster 9 are highlighted; in the *y*-axis the levels of degenerate bases are indicated as follows: A, T, C or G (0 degenerate bases or 100% conserved); R, Y, S, W, K or M (two degenerate bases or 50% conserved); D, B, H or V (three degenerate bases or 25% conserved) and N (four degenerate bases or 0% conserved)

The proposed methodology would entail a preceding screening of the taxonomic biodiversity of the studied samples. Tools in the field of molecular biology, such as high-throughput sequencing, are recommended as a preliminary screening. In addition to taxonomic

studies, microarrays [17] or *meta*-omics [22] can be carried out to detect critical genes.

Based on these screenings, the potential catabolic targets must be located in the corresponding associated node. Sequences included in this node are retrieved and aligned and the consensus sequence is generated. Finally, primers are designed.

2.2 Relating Previous Screenings with Alkane Catabolism

There is a high sequence divergence in AlkB within different bacterial taxonomic groups [10, 23]. Nevertheless, the amino acid sequences of the alkane hydroxylases clustered by taxonomic groups can be useful for primer design. These findings are summarised in the phylogenetic tree shown in Fig. 2 (the accession number of each member of this dendrogram is shown at *HyDeg*). The evolutionary relationships of AlkB are described as follows: cluster 1, including alkane hydroxylases of *Actinobacteria* related to AlkB1 (AAK97448) and AlkB2 (AAK97454) of *Rhodococcus* sp. Q15; cluster 2, including the alkane hydroxylases of *Proteobacteria* related to AlkB of *Pseudomonas fluorescens* Pf-5 (YP260041); cluster 3, with the alkane hydroxylases related to Mvan_3100 of *Mycobacterium vanbaalenii* PYR-1 (YP953908); cluster 4, the alkane hydroxylases of *Actinobacteria* related to AlkB3 (AAK97446) and AlkB4 (AAK97447) of *Rhodococcus* sp. Q15; cluster 5, comprising
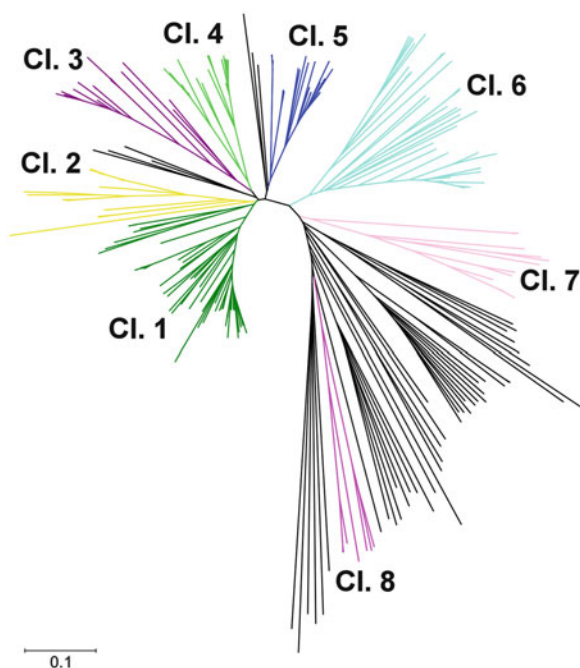
Fig. 2 Evolutionary relationships of integral-membrane alkane hydroxylases. Both accession number and bacteria harbouring the alkane hydroxylase genes are detailed at *HyDeg* (http:/www.hydeg.ugent.be)

the alkane hydroxylases of *Acinetobacter* spp. (YP046098 from *Acinetobacter* sp. ADP1, among others); cluster 6, the alkane hydroxylases related to AlkB1 of *Alcanivorax borkumensis* SK2 (BAC98365); cluster 7, including the alkane hydroxylases of *Proteobacteria* related to AlkB1 (NP250216) and AlkB2 (NP251264) of *Pseudomonas aeruginosa* PAO1; and cluster 8, with the *p*-cymene and xylene monooxygenases (NP542887).

Taxonomic bacterial diversity profiling can be a powerful screening tool to target AlkB catabolic gene variants involved in alkane degradation processes. For instance, we may have observed *Actinobacteria* enrichment in a microcosm contaminated with decane, in comparison with a control microcosm. Therefore, the alkane hydroxylases responsible for the hydroxylation of the alkane will be located most probably either in the cluster 1, cluster 3 or cluster 4 of the alkane hydroxylases dendrogram (Fig. 2).

It is important to consider that catabolic genes are species specific or even sub-species specific. This is to say, only particular strains within the *Pseudomonas* genus harbour the catabolic genes required for decane degradation. Thus, although 16S rRNA gene offers information about the location of the key genes, previous considerations beyond the scope of primer design must be contemplated.

Cytochrome P450 CYP153 alkane hydroxylases are a superfamily of enzymes involved in degradation of linear alkanes. CYP153 amino acid sequences cluster in groups depending on their sequence, although there is not clear taxa differentiation among the clusters. As shown in the phylogenetic tree of Fig. 3 (and displayed at *HyDeg* with the accession numbers of each sequence), the CYP153 superfamily may be grouped in three different clusters based on their amino acid sequences. These may correspond with different taxonomic classes as follows: cluster 1, which includes CYP153 related to α-*Proteobacteria*, β-*Proteobacteria* and *Actinobacteria*; cluster 2, which includes γ-*Proteobacteria* and *Actinobacteria*; and cluster 3, composed only of α-*Proteobacteria*.

In this way, taxonomic biodiversity profiles might not accurately indicate which branch of the CYP153 phylogenetic tree could be the potential target for primer design. Moreover, the number of genes related to CYP153 and deposited in the database is smaller than those related to AlkB. Consequently, defining the potential targets is complicated. In some cases however, preliminary screening of the taxonomic bacterial diversity may help to elucidate the CYP153 subgroups to be targeted.

So far we described how to apply our proposed strategy in controlled microcosm studies. If the aim of the study is to identify potential catabolic genes involved in the degradation of environmental linear alkanes, some techniques such as catabolic microarrays or *meta*transcriptomics (rather than metagenomics) are highly recommended as a first screening. In this manner, we might detect
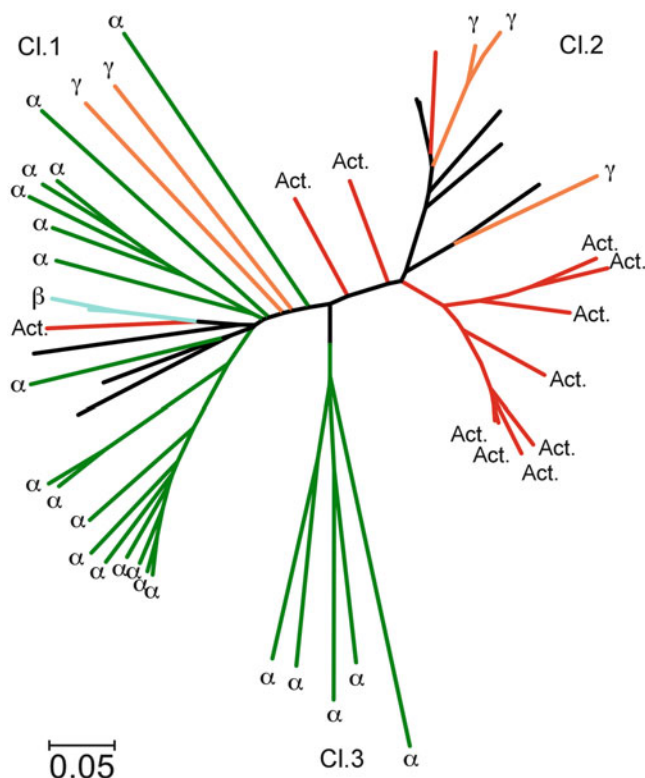
Fig. 3 Evolutionary relationships of cytochrome P450 CYP153 alkane hydroxylases. Both accession number and bacteria harbouring the cytochrome P450 CYP153 alkane hydroxylases genes are detailed at *HyDeg* (http:/www. hydeg.ugent.be)

fragments of genes of interest; we can retrieve from the database those closely related and then proceed as described below. Applying metatranscriptomics, we will detect all the genes expressed under specific conditions and not only present in the sample of interest. Currently, 300 bp reads can be retrieved from high-throughput sequencing; these can be assigned to any catabolic gene of interest upon automatic (MG-RAST) [24] and manual annotation. Following, we can acquire the sequences most closely related to those probes and design primers. This last approach will provide larger length and localise the gene in the same microorganism.

3   Primer Design

To ensure consistency, the general methodology for primer design has to follow common steps, independent of the catabolic gene family analysed. However, the complexity of the entire process can differ depending on the specific gene family cluster of interest. Let us consider the biodiversity of AlkB (Fig. 4): nodes with a low
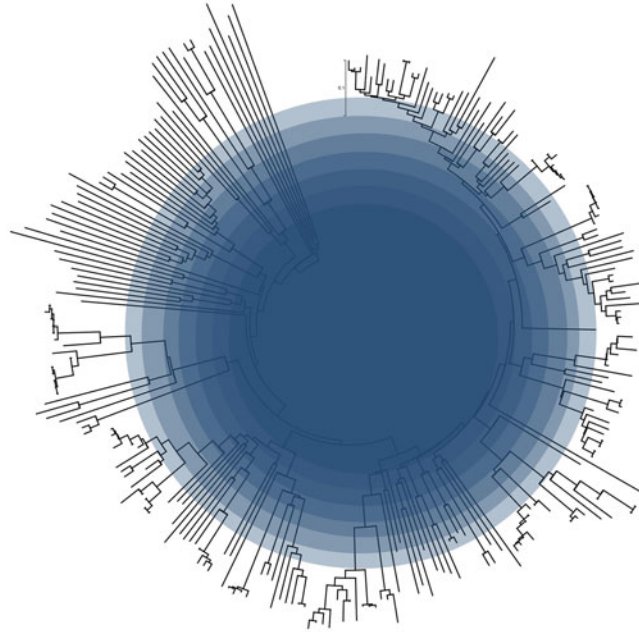
Fig. 4 Level of complexity in primer design approaches depending on the number of representatives per node. *Dark blue* denotes high level of complexity, due to low number of representatives per branch, in contrast with *light blue*

number of representatives (i.e. one member) are located in the dark blue zone; the light blue zone contains those with large number of representatives. When the number of representatives per node increases (towards the light blue zone), the sequence variability decreases; thus, the primer design is facilitated. The more branches the node has, the easier it becomes to design a primer set. In the graphical representation, the lighter area of Fig. 4 indicates the low level of complexity for designing a primer set. Nevertheless, because of the lack of a standard operating procedure that allows systematic primer design for the whole set of branches, the primer design protocol requires sequential experimental testing. It must be noticed that if we consider a large amount of representatives, the consensus sequence will contain numerous degenerate bases; as a result the probability to obtain the desired PCR products will be reduced.

Our suggested methodology is graphically represented in Fig. 5.

Based on the information resulting from the analysis of any given set of environmental samples (e.g. taxonomic biodiversity shifts, catabolic microarrays, metagenomics, metaproteomics or metatranscriptomics data), we can firstly locate potential key genes involved in the degradation of alkanes in the databases. Those databases from AlkB and CYP153, among other catabolic
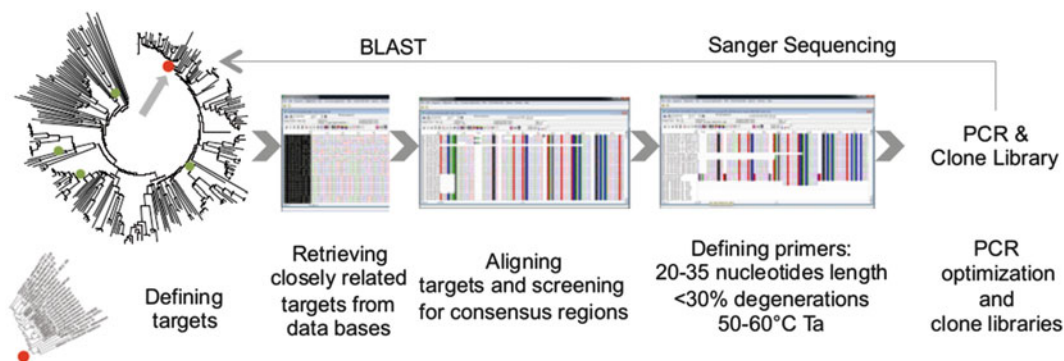
Fig. 5 Step-by-step flow for the systematic primer design methodology

genes, can be downloaded from *HyDeg* for both DNA and amino acid sequences. Then, the selection of the specific node to target can be performed following selection of the closely related representatives nearby the genes of interest. Once the node is defined, we can retrieve those amino acid sequences from the database. Each sequence will be individually blasted against the NCBI database (http://blast.ncbi.nlm.nih.gov/), using the default algorithm parameters. Further on, the nucleotide sequences of the positive matches will be retrieved, generating single *fasta* files. Finally, all sequences will be merged into a single *fasta* file, and duplicate sequences will be deleted. The complete data set for the targeted node will include unique DNA sequences of all closely related genes present in the NCBI database. Subsequently, the DNA sequences are aligned using MUSCLE [25] or Clustal Omega [26], both accessible via EMBL [27], and consensus regions are selected for primer design. We defined a node based on the frequency of the degenerated nucleotides in the consensus sequence of a given number of targets, within each superfamily of enzymes. If the frequency was higher than approximately 65%, the sequences with the lowest similarity were allocated in the adjacent node.

To illustrate this workflow: for targeting cluster 1 of AlkB (alkane hydroxylases of *Actinobacteria* related to AlkB1 (AAK97448) and AlkB2 (AAK97454) of *Rhodococcus* sp. Q15), three nodes were defined based on the sequence variability. Primers for node 1 were designed based on 69 DNA sequences; primers for node 2 were designed based on 23 DNA sequences; and primers for node 3 were designed based on 38 DNA sequences. The search for consensus regions allowed the generation of 13 different candidate primer sets; these covered the complete cluster 1 according to the general primer specifications shown in Fig. 5. All sequences for targeting cluster 1, among other clusters, can be downloaded from *HyDeg*. A summary of the proposed primers is shown in Tables 3 and 4 for targeting alkane hydroxylases and cytochrome P450 CYP153 alkane hydroxylases respectively.

Table 3
List of primers described in HyDeg for targeting alkane hydroxylases

Superfamily	Class	Subclass/ relative to target	Primer name (f_ = forward; r_ = reverse)	Sequence 5′–3′	Reference
Alkane hydroxylases	Alkane hydroxylases of *Actinobacteria*	*Micrococcus vanbaalenii* PYR 1	AlkBYP952571f_803	CACTTCTACATCGAGCACAACCG	*HyDeg* AlkB node AlkB 1
			AlkBYP952571f_803deg	CACTTCTWCATCGAGCACAACCG	
			AlkBYP952571r_1289	GCAGGTGGTAVAGGAACAGGTT	
			AlkBYP952571r_1289deg	GBAGGTGRTAVAGGAACAGRTT	
		Rhodococcus 1BN	AlkBCAC14062f_540	CACTTCTACATCGAGCACAA	*HyDeg* AlkB node AlkB 2
			AlkBCAC14062f_445	TGTCGGAGGAATCGGGATCA	
			AlkBCAC14062r_880	TGYTCGAGGTAGTTSACGGT	
		Rhodococcus erythropolis PR4	AlkBYP002764193f_546	CTTCTACVTCGAGCACAACCG	*HyDeg* AlkB node AlkB 3
			AlkBYP002764193f_546deg	YTTYTWCVTCGARCACAACCG	
			AlkBYP002764193r_862	BCCGTAGTGCTCVABGTAGTT	
			AlkBYP002764193r_862deg	BCCGTARTGCTCVABRWAGTT	
			AlkBYP002764193r_982	GTGNCGYTGCAGGTGRTAVAG	
			AlkBYP002764193r_982deg	RTGNCGYTGBARGTGRTAVAG	
	Alkane hydroxylases of *Alcanivorax*	*P. putida* TF4	AlkBAAP41820f_451	AAGCCTTTGATCGTTGGATGG	*HyDeg* AlkB node AlkB 4
			AlkBAAP41820f_451deg	ARRSYTYYGAYCGYWGGATGG	
			AlkBAAP41820r_710	GCTGGAGGATCTCATTATCG	
			AlkBAAP41820r_710deg	GYTGGAGGATYTCATTATCK	
	Cymene and xylene monooxygenases	*Pseudomonas* TW3	AlkBNP542887f_375	KCGCAYGAGYTGATGCATCG	*HyDeg* AlkB node AlkB 5
			AlkBNP542887f_375deg	KCGCAYGAGYTGWKGCATCG	
			AlkBNP542887r_980	AASAGCGAAGGCATCTGCGG	
			AlkBNP542887r_980deg	AASAGCGARGGCATCTGCGG	
			AlkBNP542887f_624	ACTYYTGYTYGCTCTRCCTGG	*HyDeg* AlkB node AlkB 6
			AlkBNP542887f_624deg	ACTCYTGYTYGCTCTGCCTGG	
			AlkBNP542887_943r	CCARAGWGGYGGAATAAGCCC	

Table 4
List of primers described in HyDeg for targeting cytochrome P450 CYP153 alkane hydroxylases

Class	Subclass/relative to target	Primer name	Sequence 5′–3′	Reference
Cytochrome P450 CYP153 alkane hydroxylases	*Rhodopseudomonas palustris* BisA53	CYP153RhPse_520f	CTBGCVACSHTBYTYGAYTTYCC	*HyDeg* CYP153 node 1
		CYP153RhPse_1165r	CCSAYRCASCGRTGVAYRCCRWARCC	
	Broad spectrum of targets (only forward)	CYP153f_467	GKNGRNAAYGAYACBACVCGNAAYWC	*HyDeg* CYP153 nodes 2,3,6,8
	Bradyrhizobium USDA 110	CYP153r_793_2	CCRAAVCCRAAVGMRATGTGGTTGCG	*HyDeg* CYP153 node 2
	Uncultured bacterium BAE47483	CYP153r_793_3	KRTGRAYRCCRAAVCCRAABGANARRTG	*HyDeg* CYP153 node 3
	Alcanivorax borkumensis SK2	CYP153r_793_6	GVAYDCCGWADCCRAAVGARAKRTG	*HyDeg* CYP153 node 6
	Rhodococcus erythropolis PR4	CYP153r_793_8	GGTGVAYRCCRWAGCCRMASGMVRDRTG	*HyDeg* CYP153 node 8
	Mycobacterium HXN1500	CYP450_BAE47485f_364	CCSAARCACGAYRWSCAGCG	*HyDeg* CYP153 node 4
		CYP450_BAE47485r_956	GTYTGCCAVCGVAYGRTCTC	
	Sphingopyxis macrogoltabida HXN200	CYP450_BAE47482f_338	GCGATGGACCMVCCMAAGCA	*HyDeg* CYP153 node 5
		CYP450_BAE47482r_1127	CGRTGRATSCCRWARCCGAA	
	Mycobacterium MCS	CYP450_CAH56120f_886	ATCKTSGGNGGHAACGACAC	*HyDeg* CYP153 node 7
		CYP450_CAH56120r_1187	GCGTTSBBBCKRTCGATGAT	

4   Perspectives

Overall, there are 14 nodes currently defined in *HyDeg*, along with the corresponding set of primers. The presence of conserved regions and similarity among the members composing each group were considered for their construction. This partitioning allowed designing primer sets that can effectively cover the complete sequence diversity reported for the families within each cluster. Nevertheless, contributions to enhance the representativeness of this proposal are welcome. Continuous upgrades would indeed refine and improve the primer design and expected coverage across family classification, in a systematic and documented manner.

We present the *HyDeg* database as a reliable tool for designing primers to target enzymes involved in hydrocarbon degradation.

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Primers: Functional Genes for Anaerobic Hydrocarbon Degrading Microbes

Tillmann Lueders and Frederick von Netzer

Abstract

The detection of anaerobic hydrocarbon degrader populations via catabolic gene markers is important for the understanding of processes at contaminated sites. The genes of fumarate-adding enzymes (FAEs; i.e., benzylsuccinate and alkylsuccinate synthases) are widely used as specific functional markers for anaerobic degraders of aliphatic and aromatic hydrocarbons. Several recent studies have shown the existence of new and deeply branching FAE gene lineages in the environment, and respective FAE gene-targeted primer systems have been advanced. Here, state-of-the art protocols for the PCR detection, T-RFLP fingerprinting as well as sequencing of FAE gene amplicons are described. These protocols can also readily be applied to other established functional markers for anaerobic degraders of petroleum hydrocarbons, such as benzoyl-CoA reductases as well as the ring-cleaving hydrolases involved in the central catabolism of aromatic hydrocarbons. In summary, these assays allow for rapid and directed insights into the diversity and identity of intrinsic degrader populations and degradation potentials in hydrocarbon-impacted systems.

Keywords: Benzoyl-CoA reductase, Benzylsuccinate synthase, Fingerprinting, Fumarate-adding enzymes, PCR amplification, Sequencing

1 Introduction

1.1 Anaerobic Hydrocarbon Degrading Microbes in the Environment

Biodegradation is the key process reducing hydrocarbon contamination in natural environments. In the absence of oxygen, the initial activation is the crucial step in the catabolism of aliphatic or aromatic hydrocarbons. Similar to aerobic catabolic pathways, anaerobic hydrocarbon degradation is based on funneling pathways, where a compound is initially activated and converted to central metabolites, which are then further degraded to assimilatory units or completely oxidized to CO_2 [1].

Currently, three general strategies for the anaerobic activation of petroleum hydrocarbons are known [2]: (i) addition of a methyl or methylene group of the hydrocarbon substrate to fumarate via a glycyl radical enzyme; (ii) oxygen-independent hydroxylation, known to be involved in the degradation of ethylbenzene and

related substituted benzenes [3, 4]; and (iii) carboxylation, proposed for alkanes [5, 6], methylnaphthalenes [7], naphthalene and benzene [8, 9] as well as for phenanthrene [10].

Fumarate addition by benzylsuccinate synthase (BSS) was first reported for the activation of toluene by *Thauera aromatica* strain K172 [11]. BSS adds an enzyme-bound benzyl radical formed from toluene to the double bond of fumarate (Fig. 1). Technically, this process is often referred to as "fumarate addition." The thus formed benzylsuccinate is subsequently degraded via further activation to CoA-thioesters and via reactions similar to β-oxidation to benzoyl-CoA, the central metabolite in anaerobic aromatic hydrocarbon degradation [1]. The substrate range of fumarate-adding enzymes (FAE) is not only limited to the activation of aromatic compounds such as toluene, xylenes, and also ethylbenzene [2]. The same activation reaction is also used in alkylsuccinate synthases (ASS, also called methylalkylsuccinate synthase MAS [12, 13]) for long and short chain alkanes [14–16], as well as in naphthylmethyl-succinate synthases (NMS) for 2-methylnaphthalene activation [17]. The analogous activation reactions for BSS, NMS, and ASS are illustrated in Fig. 1.

Cyclic alkanes may also be activated by addition to fumarate [18, 19]. Furthermore, cresols [2] and also linear alkylbenzene sulfonate detergents [20] have been reported to be activated via fumarate addition. Therefore, fumarate addition can be considered as widely spread key reaction for anaerobic hydrocarbon degradation [21].

All aforementioned anaerobic aromatics activation pathways funnel the monoaromatic (and possibly also polyaromatic) compounds to benzoyl-CoA. The aromatic ring is desaturated in several steps by benzoyl-CoA reductases. There are two systems known for the initial dearomatization step [1]: either the ATP-dependent benzoyl-CoA reductase BcrABCD in facultative anaerobes like *T. aromatica* and *Azoarcus* spp. or the ATP-independent benzoyl-CoA reductase BamBCDEFGHI in strict anaerobes like *Geobacter metallireducens*. Subsequently, the ring-cleaving hydrolase (BamA) precedes subsequent β-oxidative-like reactions, yielding CO_2 and three molecules of acetyl-CoA which can be funneled into central metabolism (Fig. 1).

A wide diversity of bacterial cultures and enrichments is known to use fumarate addition for activating hydrocarbons and related substances while respiring different electron acceptors. We cannot go into extensive detail here, but important degraders are to be found within the *Rhodocyclaceae* (*Betaproteobacteria*), *Geobacteraceae*, *Desulfobacteraceae*, *Syntrophobacteraceae* (*Deltaproteobacteria*), and *Peptococcaceae* (*Clostridia*) [22, 23].
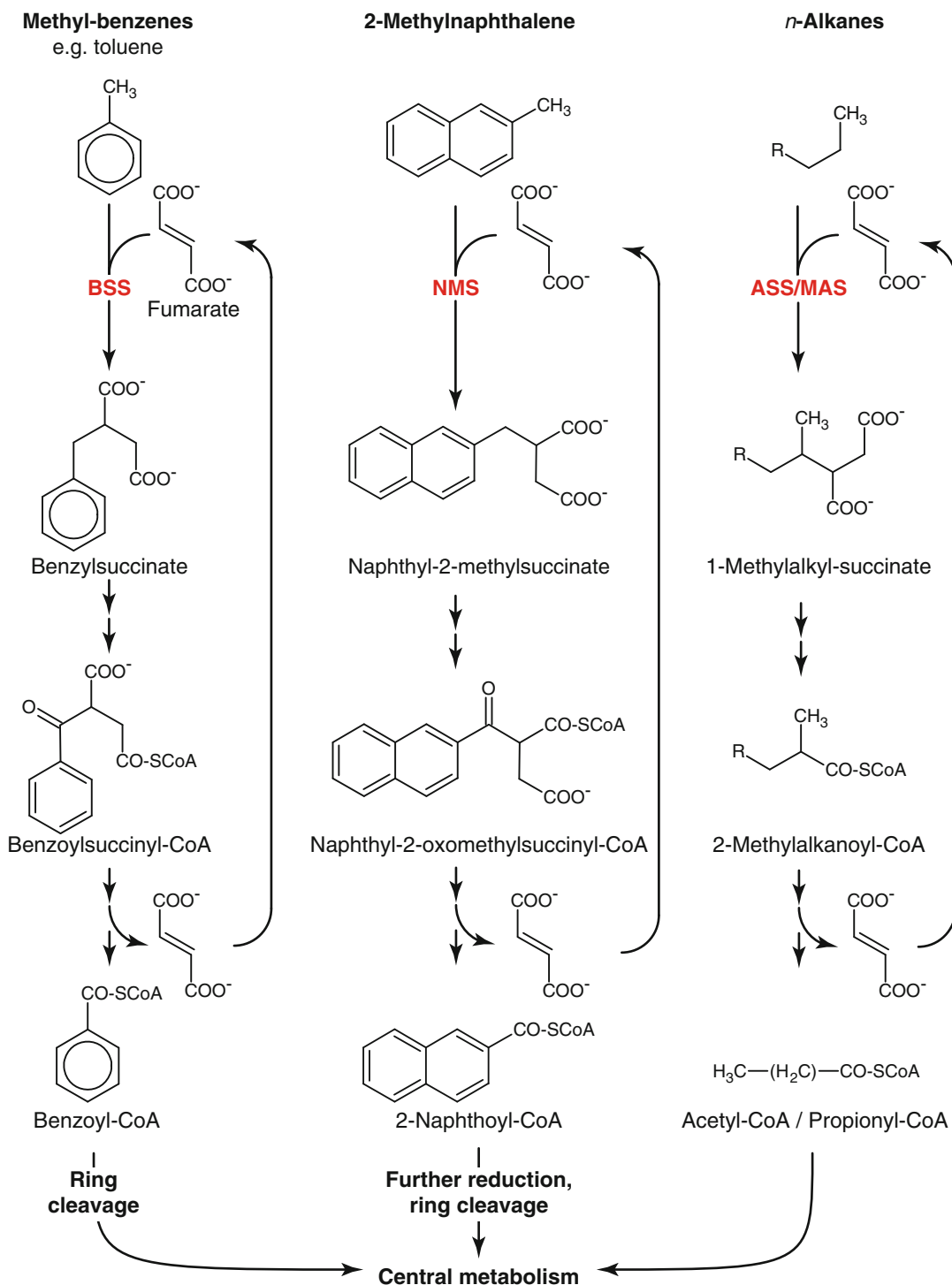
Fig. 1 Initial activation of toluene, 2-methylnaphthalene, and *n*-alkanes by the FAEs benzylsuccinate synthase (BSS), naphthylmethylsuccinate synthase (NMS), and alkylsuccinate synthase (ASS)/methylalkylsuccinate synthase (MAS). Subsequent degradation steps and funneling into central metabolism are simplified, as well as the recycling of fumarate

1.2 Detection Assays for Anaerobic Hydrocarbon Degradation Genes

Because of their widespread occurrence and strict functional affiliation, FAE genes are well suited for tracing natural populations of anaerobic hydrocarbon degraders [24, 25]. Although FAE display wide substrate ranges, due to their unique biochemistry – addition of a hydrocarbon substrate radical to fumarate – they harbor specific protein motifs whose gene sequences are ideal for creating functional gene PCR assays. Several primers targeting the genes for the α-subunit of BSS and ASS are currently in use for the detection of hydrocarbon degrader lineages (*see* Table 1 and Fig. 2 for an overview).

The first primers for qualitative (and quantitative) PCR targeting of *bssA* genes of nitrate-reducing *Betaproteobacteria* were introduced by Beller and colleagues [26]. This assay was later updated also for sulfate-reducing bacteria [27]. The primers of Washer et al. [28] were specifically designed for a toluene-degrading, methanogenic microcosm. The assay of Winderl and co. [24] extended the range of detectable hydrocarbon-degrading microbes to iron- and sulfate-reducing *Deltaproteobacteria*. Staats et al. [29] applied altered primers first developed by Botton and co. [30], which targeted *bssA* of iron- and nitrate-reducing degraders in an aquifer contaminated by landfill leachate. The retrieved *bssA* sequences were related to the betaproteobacterial *bssA* sequence of *Georgfuchsia toluolica* [31], rather than to the *Geobacter* populations expected from in situ 16S rRNA gene studies.

Recently, Callaghan and co. [25] introduced assays also for ASS genes, evolved from existing *bssA* primers, on the basis of the small number of pure culture *assA* sequences available. These optimized primer sets were applied to DNA extracted from propane- and paraffin-degrading enrichments as well as several aquifer, freshwater, and estuarine habitats contaminated with alkanes, revealing for the first time a similar diversity of *assA* genes in the environment as already known for *bssA* genes. Also in contaminated marine sediments, some of these primers have been proven to successfully recover hydrocarbon-degrading potentials [32].

However, most of these established *bssA* and *assA* gene detection assays were not designed to target novel, deeply branching FAE gene sequences. Already Winderl et al. [24] retrieved several unassigned, deeply branching *bssA* lineages, the so-called T- and F-clusters (Fig. 3). Using the same primers on a tar-oil-contaminated aquifer, Yagi and coworkers found new sequences related to the F2-cluster [33]. Similarly, Hermann et al. reported sequences related to T-cluster *bssA* homologues and *nmsA* sequence clusters for xylene-degrading enrichments [34]. These studies emphasize the existence of new and deeply branching FAE lineages, in addition to the known BSS, NMS, and ASS lineages.

Furthermore, several novel anaerobic hydrocarbon degraders within the *Clostridia* were recently discovered: *Desulfitobacterium aromaticivorans* UKTL, using fumarate addition for toluene

Table 1
Primers published for the detection of *bssA* and *assA* genes in environmental samples

Reference	Primer name		Gene target	Lineage specificity (if demonstrated)	Primer sequence (5′–3′)	Amplicon (bp)	qPCR tested
Beller et al. [26]			*bssA*	Denitrifying *Betaproteobacteria*	ACGACGGYGGCATTTCTC GCATGATSGGYACCGACA	130	Yes
Winderl et al. [24]	7772f 8546r		*bssA*	*Beta-* and *Deltaproteobacteria, Clostridia*	GACATGACCGACGCSATYCT TCGTCGTCRTTGCCCCAYTT	800	Yes
Beller et al. [27]	SRBf SRBr		*bssA*	Sulfate-reducing *Deltaproteobacteria*	GTSCCCATGATGCGCAGC CGACATTGAACTGCACGTGRTCG	100	
Callaghan et al. [25] (Set 2: Callaghan et al. [13])	Primer Set 1		*assA*	Designed to target also *bssA*	TTTGAGTGCATCCGCCAYGGICT TCGTCRTTGCCCCATTTIGGIGC	700	
	Primer Set 2		*bssA*	Proteobacterial *bssA*	GACATGACCGAYGCCATYCT TCRTCGTCRTTGCCCCAYTT	800	
	Primer Set 5		*assA*	Deltaproteobacterial *assA*	TTYGAGTGYATNCGCCASGGC TCRTCATTNCCCCAYTNGG	700	
	Primer Set 7		*assA*		CCNACCACNAAGCAYGG TCRTCATTNCCCCAYTTNGG	520	
	Primer Set 9		*assA*		CCNACCACNAAGCAYGG TCGTCRTTGCCCCATTTIGGIGC	500	
Staats et al. [29]	bssA3f bssAr		*bssA*	Iron- and nitrate-reducing *Proteobacteria*	TCGAYGAYGGSTGCATGGA TTCTGGTTYTTCTGCAC	500	Yes
von Netzer et al. [21]	FAE-B	7768f 8543r	*bssA* s.l.	Clostridial *bssA*, *lbssA* sensu lato, *nmsA*	CAAYGATTTAACCRACGCCAT TCGTCRTTGCCCCAYTTNGG	800	
	FAE-N	7363f 7374f 8543r	*nmsA*	*nmsA* sensu stricto	TCGCCGAGAATTTCGAYTTG TTCGAYTTGAGYGGACAGCGT TCGTCRTTGCCCCAYTTNGG	1200	
	FAE-KM	7757f-1 7757f-2 7766f 8543r	*assA* (*nmsD*)	Deltaproteobacterial *assA*	TCGGACGCGTGCAACGATCTGA TCGGACGCGTGCAACGCCCTGA TGTAACGGCATGACCATTGCGCT TCGTCRTTGCCCCAYTTNGG	800	

(continued)

Table 1
(continued)

Reference	Primer name	Gene target	Lineage specificity (if demonstrated)	Primer sequence (5′-3′)	Amplicon (bp)	qPCR tested
Aitken et al. [41]	assA2F / assA2R	assA	Sulfate-reducers and syntrophs	YATGWACTGGCACGGMCA / GCRTTTTCMACCCAKGTA	500	
	assA3F / assA3R	assA	Sulfate-reducers and syntrophs	CCGCACCTGGGTKCAYCA / GKCCATSGTGTAYTTCTT	500	
	assA2Fq / assA2Rq	assA		ATGTACTGGCACGGACA / GCGTTTTCAACCCATGTA	500	Yes
	assA 3Fq / assA 3Rq	assA		CGCACCTGGGTTCATCA / GGCCATGGTGTACTTCTT	500	Yes
Song and Ward [43]	bzAQ41F / bzAQ4R	bcrA s.l.	bcrA homologues of Alpha-, Beta-, and Gammaproteobacteria	GTGGGCACCGGNTAYGGNMG / GGTTCTTGGCGAYNCCNCCNGT	450	
Hosoda et al. [42]	bcr-1f / bcr-2r	bcrA	Thauera aromatica, Azoarcus evansii, Rhodopseudomonas palustris	GTYGGMACCGGCTACGGCCG / TTCTKVGCIACICCDCCGG	480	Yes
Kuntze et al. [45]	bamA-SP9-f / bamA-ASP1-r	bamA	Geobacter metallireducens, Syntrophus aciditrophicus	CAGTACAAYTCCTACACVACBG / CMATGCCGAIYTCCTGRC	300	Yes
Löffler et al. [44]	bamBf / bamBr	bamB	Deltaproteobacteria, Clostridia	ATGMGGTAYGSAGARACHGG / CCSGCRWRYTTCADYTCCG	320	
Staats et al. [29]	oah_f / oah_r	bamA	Iron- and nitrate-reducing Proteobacteria	GCAGTACAAYTCCTACACSACYGABATGGT / CCRTGCTTSGGRCCVGCCTGVCCGAA	350	Yes
Kuntze et al. [45]	bamA-ASP23-r / bamA-ASP33-r	bamA (with bamA-SP9-f)	GMT cluster SA cluster	TTTTCCTTGTTGVSRTTCC / CAKYSGGGAASAGRTTKG	800 700	Yes
	bzdNf / bzdNr	bzdN	Azoarcus-type class I benzoyl-CoA reductases	GAGCCGCACATCTTCGGCAT / TRTGVRCCGGRTARTCCTTSGTCGG	700	
	bcrCf / bcrCr	bcrC	Thauera-type class I benzoyl-CoA reductases	CGHATYCCRCGSTCGACCATCG / CGGATCGGCTGCATCTGGCC	600	
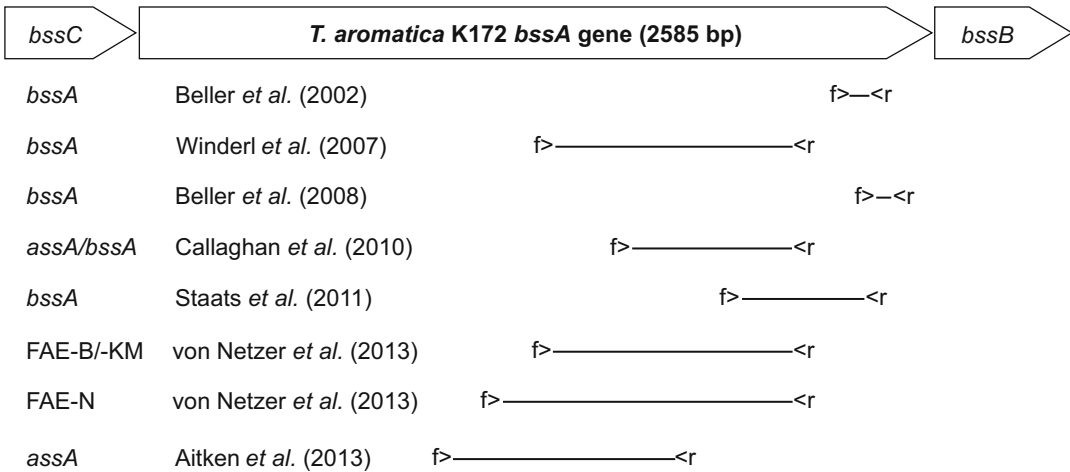
Fig. 2 Localization and overlap of selected published *bssA* and *assA* primer sets on the *bssA* gene of *Thauera aromatic* K172 (AJ001848). Naming of primer sets and amplicon length are as in Table 1. Modified from [24]
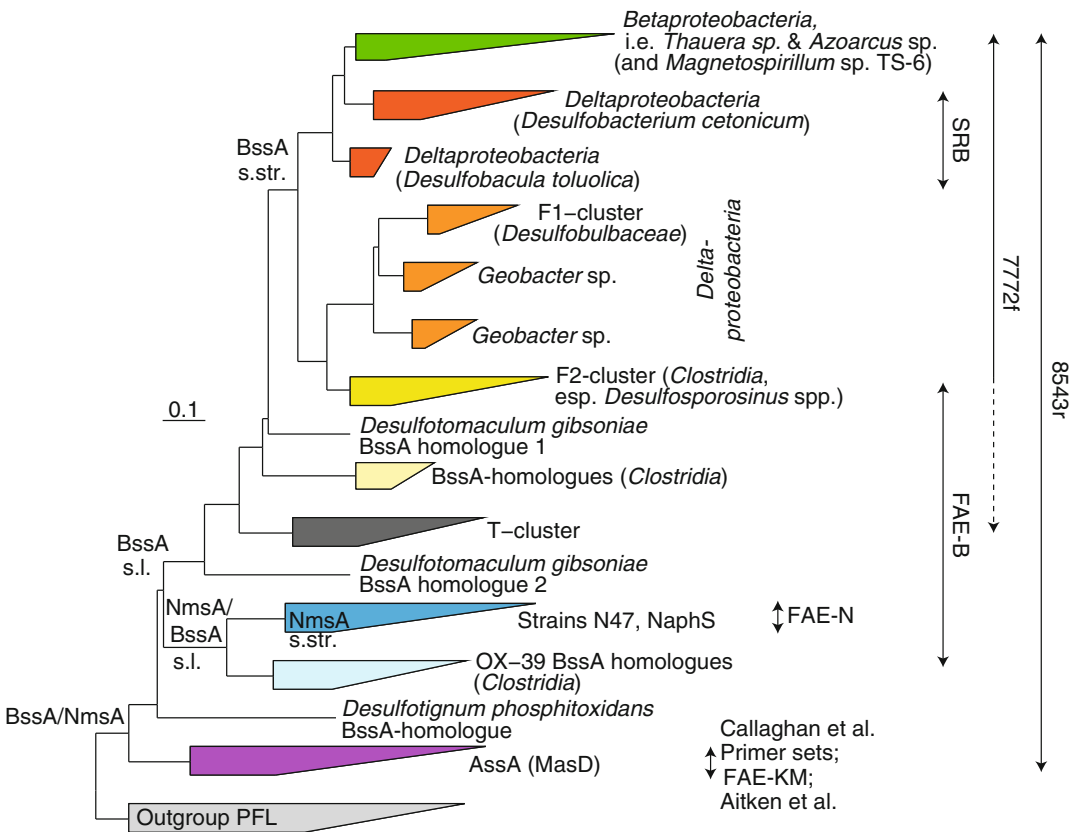
Fig. 3 Overview of the phylogeny of known pure culture and environmental FAE gene sequences. Several lineages are collapsed with only a few representatives named. Additionally, the demonstrated range of coverage for selected primer pairs is indicated. *S.str.* sensu stricto, *s.l.* sensu lato. The *scale bar* represents 10% amino acid sequence divergence

activation [35], and strain BF, possessing a *bss*-homologous operon [9] despite utilizing benzene and not toluene. Their FAE genes were not detectable with the aforementioned *bssA* assays. Moreover, the NMS genes of recently described naphthalene-degrading marine strains NaphS2, NaphS3, NaphS6 [36], and aquifer sediment enrichment strain N47 [37] were not targeted by these primers. Also for the syntrophic *Smithella* spp. found in several methanogenic alkane-degrading systems, a new clade of *assA* genes has recently been identified [38–40]. Consequently, the optimization of assays for a more comprehensive recovery of FAE genes with primers less biased towards known proteobacterial FAE gene sequences is still an ongoing process [21, 41].

Apart from FAE genes, several other functional markers have also been employed successfully for detecting the functional guild of anaerobic aromatic degraders: the benzoyl-CoA reductases *bcr/bzd* [42, 43] or *bamB* [44] as well as the ring-cleaving hydrolase *bamA* [29, 45, 46] of the central metabolism of aromatic hydrocarbon degradation. Although less strictly targeted towards anaerobic pollutant degraders [29], these assays allow for insights into the diversity and identity of intrinsic aromatics degrader populations also when fumarate addition is not involved.

1.3   Primer Selection   The primary protein structure of the alpha subunit of succinate synthases is more conserved towards the C-terminus [47]. Therefore, it is easier to find conserved primer motifs towards the 3′-end of fumarate-adding enzyme genes (Fig. 2). At the moment, we recommend the 8543r primer [21], adapted for more optimal performance compared to prior versions [24, 25, 28], as most suited for covering a wide selection of FAE gene lineages (Fig. 3). The lineage specificity of the PCR reaction must then be guided by the forward primer and also the annealing temperature [21]. For detecting a wide range of *bssA* genes sensu stricto (s.str.), the forward primer 7772f has been used and tested extensively [24, 34, 48–50]. Other forward primers are needed for more deeply branching *bssA* sensu lato (s.l.) and homologues (i.e., the FAE-B primer for clostridial *bssA* homologues in Fig. 3) and especially for the *nmsA* lineage. The *assA*-specific primer sets of Callaghan et al. [25] and Aitken and coworkers [41], as well as the FAE-KM primers [21] are efficient and specific for recovering *assA* sequences [32, 41, 51].

Other primers are also available for the tracing of specific sublineages of *bssA* s.str., such as the primer set of Staats and colleagues [29] for iron- and nitrate-reducing *Proteobacteria*, the original *bssA* primer set designed for denitrifying *Betaproteobacteria* [26], as well as the SRB primers for sulfate-reducing *Deltaproteobacteria* [27].

1.4 Methods for the Screening of Anaerobic Hydrocarbon Degradation Genes

The screening for hydrocarbon degradation genes always starts with a simple PCR as a quick and qualitative check for the presence of potential degraders but should be followed by further downstream processing for more detailed characterization. For phylogenetic dissection of degrader communities, cloning and sequencing of FAE genes and also other markers is well established [24, 25, 29, 45]. The cloning yield is directly dependent on the specificity and diversity coverage of the chosen primer pair for a given site. PCR artifacts of low stringency primers will reduce the cloning yield drastically.

qPCR with FAE gene primers has also been repeatedly used for quantifying hydrocarbon degradation genes in environmental samples [26, 27, 41, 52]. However, compared to cloning and sequencing, qPCR reactions need to be performed under even more stringent conditions. The detection relies either on SYBR-Green [27, 41] or also on lineage-specific qPCR probes [26, 52]. However, due to elevated stringency needs, only distinct sublineages may be readily quantifiable via qPCR. Some of the more general primers given in Table 1 are certainly too degenerate for providing reliable quantification results. Table 1 indicates the primers where qPCR has been performed successfully with either high stringency settings or for samples with low diversity.

Fingerprinting, such as terminal restriction fragment length polymorphism (T-RFLP) analysis, offers a means for the rapid screening of large numbers of amplicon pools. Although T-RFLP analysis is not diagnostic, the identity of T-RFs may be elucidated with caution by cross-referencing of fragment lengths to in silico digested sequences. A dual-digest T-RFLP fingerprinting method for sequencing-independent diagnostics of major FAE gene lineages in environmental samples has recently been introduced [21]. In this manner, clear T-RF identification is facilitated, as some FAE gene lineages may share the same conserved restriction sites. Using a second digest with an alternative restriction enzyme provides a better means of peak identification, as the combined restriction fragment lengths are unique.

Recently, next-generation sequencing of marker gene amplicons is more and more widely used for targeting the diversity of environmental gene pools. Next-generation sequencing of 16S rRNA gene amplicons is already a standard method [53], and there are well-established, ready-to-use bioinformatics resources publicly available for automated 16S rRNA sequence classification [54–57]. In contrast, next-generation sequencing of functional genes is currently still emerging, requiring the setup of dedicated pipelines and well-curated classification databases, and has been employed mostly for aerobic monooxygenase genes [58, 59]. A first next-generation sequencing pipeline for FAE genes, enabling the high-throughput characterization of anaerobic hydrocarbon degrader communities, is currently under development.

2 Materials

2.1 PCR Amplification of FAE Gene Markers

1. Nuclease-free water (Promega (www.promega.com)), stable at room temperature but better kept at −20°C for lower contamination risk.

2. Taq Polymerase and PCR Kit (Fermentas (www.thermoscientificbio.com/fermentas/)), stable at −20°C.

3. Bovine serum albumin (BSA; Roche (www.roche.com/products.htm)), stable at −20°C.

4. dNTPs (Fermentas (www.thermoscientificbio.com/fermentas/)), stable at −20°C.

5. *bssA* 7772f and FAE-B 8543r PCR primers (*see* Table 1, biomers.net (www.biomers.net)), stable at −20°C.

6. Any PCR cycler and standard agarose gel electrophoresis unit available in your lab.

2.2 T-RFLP Fingerprinting of FAE Gene Amplicons

1. PCR reagents and equipment as listed above.

2. 5′-FAM-labeled FAE-B 8543r reverse primer (biomers.net (www.biomers.net)), stable at −20°C; keep exposure to light minimal.

3. MinElute PCR cleanup columns incl. 10 mM Tris (pH 8.5) elution buffer (Qiagen (www.qiagen.com/products/)).

4. Any UV–spectrophotometer capable of quantifying DNA in small (~2–5 μl) volumes of DNA available in your lab.

5. *TaqI* and *HaeIII* restriction enzyme kits incl. buffers (New England Biolabs (www.neb.com/products)), stable at −20°C.

6. Dye-Ex Sephadex spin columns (Qiagen (www.qiagen.com/products/)).

7. Any table-top micro-centrifuge available in your lab.

8. Hi-Di formamide (Life Technologies (www.lifetechnologies.com)), stable at −20°C, toxic.

9. ROX-labeled MapMarker 1000 fragment ladder (BioVentures (www.bioventures.com)), stable at 4°C.

10. Any capillary electrophoresis genetic analyzer and GeneMapper fragment analysis software (both Life Technologies (www.lifetechnologies.com)) available in your lab.

2.3 Sequencing of FAE Gene Amplicons

1. PCR reagents and equipment as listed above.

2. Any routine TA-cloning kit and competent *E. coli* cells used in your lab (e.g. TOPO-XL Kit, Invitrogen (www.lifetechnologies.com/de/en/home/brands/invitrogen.html)).

3. Appropriate agar plate media for selection of insert-positive clones as suggested by the manufacturer of the cloning kit.

4. Vector-targeted PCR primers or plasmid isolation kit as specified by the manufacturer of the cloning kit.

5. Any capillary electrophoresis genetic analyzer and Sanger sequencing analysis software (both Life Technologies (www.lifetechnologies.com)) available in your lab.

6. Any standard sequencing software suite (e.g., SeqMan, DNAStar (www.dnastar.com)) available in your lab.

7. Any sequences alignment database and phylogeny package available in your lab (e.g., ARB [60]).

3   Methods

The protocols below describe the PCR amplification, T-RFLP fingerprinting, cloning, and sequencing of FAE gene amplicons generated with established FAE gene primers [21, 24]. Basic workflows used in our lab are explained, but preferred kits and available instruments may differ in others. The general workflows can also easily be adapted to any other of the catabolic gene primer sets listed in Table 1.

Start with the DNA extraction protocol established in your lab and optimize for the respective samples (water, soil, sediments, enrichments). For the plethora of distinct DNA extraction protocols, further detail cannot be given here. Prior to FAE gene PCR, the quality and integrity of the recovered genomic DNA must be verified (e.g., by standard agarose gel electrophoresis).

3.1   PCR Amplification of FAE Gene Markers

1. Set up a PCR reaction within a total volume of 50 µl containing nuclease-free water, $1\times$ PCR buffer, 1.5 mM $MgCl_2$, 10 µg BSA, 0.1 mM of each dNTP, and 1.25 U Taq polymerase.

2. Add 0.3 µM of each primer (*see* **Note 1**).

3. Add 1–2 µl of DNA extract (~2–20 ng) (*see* **Note 2**).

4. Run respective negative and positive controls as well (*see* **Note 3**).

5. Amplify in a PCR cycler under the following conditions: 3 min initial denaturation at 94°C, 30 cycles of amplification (30 s at 94°C, 30 s at 52°C, 60 s at 72°C), and 5 min at 72°C for terminal extension (*see* **Note 4**).

6. Visualize the resulting FAE gene amplicons by standard agarose gel electrophoresis (*see* **Note 5**).

3.2 T-RFLP Fingerprinting of FAE Gene Amplicons	1. Generate FAE gene amplicons from an environmental DNA extract as described in Sect. 3.1 using a 5′-FAM-labeled FAE-B 8543r reverse primer. Alternative fingerprinting assays with a labeled forward primer are also conceivable.

3.2 T-RFLP Fingerprinting of FAE Gene Amplicons

1. Generate FAE gene amplicons from an environmental DNA extract as described in Sect. 3.1 using a 5′-FAM-labeled FAE-B 8543r reverse primer. Alternative fingerprinting assays with a labeled forward primer are also conceivable.

2. After gel visualization, selected amplicons are purified using PCR cleanup columns following the manufacturer protocols.

3. Re-elute amplicons in 25 μl of elution buffer (*see* **Note 6**).

4. Quantify purified amplicons using UV–photometry (*see* **Notes 7** and **8**).

5. Separate dual digests can be set up per amplicon to increase diagnostic strength of downstream T-RF analyses. Thus, steps 7–13 are done in parallel for each sample with separate restriction enzymes.

6. Digest 60 ng of the purified amplicon in a 10 μl reaction by adding 0.3 μl (10 U μl^{-1}) restriction enzyme and 1 μl 10 × restriction buffer, and add nuclease-free water to a total volume of 10 μl.

7. Incubate for 2 h at 65°C (*TaqI*) or at 37°C (*HaeIII*-digests), respectively.

8. Desalt digested amplicons via Sephadex spin columns by centrifugation at 750 × *g* and at room temperature following manufacturer protocols (*see* **Note 9**).

9. Load 1 μl of desalted digest into 13 μl of Hi-Di formamide, containing a 1:400 dilution of ROX-labeled MapMarker 1000 fragment ladder (*see* **Note 10**).

10. Denature fragments at 95°C for 5 min, place immediately on ice.

11. Separate fragments by capillary electrophoresis (*see* **Note 11**).

12. Evaluate electropherogram raw data with fragment analysis software (*see* **Note 12**) to size-call T-RFs prior to downstream data handling (*see* **Note 13**).

3.3 Sequencing of FAE Gene Amplicons

1. Generate FAE gene amplicons from an environmental DNA extract as described in Sect. 3.1.

2. After gel visualization, purify selected amplicons using PCR cleanup columns following the manufacturer protocols, re-elute amplicons in elution buffer, and quantify by UV-measurement (*see* **Notes 5–7**, **14**).

3. Ligate ~35 ng of selected purified FAE gene amplicons into vector and transform competent *E. coli* cells using routine TA-cloning following manufacturer protocols.

4. Pick ~50–100 insert-positive clones on appropriate selective agar plate media.

5. Perform vector-targeted PCR of cloned inserts or isolate plasmids and screen for expected insert size.

6. Sequence inserts from both ends with vector primers on a Sanger sequencer following manufacturer's protocols.

7. Assemble resulting forward and reverse sequence reads for each clone individually with a standard sequencing software suite. Perform standard quality checks and crop remaining vector sequences.

8. Preliminarily identify contigs by BLAST (http://blast.ncbi. nlm.nih.gov/Blast.cgi) for similarities to published FAE genes.

9. Introduce generated FAE gene sequences into a database alignment for comprehensive downstream phylogenetic analysis.

4 Notes

1. Table 1 should assist the reader to select the optimal catabolic marker gene assay considering the specific research question and the anaerobic degrader lineages expected for the investigated samples.

2. Prior to FAE PCR, it is advised to check respective DNA extracts for absence of inhibitory humics concentrations and general amplifiability using routine bacterial 16S rRNA gene PCR.

3. Any DNA extract of an anaerobic toluene-degrading pure culture can be used as positive control. *Thauera aromatica* K172 or *Geobacter metallireducens* can be obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ (www.dsmz.de)) and are easily cultured.

4. Alternative thermal cycling programs can be found in the references for the other primers given in Table 1.

5. Owing to their high degeneracy, FAE gene primers tend to produce considerable PCR artifacts (smears of unspecific amplification products). Especially if more deeply branching, atypical FAE gene lineages are present. To alleviate this, reconsider primer choice or modify PCR annealing temperature. Gel purification of the desired amplicon is also a choice to improve downstream handling.

6. The elution volume may be lower or higher (15–50 μl), depending on the amplicon quality.

7. A NanoDrop spectrophotometer (Thermo (www. thermoscientific.com)) is well suited.

8. Purified amplicons can be stored frozen (−20°C) at this point.

9. Desalted digests can be stored frozen (−20°C) at this point.

10. The ratio of necessary ladder amendment may vary depending on the sequencer used.

11. Our lab uses an ABI 3730 genetic analyzer (Life Technologies (www.lifetechnologies.com)). Electrophoresis is done with POP-7 polymer in a 50 cm capillary array under the following conditions: 10 s injection time, 2 kV injection voltage, 7 kV run voltage, 66°C run temperature, and 63 min analysis time.

12. We use GeneMapper 5.1 (Life Technologies (www.lifetechnologies.com)). Usually, the fragment analysis window is set between 50 bp and the maximum amplicon length. Still, it is relevant to also consider the raw data, to find potential FAE T-RFs outside these cutoffs [21].

13. For example, the T-REX software [61]. Noise filtering can be done on the basis of the peak height with the standard deviation multiplier set to 1. Terminal restriction fragments (T-RFs) should be defined by aligning peaks with a clustering threshold of 1 bp.

14. For cloning of difficult FAE gene amplicons, further amplicon purification via gel extraction (QIAquick Gel Extraction kit, Qiagen (www.qiagen.com/products/)) might be necessary.

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Primers: Functional Marker Genes for Methylotrophs and Methanotrophs

Marc G. Dumont

Abstract

Methylotrophs are a diverse group of microorganisms that use compounds without a carbon–carbon bond as a sole source of carbon and energy for growth. Methylotrophs play an important role in most environments, including terrestrial, aquatic, and marine habitats. Several approaches to detect and identify methylotrophs in environmental samples have been developed. A common approach is to target protein-encoding genes since methylotrophs are phylogenetically diverse, making the design of 16S rRNA primers and probes with wide coverage difficult or impossible. The *mxaF* gene encoding the active site subunit of the methanol dehydrogenase is one of the more universal targets for methylotrophs, as are some of the genes involved in C1-transfer reactions, such as *fhcD* gene of methanopterin-linked pathway. The *pmoA* gene, encoding the β-subunit of the particulate methane monooxygenase, is a common target for methanotrophs. In many cases the evolution of these functional genes is congruent with the 16S rRNA and other phylogenetic markers, making them suitable for inferring taxonomy. This chapter summarizes the available primers and methods to detect or quantify various aerobic methylotrophs in environmental samples.

Keywords: *fhcD*, Methane, Methanol, Methylamine, Microbial ecology, *mxaF*, PCR, *pmoA*

1 Introduction

Methylotrophs have the ability to grow using one-carbon compounds as a sole source of carbon and energy. These include a wide diversity of microorganisms in all domains of life, including Archaea, Bacteria, and fungi [1–3]. This chapter focuses on aerobic methylotrophs, which are widely distributed across several bacterial phyla including Alpha-, Beta-, and Gammaproteobacteria, Verrucomicrobia, Cytophagales, Bacteroidetes, and Actinobacteria [1, 3].

A variety of compounds lacking carbon–carbon bonds are used as substrates for aerobic methylotrophs, including methanol, methane, methylamine, methanesulfonate, and chloromethane. Methylotrophs capable of aerobic growth with methane are referred to as methanotrophs. Methanotrophs first oxidize methane to methanol, which can then be completely oxidized to carbon dioxide with a

net gain in energy or assimilated into biomass at the level of formaldehyde or formate [1, 4]. Except for the oxidation of methane, one-carbon metabolism in methanotrophs and Gram-negative methanol utilizers is similar. Many methylotrophs use either the RuMP or serine pathway for carbon assimilation, and some also possess the Calvin–Benson–Bassham (CBB) cycle enzymes [5].

In many cases, methylotrophs can be detected in environmental samples by targeting functional marker genes encoding enzymes involved in one-carbon metabolism. For many purposes this is superior to trying to identify methylotrophs with 16S rRNA since methylotrophy is polyphyletic. One exception is for detection by conventional fluorescence in situ hybridization (FISH) since the high ribosome abundance in the cells results in many probe targets and strong fluorescence signals, which facilitates the procedure [6]. FISH using 16S rRNA probes has been widely used to detect methylotrophic organisms [7–13]. In addition, 16S rRNA targeted PCR primers have been designed to target individual genera of methylotrophs, such as *Methylobacterium* [14], or wider groups such as methanotrophs belonging to either the family Methylococcaceae (traditionally referred to as Type I methanotrophs) or Methylocystaceae (Type II methanotrophs) [15].

This chapter focuses on the PCR primers and assays used to target aerobic methylotrophs in environmental samples. Functional marker genes covering broad groups of methylotrophs are summarized, as well as those specific for specialized classes including methane, methylamine, methylated sulfur, and halomethane utilizers. Primers targeting the functional marker genes discussed in the chapter are shown in Table 1.

2 Functional Marker Genes for the Comprehensive Detection of Methylotrophs

There is no gene that is both unique to methylotrophs and found in all methylotrophic organisms; however, there are some metabolic modules that are present in diverse classes of methylotrophs [52]. PCR assays have been designed targeting functional genes encoding several of these enzymes diagnostic for methylotrophy, such as those responsible for methanol oxidation, the tetrahydromethanopterin-linked C1 transfer pathway and C1 assimilation. The assays for assimilation pathways are not discussed here since *cbbL* of the Calvin–Benson–Bassham cycle is not unique to methylotrophs, attempts to design primers for *hps* (3-hexulose 6-phosphate synthase) of the RuMP pathway were not successful [44], and assays for *hpr* (encoding hydroxypyruvate reductase) have not been extensively tested [44].

Table 1
Selection of PCR primers for the detection of methylotroph functional genes

Primer combination Forward/reverse	Gene target	Specificity[a]	Sequences (5′–3′) Forward/reverse	Product Product length (bp) [b]	Reference
mxaf1003/ mxar1561	*mxaF*		GCGGCACCAACTGGGGCTGGT/ GGGCAGCATGAAGGGCTCCC	560	[16]
1003 F/1555R	*mxaF*		GCGGCACCAACTGGGGCTGGT/ CATGAABGGCTCCCARTCCAT	550	[17]
F1003degen/ R1561degen	*mxaF*		GGNCANACYTGGGGNTGGT/ GGGARCCNTTYATGCTNCCN	560	[18]
mch1/mch2	*mch*		CTDCGCGMTCGGCTCSGGBCC/ ACGAAATCGGGHTGGGGCGG	340	[19]
mtdB1f/mtdB1r[c]	*mtdB*		CCGTTKGAYGTGAACATGGC/CTGGTAYTTGACGTTGCCGA		[19]
mtdB2f/mtdB2r[c]	*mtdB*		ACCGGCATCTTYATCGGCGGC/ GGCGGCACGGCGTTGACGTC	510	[19]
fae1f/fae1r[c]	*fae*		GTCGGCGACGGCAAYGARGTCG/GTAGTTGWANTYCTGGATCTT		[19]
fae2f/fae2r[c]	*fae*		GCACACMATCGACCTSATCATSGG/ CCAGTGRATGAAVACGGCRAC	305	[19]
planc-faef/ planc-faer	*fae*	Planctomycetes	CYCACATCGACCTGYTSATCGG/ CTTCACGGCTTCGTAGTTGTAC	360	[19]
Gamma-fae1f/ gamma-fae1r[c]	*fae*	Type I methanotrophs	AACHTGCGCGTHGGCGAATCWTTG/ GTTGTTAGCAGCAAATGGGTGTGTTGC		[19]
Gamma-fae2f/ gamma-fae2r[c]	*fae*	Type I methanotrophs	GGCAACGAARTYGCKCACATCG/ GCCGGCAACVGCGCGMKCGATGGC	395	[19]
fhcD105/fhcD947	*fhcD*		GACACCTTYGCNGARGCSTTYSC/ CCSAGNTTRCCGCCGTAGTTGCC	800	[20]

(continued)

Table 1
(continued)

Primer combination Forward/reverse	Gene target	Specificity[a]	Sequences (5′–3′) Forward/reverse	Product Product length (bp)[b]	Reference
A189f/A682r	pmoA/amoA		GGNGACTGGGACTTCTGG/ GAASGCNGAGAAGAASGC	530	[21]
A189f/mb661	pmoA		GGNGACTGGGACTTCTGG/ CCGGMGCAACGTCYTTACC	510	[22]
A189f/A650r[d]	pmoA		GGNGACTGGGACTTCTGG/ ACGTCCTTACCGAAGGT	480	[23]
pmof1/pmor	pmoA		GGGGGAACTTCTGGGGITGGAC/ GGGGGRCIACGTCITTACCGAA	330	[24]
pmof2/pmor	pmoA		TTCTAYCCDRRCAACTGGCC/ GGGGGRCIACGTCITTACCGAA	180	[24]
pmoAfor/pmoArev	pmoA		TTCTGGGGNTGGACNTAYTTYCC/ CCNGARTAYATHMGNATGGTNGA	280	[25]
f326/r643	pmoA		TGGGGYTGGACCTAYTTCC/ CCGGCRCRACGTCCTTACC	360	[26]
A189F/Mb601R	pmoA	Mbact/Msarc[e]	GGNGACTGGGACTTCTGG/ ACRTAGTGGTAACCTTGYAA	430	[27]
A189f/Mc468R	pmoA	Methylococcus	GGNGACTGGGACTTCTGG/ GCSGTGAACAGGTAGCTGCC	300	[27]
II223F/II646R	pmoA	Mcystis/Msinus[f]	CGTCGTATGTGGCCGAC/ CGTGCCGGCTCGACCATGYG	445	[27]
A189f/Mcap630	pmoA	Methylocapsa	GGNGACTGGGACTTCTGG/ CTCGACGATGCGGAGATATT	460	[27]
V170f/V613b	pmoA	"Methylacidiphilum"	GGATWGATTGGAAAGATMG/ GCAAARCTYCTCATYGTWCC	445	[28]

Primer	Gene	Target	Sequence	Size	Ref
Verruco120f/Verruco391r[g]	*pmoA*	"Methylacidiphilum"	GCCYATAGGWGCRACMT/GTCCATAGTATTCCAC	270	[29]
Verruco29f/Verruco500r[g]	*pmoA*	"Methylacidiphilum"	AAGAYMGRATGTGGTG/ACDCCHCCNGCAAARCT	470	[29]
VpmoA216/VpmoA622	*pmoA*	"Methylacidiphilum"	GGAAAGAYMGRATGTGGTGGCC/GTTTCNACCATNCGNATRTAYTCAGG	405	[30]
LVpmoAf/LVpmoAb	*pmoA*	Strain LP2A[h]	GGRTKGACTGGAAAGAYCG/GCGAARCTYCGCATCGTTCC	475	[31]
A189b/cmo682[c]	*pmoA*	"Methylomirabilis"	GGNGACTGGGACTTYTGG/AAAYCCGGCRAAGAACGA		[32]
cmo182/cmo568[c]	*pmoA*	"Methylomirabilis"	TCACGTTGACGCCGATCC/GCACATACTCCATCCCCATC	390	[32]
A189f/Forest675R	*pmoA*	USCα	GGNGACTGGGACTTCTGG/CCYACSACATCCTTACCGAA	505	[27]
USCa-346f/A682R	*pmoA*	USCα	TGGGGYGATCCTNGCNC/GAASGCNGAGAAGAASGC	185	[29]
A189f/Gam634r	*pmoA*	USCγ	GGNGACTGGGACTTCTGG/ACGAAGCGGATGTACTCGGG	465	[33]
A189f/C7-128r	*pmoA*	AOB-related[i]	GGNGACTGGGACTTCTGG/CCAATGGGGAGCCTAAAT	130	[29]
pxmA230F/pxmA732R.v1[j]	*pxmA*[k]		GGCARTGGTGGCCNTTGGT/TGGCGAACCATTTACCGATGTAC	550	[34]
pxmA230F/pxmA732R.v2[j]	*pxmA*[k]		GGCARTGGTGGCCNTTGGT/TSGCAAACCACTTGCCGATRTRC	550	[34]
A166f/B1401r	*mmoX*		ACCAAGGARCARTTCAAG/TGGCACTCRTARCGCTC	1,230	[35]
mmoX206f/mmoX886r	*mmoX*		ATCGCBAARGAATAYGCSCG/ACCCANGGCTCGACYTTGAA	720	[36]
534f/1393r	*mmoX*		CCGCTGTGGAAGGGCATGAA/CACTCGTAGCGCTCCGGCTC	865	[37]
met1/met4	*mmoX*		ACCAAGGAGCAGTTC/TCCAGAAGGGGTTGTT	665	[38]

(continued)

Table 1
(continued)

Primer combination Forward/reverse	Gene target	Specificity[a]	Sequences (5′–3′) Forward/reverse	Product length (bp)[b]	Reference
mmoX1/ mmoXr901b	mmoX		CGGTCCGCTGTGGAAGGGCATGAAGCGCGT / TGGGTSAARACSTGGAACCGCTGGGT	395	[39]
mmoXf882/ mmoXr1403	mmoX		GGCTCCAAGTTCAAGGTCGAGC / TGGCACTCGTAGCGCTCCGGCTCG	535	[40]
mmoX1/mmoX2	mmoX		CGGTCCGCTGTGGAAGGGCATGAAGCGCGT / GGCTCGACCTTGAACTTGGAGCCATACTCG	370	[41]
536f/877r	mmoX		CGCTGTGGAAGGGCATGAAGCG / GCTCGACCTTGAACTTGGAGCC	340	[42]
MmoXAf/ Mcel422r	mmoX	*Methylocella*	ACCAAGGARCARTTCAA/GAAGCCGCATTGATGGGT	295	[33]
mmoXLF/ mmoXLR	mmoX	*Methylocella*	GAAGATTGGGCGGCATCTG/ CCCAATCATCGCTGAAGGAGT	450	[43]
mauAf1/mauAr1	mauA		ARKCYTGYGABTAYTGGCG/ GARAYVGTGCARTGRTARGTC	310	[17]
mauA1-252 F/ mauA1-490R	mauA		GCACTGTTCCATCGACGGCA/ GCGCCGAAGCACCAGATGAT	240	[44]
mauA2-232 F/ mauA-526R	mauA		AAGTCTTGCGATTACTGGCG/ GACCGTGCAATGGTAGGTCA	295	[44]
mauAI-252 F/ mauAI-490R	mauA		GCACTGTTCCATCGACGGCA/ GCGCCGAAGCACCAGATGAT	240	[44]
mauAII-232 F/ mauAII-526R	mauA		AAGTCTTGCGATTACTGGCG/ GACCGTGCAATGGTAGGTCA	295	[44]
gmaS_557f/ α_gmaS_970r	gmaS	Alphaproteobacteria	GARGAYGCSAACGGYCAGTT/ CSGGCAAYAAYCGSACCCA	410	[45]

Primer	Gene	Specificity[a]	Sequence (5′–3′)	Length[b]	Reference
gmaS_557f/β_γ_gmaS_1332r	*gmaS*	Beta-/Gammaproteobacteria	GARGAYGCSAACGGYCAGTT/CATGGARTGGRTSGAKTAC	770	[45]
gmaS_F/R	*gmaS*	Marine *Roseobacter* clade	CCNGCBCAYCCSGAYATGCT/GTGCGGTTRTTGCCVGHCCA	790	[46]
ForA/B1rev2	*msmA*		TGAATGGGTTGATAGCCG/CCACTGGTTCGGCGGCAGATG	785	[47]
msmA_for/msmA_rev	*msmA*		AGGAAGGCTATCAGGACCG/GTTATGGTAGTGCATGAAGTC	235	[48]
msmA-202 F/msmA-854R	*msmA*		GCATCGCCAACGAGCCGATC/CCACTGGTTCGGCGGCAGAT	650	[44]
msmA-421 F/msmA-686R	*msmA*		AAGGAAGGCTATCAGGACCG/CCGGTTATGGTAGTGCATGA	265	[44]
cmuA802f/MF2	*cmuA*		TTCAACGGCGAYATGTATCCYGG/CCRCCRTTRTAVCCVACYTC	165	[49]
cmuA802f/cmuA1609R	*cmuA*		TTCAACGGCGAYATGTATCCYGG/TCTCGATGAACTGCTCRGGCT	785	[50]

[a]A specificity is indicated only in cases where the primers were designed to target genes from a restricted group of organisms rather than being universal in their specificity

[b]Lengths are approximate

[c]Amplified using a nested PCR method – the amplicon size is indicated for the final product only

[d]This primer was designed for use in DGGE, and therefore degeneracies were not included; also note that this primer set was shown to suffer from severe bias with environmental DNA templates [23]

[e]*Methylobacter* and *Methylosarcina pmoA* specific

[f]*Methylocystis* and *Methylosinus pmoA* specific

[g]These primers were used in all combinations – however, not tested using positive control DNA template [29]

[h]Targets the *pmoA* gene of the mesophilic "Methylacidiphilum infernorum" strain LP2A

[i]Targets the AOB-related clade as shown in Fig. 3 – but referred to originally as Cluster 7 [29]

[j]PCR is performed using a cocktail of the two versions of the reverse primer [34]

[k]*pxmA* is a *pmoA*-like gene found in some Methylococcaceae [34]

2.1 Quinoprotein Methanol Dehydrogenase (mxaF/xoxF)

Pyrroloquinoline quinone (PQQ)-containing methanol dehydrogenase (MDH) is found in diverse Alpha-, Beta-, and Gammaproteobacteria methylotrophs that have methanol as central metabolite [18]. Detailed phylogenetic trees of *mxaF* and related sequences have been shown and analyzed in the recent literature [53]. In methanotrophs, the MDH enzyme catalyzes the second step in the oxidation of methane, whereas in methanol utilizers it is the first step of carbon metabolism. The *mxaF* gene, encoding the large subunit of MDH, has been widely used as a functional marker [16, 18].

A homolog of *mxaF*, called *xoxF*, is found in some methylotrophic members of the Proteobacteria [54, 55] and Verrucomicrobia [56]. In *Methylobacterium*, *xoxF* is present in addition to *mxaF* [57] and is necessary for transcription of MDH and growth on methanol [58]. Recent evidence has shown that the enzyme is a bona fide methanol dehydrogenase but requires rare earth metals, such as La^{3+}, as a cofactor [59, 60].

In 1997, McDonald and Murrell [16] described the first PCR primers for detection of *mxaF* in environmental samples. The primers were reevaluated and the reverse primer modified to account for divergence detected in genome sequences [17]. These primers also detect some, but not all, *xoxF* genes. Most recently, highly degenerate versions of *mxaF* primers were developed and tested [18]. A competitive PCR assay has been used to quantify *mxaF* in environmental samples [61].

Care must be taken when interpreting the phylogeny of *mxaF* sequences retrieved from environmental samples because of evidence of extensive horizontal gene transfer, as demonstrated by Kist and Tate [62]. Despite this, the study showed that *mxaF* could be used as a reliable phylogenetic marker for some genera, such as *Methylobacterium*.

2.2 Tetrahydromethanopterin (H₄MPT)-Dependent Enzymes

Many methylotrophs oxidize formaldehyde via the (H4MPT)-linked pathway, including *Candidatus* "Methylomirabilis oxyfera" of the "NC10" phylum and most Proteobacteria methylotrophs including *Methylobacterium*, *Methyloversatilis*, and members of the Methylophilaceae, such as *Methylophaga* [52, 63]. These enzymes and coenzymes are also found in methanogens and sulfate-reducing archaea [64]. Among bacteria, this pathway is mostly unique to known methylotrophs, with the exception of being present in a few organisms in which methylotrophic growth has not been clearly demonstrated, such as *Burkholderia*, *Rubrivivax*, and some Planctomycetes [65]. It is possible that methylotrophy occurs in these organisms in natural environments under conditions that have not yet been replicated in the laboratory. For example, *Beggiatoa alba* which possesses the genes for the pathway was shown to use methanol only under microoxic conditions in a sulfide gradient system [66].

PCR assays have been designed for four genes of the (H_4MPT)-linked pathway, namely, *fae*, *mtdB*, *mch*, and *fhcD* [19, 20]. The *fae* gene of methylotrophs encodes formaldehyde activating enzyme, *mtdB* encodes methylene H_4MPT dehydrogenase, *mch* encodes methylene H_4MPT cyclohydrolase, and *fhcD* a subunit of formyltransferase/hydrolase complex (Fig. 1). The *fhcD* was identified as the best of the four genes for environmental surveys since it showed no obvious bias, amplified nearly the complete gene sequence providing substantial information for phylogeny, and could be amplified in a single PCR step rather than a two-step PCR necessary for the others [20, 65]. The authors designed primers targeting bacterial *fhcD* genes, which they tested using Lake Washington sediment DNA. The assay retrieved a wide diversity of bacterial *fhcD* that diverged as much as 43 % at the amino acid level. A phylogenetic analysis of FhcD protein sequences retrieved from Genbank demonstrates its ability to resolve genera
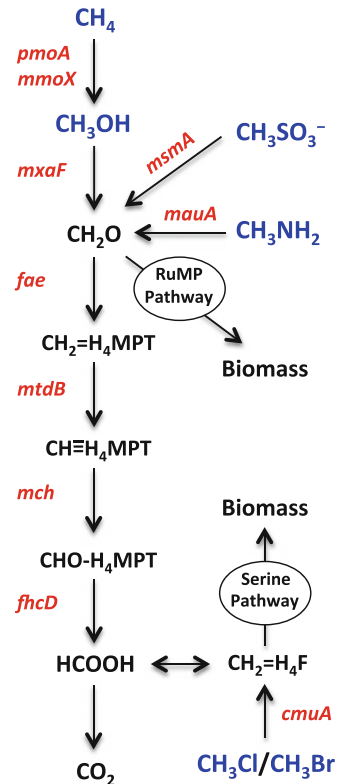
Fig. 1 Simplified composite depiction of C1 metabolism relevant to the discussion in this chapter. C1 substrates (*blue*) and functional genes (*red*) described in the text are *highlighted*. The functional genes are positioned based on the enzymatic transformation mediated by the encoded enzyme

of methylotrophs with no evidence of horizontal gene transfer (Fig. 2). Relatively few studies have targeted *fhcD* in environmental samples, suggesting it has been underappreciated as a functional marker gene for methylotrophy.

3   Methanotrophs

3.1   Methane Monooxygenases (pmoA, mmoX)

The *pmoA* gene encoding the β-subunit of the particulate methane monooxygenase enzyme (pMMO) is the most commonly used functional marker for identifying methanotrophs in environmental samples. There is a relatively large and well-curated database of *pmoA* [51] and methods available to classify *pmoA* sequences obtained by high-throughput sequencing [67]. In addition, there is a comprehensive microarray available for identifying *pmoA* phylotypes in environmental samples [68].

The pMMO is found in nearly all methanotrophs, including all characterized members of the Methylococcaceae, Methylocystaceae, "NC10" phylum methanotrophs (i.e., *Candidatus* "Methylomirabilis oxyfera"), and Verrucomicrobia methanotrophs (e.g., "Methylacidiphilum infernorum"). The only methanotrophs known to lack pMMO are some genera within the family Beijerinckiaceae, such as *Methylocella* [69] and *Methyloferula* [70]. These methanotrophs lacking pMMO possess the soluble methane monooxygenase (sMMO). The A189f/A682r primer set [21] is still the gold standard for retrieving the broadest diversity of *pmoA* sequences. These primers were designed to amplify both *pmoA* and the *amoA* gene of the bacterial ammonia monooxygenase enzyme, which belong to the same class of copper-containing monooxygenases [71]. Despite their broad coverage, they do not target the *pmoA* of "NC10" and Verrucomicrobia methanotrophs, which require specific assays as described later in this chapter. Environmental surveys using A189f/A682r primers have recovered a large diversity of *pmoA*-related genes from uncultivated microorganisms (Fig. 3). Several of these novel clades are believed to belong to as yet uncultivated methanotrophic organisms. For example, the USCα, USCγ, and JR3 clades (Fig. 3) have been proposed to belong to uncultivated atmospheric methane oxidizers [72, 73]. The enzymatic function associated with some *pmoA*-like genes is currently unknown, such as the *pxmA* genes (e.g., M84-P105, *Methylomonas*) [34].

In some samples, PCR products obtained with the A189f/A682r primer set can be dominated by *amoA* sequences or by spurious amplification products [23]. The A189f/mb661r primer combination was designed to offer greater specificity towards *pmoA* genes with less co-amplification of *amoA* and other *pmoA*-like sequences [22]. In addition, these primers generally have fewer problems with spurious amplification of nontarget sequences. A semi-nested PCR approach using A189f/A682r first followed by A189f/mb661r has been used to improve PCR success and further
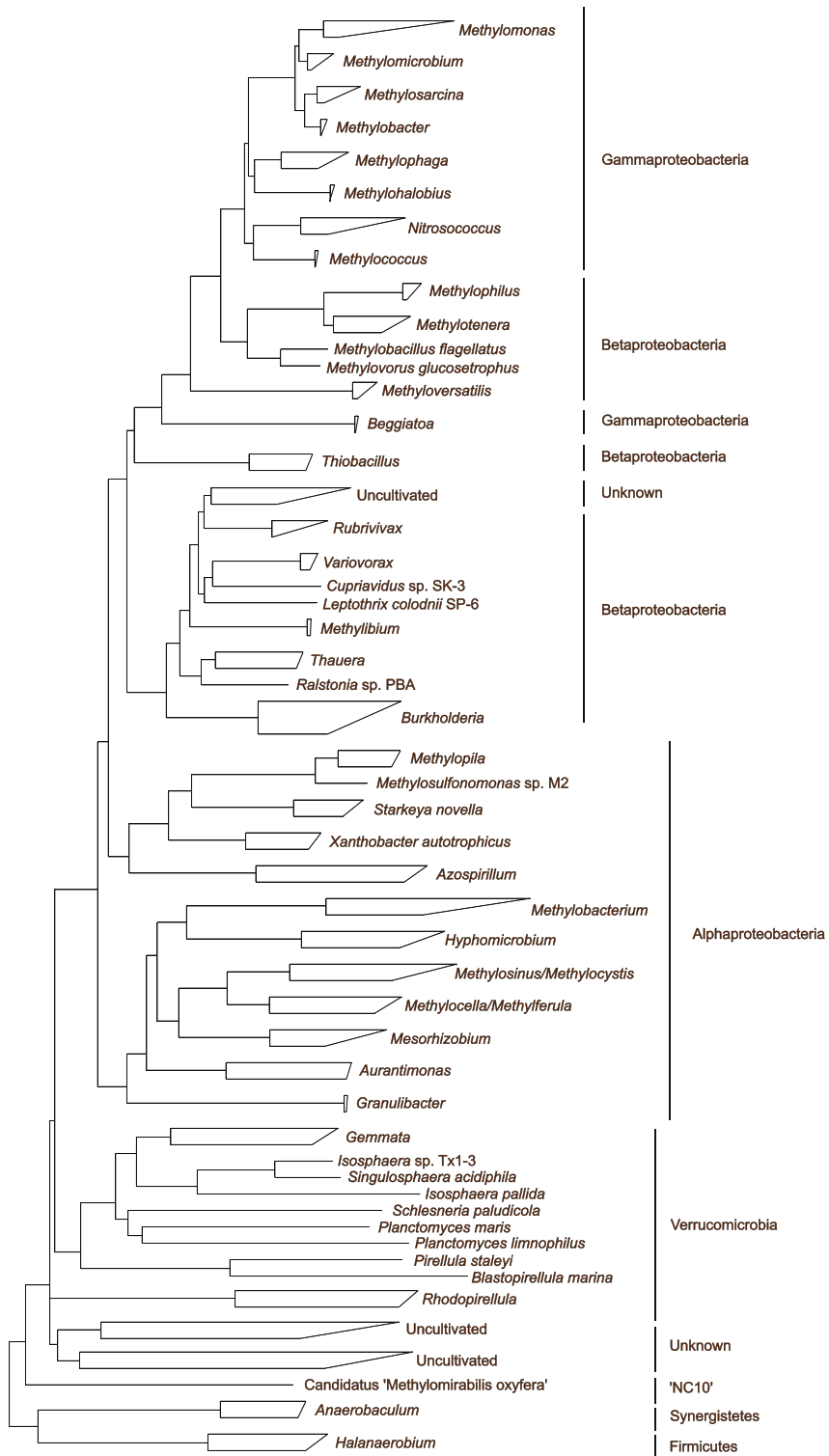
Fig. 2 Neighbor-joining phylogenetic tree of 270 representative FhcD protein sequences retrieved from the NCBI database (May 2014). Archaeal FhcD were used as an outgroup during tree construction (not shown)
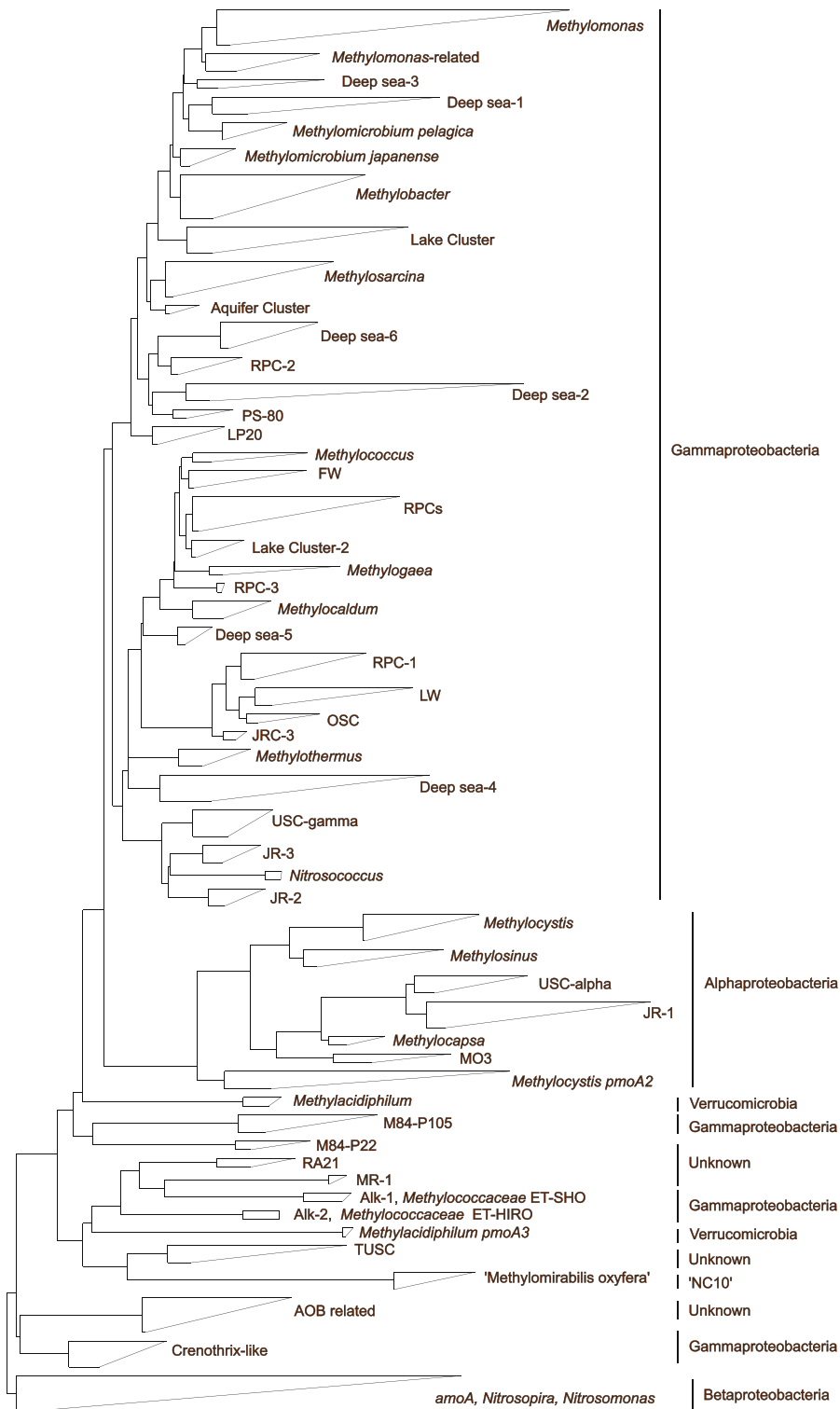
Fig. 3 Neighbor-joining phylogenetic tree of approximately 2,500 PmoA protein sequences. Clades corresponding to sequences of cultivated methanotrophs are named accordingly to the genera or species. The phylogenetic affiliation corresponding to uncultivated clades is tentative and will need to be confirmed by phylogenetic analysis of an isolated representative. Adapted from Lüke and Frenzel [51]

reduce the amplification of nonspecific targets [74]. Conditions for qPCR using A189f/mb661r primers have also been established [27], as well as additional primers and assays designed for the enumeration of *pmoA* from subsets of methanotroph genera (Table 1).

As mentioned above, the sMMO is an alternative methane monooxygenase enzyme present in some methanotrophs [75]. It is the sole enzyme catalyzing methane oxidation in the Beijerinckiaceae methanotrophs lacking pMMO. Some species within the families Methylococcaceae and Methylocystaceae possess both enzymes, which are differentially expressed [76]. The *mmoX* gene encodes the active site subunit of sMMO and several primer sets are available to target *mmoX* in environmental samples (Table 1). Quantitative assays have been designed to specifically enumerate *Methylocella mmoX* sequences [33, 43]. In addition, a competitive reverse transcription PCR assay was developed for the quantification of *mmoX* mRNA from diverse methanotrophs [77].

3.2 Uncultivated Atmospheric Methane Oxidizers

The *pmoA* clades associated with putative atmospheric methane oxidizers in upland soils, such as USCα, USCγ, and JR1 [73, 78, 79], can be detected with the A189f/A682r primer set; however, amplification products can be difficult to obtain with these primers or can sometimes be dominated by bacterial *amoA* or spurious products, as described above. The A189f/mb661r primer combination is generally poor at recovering these *pmoA* clades and therefore is not a good choice for analyzing soils with atmospheric methane uptake activity. Horz and colleagues [74] found that PCR using A189f with a mixture of mb661r/A650r primers retrieved *pmoA* associated with cultivated methanotrophs as well as the uncultivated JR1 clade. PCR primers have been designed to specifically target USCα, for example, A189f/forest675r that was also optimized for qPCR [27]. Another primer set is USCα-346f/A682r, which the authors reported to offer slightly better coverage and result in higher copy number estimations than the A189f/forest675r assay [29]. USCγ *pmoA* sequences can be targeted with the primers A189f/Gam634r, which was also optimized for qPCR [33].

3.3 "NC10" Phylum Methanotrophs

These methanotrophs are represented by *Candidatus* "Methylomirabilis oxyfera" [80]. They are remarkable in a number of respects, most notably that they generate their own oxygen presumably via the dismutation of nitric oxide. Although they oxidize methane with oxygen, they are active in anoxic environments. They are evolutionarily very distant from all other known methanotrophs, but oxidize methane with a pMMO enzyme. A PCR assay has been designed to amplify *pmoA* sequences from this clade [32]. It requires a nested approach, with a first round of PCR with modified versions of the A189f/A682r *pmoA* primers (A189b/cmo682), followed by PCR with internal primers (cmo182/cmo568) (Table 1).

3.4 Verrucomicrobia Methanotrophs

The Verrucomicrobia methanotrophs, represented by "Methylacidiphilum" species [81], are another unique group of methanotrophs that were discovered in hot (>50°C) and acidic (pH <2) geothermal environments [82, 83]. An environmental survey of geothermal springs and acidic wetlands indicated that they are only present in acidic geothermal spring environments [31]. "Methylacidiphilum" oxidize methane using a pMMO enzyme. Genome sequencing of "Methylacidiphilum" representatives indicate the presence of three copies of *pmoA*, two of which are nearly identical (*pmoA1*, *pmoA2*) and a third highly divergent gene (*pmoA3*) (Fig. 3). Different primer sets targeting *pmoA1* and *pmoA2* genes of Verrucomicrobia have been developed by independent research groups (Table 1) [28–31]. As noted by Sharp et al. [31], there are few representative "Methylacidiphilum" isolates making it difficult to design what could be universal Verrucomicrobia *pmoA* primers that are likely to detect the complete diversity in environmental samples.

4   Methylamine Utilizers

A variety of Gram-positive and negative bacteria can grow using methylamine. Many of these species, such as *Methylobacterium extorquens*, *Pseudomonas denitrificans*, *Methyloversatilis universalis*, and *Methylophaga flagellatus*, also grow using methanol [52]. Many of the Gram-negative methylamine utilizers convert methylamine to formaldehyde via methylamine dehydrogenase (MADH) encoded by *mauFBEDAGLMN* genes [84]. In contrast, *M. flagellatus* uses the indirect gamma-glutamylmethylamide / *N*-methylglutamate (GMA/NMG) pathway for methylamine utilization [85]; this pathway has also been shown to enable the use of methylamine as a nitrogen source in a non-methylotrophic organism [86]. Gram-positive organisms such as *Arthrobacter* P1 use a methylamine oxidase to convert methylamine to formaldehyde [87].

PCR assays targeting the *mauA* gene has been used as a functional marker to detect methylamine utilizers that operate via the MADH pathway [17, 44]. This has had some success. However, failure to obtain PCR products from methylamine enrichments [88] and cultures of methylamine utilizers [44] were also reported, indicating that the PCR conditions were not optimal or that alternative enzyme systems might be prevalent. Using marine *Roseobacter* clade genomes as a template, functional gene primers were designed to amplify glutamylmethylamide synthetase (*gmaS*) of the GMA/NMG pathway [46]. The same study also reported PCR primers targeting *tmm* encoding the trimethylamine monooxygenase of these organisms. Most recently, new *gmaS* primers were designed that are not limited to targeting sequences from marine bacteria [45] (Table 1).

5   Methylated Sulfur Utilizers

Organisms have been isolated that are able to grow using methylated sulfur compounds such as dimethylsulfoxide (DMSO), dimethylsulfide (DMS), dimethyldisulfide (DMDS), and methanesulfonate [89]. These organisms are widely distributed phylogenetically, for example, known DMS utilizers are found in the Alpha-, Beta-, and Gammaproteobacteria, Firmicutes, and Actinobacteria [89]. Two pathways for DMS utilization include a DMS monooxygenase [90] characteristic of *Hyphomicrobium* and methyltransferase found in *Thiobacillus* [91]. *Methylosulfonomonas*, *Marinosulfonomonas*, and strains of *Hyphomicrobium* and *Methylobacterium* can use methanesulfonate as a carbon and energy source for growth [92]. This is performed via a methanesulfonate monooxygenase. The initial demethylation reactions result in formaldehyde, which is metabolized via classic methylotrophic pathways [5].

Assays targeting methanesulfonate utilizers have been developed using the *msmA* gene, encoding the α-subunit of the hydroxylase of methanesulfonate monooxygenase [47]. The primers (ForA/B1rev2) amplify a ~785 bp fragment of the gene (Table 1). Moosvi and colleagues [48] found that the primers coamplified nonspecific targets from some environmental samples. Therefore, they designed internal primers (msmA_for/msmA_rev) to perform a nested PCR that amplifies a ~235 bp internal fragment. Studies have yet to fully explore the diversity of *msmA* and compare its evolution in pure cultures with the 16S rRNA. Hung et al. [44] reported that five proteobacterial isolates in their culture collection known to grow with methanesulfonate did not yield PCR products with *msmA* primers, indicating suboptimal PCR conditions or an alternate enzyme system in those organisms. To date, assays targeting genes encoding DMS uptake systems in methylotrophs have not been reported.

6   Halomethane Utilizers

Some specialized methylotrophs can grow using monohalogenated or dihalogenated methane. Microorganisms using dichloromethane first perform a dehalogenation using a glutathione-linked dehalogenase, which results in formaldehyde that is then further oxidized or assimilated using standard methylotrophic pathways [93]. Methylotrophs such as *Methylobacterium* strain CM4 and *Hyphomicrobium chloromethanicum* that grow on chloromethane or bromomethane use two enzymes, CmuA and CmuB, which transfer the methyl group first to a corrinoid protein and then to tetrahydrofolate (H_4F) [94, 95]. The methyl-H_4F can directly enter the serine pathway for carbon fixation. The *cmuA* gene has

been used as a functional marker gene target for chloromethane utilizers [49, 96]. A qPCR system was developed using the primers cmuA802f and MF2, as described by Nadalig et al. [49] and offers a means to identify and quantify these organisms in environmental samples.

7 Perspectives

Many of the PCR assays described in this chapter were designed at a time when amplicons were typically analyzed by cloning followed by Sanger sequencing. In most cases, researchers would now choose to analyze functional marker gene amplicons from an environmental sample using suitable next-generation sequencing (NGS) technology, such as MiSeq Illumina. The length of the amplicon becomes an important consideration for MiSeq since merging paired-end reads requires an overlap, which would limit the length to a maximum of ~550 bp with the current technology. Several pipelines are available for analysis of NGS data of protein-encoding genes, such as FunGene [97], FunFrame [98], and HMM-FRAME [99]. In addition, specialized classifiers can also be established, as shown for *pmoA* [67].

One of the limitations of PCR assays to investigate gene diversity is that it will be limited by the specificity of the primers. Therefore, primers should be regularly reassessed and revised to account for new sequence diversity uncovered in genome and metagenome sequences. In addition, there is still scope to develop new assays for methylotroph functional markers, particularly for methylotrophs that are not detected using existing assays. Several of these potential functional marker genes for methanol utilizers were highlighted in the recent literature [100]. Despite the current limitations, the analysis of methylotroph diversity by targeting functional marker genes remains an excellent means to characterize their diversity in environmental samples.

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Primers: Functional Genes and 16S rRNA Genes for Methanogens

Takashi Narihiro and Yuji Sekiguchi

Abstract

To date, a great number of oligonucleotide probes/primers targeting phylogenetic markers of methanogenic archaea (methanogens), such as 16S rRNA and the gene for the α-subunit of methyl-coenzyme M reductase (*mcrA*), have been developed and used for the identification and quantification of individuals and groups of methanogens in environmental samples. These probes/primers were designed for different taxonomic levels of methanogens and have been used for studies in environmental microbiology as hybridization probes or PCR primers of qualitative and quantitative molecular techniques, such as high-throughput sequencing, quantitative PCR, fluorescence in situ hybridization (FISH), and rRNA cleavage method. In this chapter, we present a comprehensive list of known oligonucleotide probes/primers, which enable us to decipher methanogen populations in an environment quantitatively and hierarchically, with examples of the practical applications of probes/primers.

Keywords: Methanogenic archaea, Methyl-coenzyme M reductase gene, Oligonucleotide probe, PCR primer, SSU rRNA

1   Introduction

Methanogenic archaea (methanogens) play a key role in the anaerobic biodegradation of complex carbon in many ecosystems on Earth [1–3]. Methanogens utilize a limited number of substrates. These are primarily hydrogen, formate, and acetate, but occasionally also secondary alcohols, and methyl-group-containing compounds for methanogenesis [1]. These microorganisms are frequently found in anoxic environments where external electron acceptors other than carbon dioxide are limited; examples of such environments are rice paddy fields [4–6], wetlands [7, 8], permafrost [9, 10], landfills [11], subsurfaces [12, 13], and ruminants [14], all of which are considered to be the major sources of atmospheric methane [15]. Recently, a growing number of studies have shown that methanogens are also active in many oxic environments such as desert crusts and grasslands [16]. The annual global source

of methane is estimated to be 542–852 Tg, and atmospheric methane concentration has risen threefold over the past 200 years, which is now considered one of the most prevalent greenhouse gasses [17]. With increased interests in the global climate change, studies on the diversity, physiology, and ecological functions of methanogens in these environments have been extensively performed using cultivation-dependent and cultivation-independent approaches [1, 18]. Methanogens are also considered key organisms underpinning anaerobic digestion biotechnology [19–22]. This technology is widely used for the treatment of municipal and industrial waste/wastewater, often generating methane from those wastes as reusable energy resource [23]. From engineering viewpoints, studies deciphering the methanogenic populations in anaerobic digestion processes have also been topics of focus. For populations in both natural and engineered ecosystems, methanogen-specific oligonucleotide probes/primers have long been used for clone library construction, fingerprinting (e.g., denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), and length heterogeneity-PCR (LH-PCR)), high-throughput sequencing, quantitative PCR (qPCR), hierarchical oligonucleotide primer extension (HOPE), membrane hybridization, fluorescence in situ hybridization (FISH), cleavage method with ribonuclease H (RNase H method), DNA microarray, and stable isotope probing (SIP) [24]. Although next-generation sequencing-based molecular methods without using methanogen-specific primers, such as high-throughput 16S rRNA gene amplicon sequencing with primers targeting regions broadly conserved in *Bacteria* and *Archaea* [25, 26] and shotgun-sequencing-based metagenomics [27], are being used as powerful methods for the same purpose, methanogen-specific oligonucleotides are also required when studying environments where methanogens are a minority in the general archaeal population. In this chapter, we list previously developed oligonucleotide probes/primers targeting genes of methanogens. Particular emphasis is placed on the probes/primers for 16S rRNA and the gene for the α-subunit of methyl-coenzyme M reductase (*mcrA*), which are the markers generally used for the taxonomic classification of methanogens [1, 28].

2   Phylogeny and Classification of Methanogens

All of the known methanogens have been classified into the archaeal phylum *Euryarchaeota* [29]. The known methanogens are assigned into five classes *Methanomicrobia*, *Methanobacteria*, *Methanococci*, *Methanopyri*, and *Thermoplasmata* (Fig. 1). The class *Methanomicrobia* comprises three orders (*Methanosarcinales*, *Methanomicrobiales*, and *Methanocellales*) and 23 genera belonging to nine families.

Fig. 1 16S rRNA gene-based phylogeny of known methanogens. The neighbor-joining tree was constructed on the basis of 16S rRNA gene sequences using the ARB package [30] with the dataset provided from Greengenes databases [31], showing representative species of methanogens that have been described to date

Methanocorpusculaceae

Methanocorpusculum sinense China Z, AF095268
Methanocorpusculum parvum DSM3823, M59147
Methanocorpusculum aggregans DSM3027, HG794418
Methanocorpusculum labreanum DSM 4855, AY260436
Methanocorpusculum bavaricum SZSXXZ, AY196676

F6SC393,
GMCP489,
GMCP490fm,
Msco183/583/
832/843

Rice cluster II

'*Ca.* Methanoflorentaceae'

'*Ca.* Methanoflorens stordalenmirensis RC2, SRA096214

RC281r2 ¦ UncM283/301/
¦ 385/869

Methanomassiliicoccales

Methanomassiliicoccaceae

'*Ca.* Methanogranum caenicola' Kjm51a, AB749767
'*Ca.* Methanomethylophilus alvus' Mx1201, KC412010
'*Ca.* Methanomassiliicoccus intestinalis Issoire–Mx1, CP005934
Methanomassiliicoccus luminyensis B10, HQ896499

RC-III131,
B10-dir, B10-VIC
B10-rev

Thermoplasmata

WSA2

mesophilic UASB sludge clone YkA78fl, AB266909
mesophilic anaerobic digester sludge clone, CU917458.
submarine sediment clone KZNMV–25–A37, FJ712390

ArcI-F, ArcI-Taq,
ArcI-R, Arc864

Methanobacteriaceae

Methanobrevibacter thaueri CW, U55236
Methanobrevibacter millerae ZA–10, AY196673
Methanobrevibacter gottschalkii HO, U55238
Methanobrevibacter smithii PS, U55235
Methanobrevibacter woesei GS, U55237
Methanobrevibacter wolinii SH, U55240
Methanobrevibacter acididurans ATM, AF242652
Methanobrevibacter boviskoreani JH1, KC608769
Methanobrevibacter olleyae KM1H5–1P, AY615201
Methanobrevibacter ruminantium M1, AY196666
Methanobrevibacter cuticularis RFM–1, U41095
Methanobrevibacter arboriphilus DH–1, AY196665
Methanobrevibacter filiformis RFM–3, U82322
Methanobrevibacter curvatus RFM–2, U62533
Methanobacterium movens TS2, EU366499
Methanobacterium alcaliphilum NBRC 105226, AB496639
Methanobacterium flexile GH, EU333914
Methanobacterium ivanovii OCM140, AF095261
Methanobacterium espanolae GP9, AF095260
Methanobacterium bryantii DSM 863, M59124
Methanobacterium arcticum M2, DQ517520
Methanobacterium veterum MK4, EF016285
Methanobacterium bryantii MOH, AY196657
Methanobacterium oryzae FPi, AF028690
Methanobacterium aarhusense H2–LR, AY386124
Methanobacterium movilense MC–20, JF812256

Msmithii probe, Rum16S 862R, Mbre364/731/178
fMbb1,
GMB406,
Msmithii reverse,
GMB376rm,
AbM4-F,
AbM4-R
Rum16S 740F,
Rum16S FAM,
NestMbb-F/R,
MBR1001
Mbre364/377/406
Acrh21f-Mvb
Mbre846

fMbium,
GMBA755,
S-F-Mbac-0398-S-a-20,
S-G-Mbac-0526-A-a-33,
S-G-Mbac-0578-A-a-22,
Mbt-210F,
Mbt-341Taq,
Mbt-359R,

Methanobacteriales

Methanobacteria

MBT929F,
MBT-NR,
MBT-N-probe

Methanobacteriaceae

MB310,
MB311,
Mbac I331,
MB1174,
MB1167fm,
MBT857F,
MBT1196R,
1401R,
MB1175m,

Fig. 1 (continued)

Methanococci

Methanopyri

Methanococcales

MCC495F,
MCC686F,
MCC832R,
1202R,
MC504,
MCMT1008fm

Methanopyrales

Methanothermaceae

Methanocaldococcaceae

S-F-Mcc-1109-b-A-20,
FMCMT1044,
Mcald444

Methanococcaceae

MC1109,
M(CO/BA)377,
Mcc r

Methanopyraceae

MEB859,
uniMet1-F,
uniMet1-R,
MBT-NF,
Mbac855,
Mbac313,
MMMM817,
MbM405

Mbc263
Mbc542
Mbc644
Mbc744

MbcF733/745

MMbac183

Stad-R, Stadt, 16SF,
Msph599/743/673

6 probes

3 probes

FMTH1183

Mt392f, Mt578r, 410F,
667R, GMTB541,
GMTB645f,
S-F-Mbac-0398-S-a-20,
S-G-Mthb-0549-S-a-32,
S-G-Mthb-0589-A-a-25,
Mthe167/190/426/643,
MtheT193/176

GMCC829f,
Mcald179,
Mcald276,
Mcald822

GMC728, GMC231,
GMC416, GMC735rm

GMTL416

FMCMT1044, S-G-Mp-0431-a-A-20, GMPK1331

Methanobacterium congolense C, AF233586
Methanobacterium paludis SWAN1, CP002772
Methanobacterium beijingense 8–2, NR_028202
Methanobacterium lacus 17A1, HQ110085
Methanobacterium subterraneum A8p, X99044
Methanobacterium palustre DSM3108, AF093061
Methanobacterium formicicum DSMZ1535, AF169245
Methanobacterium kanagiense 169, AB368917
Methanobacterium petrolearium Mic5c12, AB542742
Methanobacterium ferruginis Mic6c05, AB542743
Methanosphaera stadtmanae DSM 3091, CP000102
Methanosphaera cuniculi DSM4103, HE582783
Methanothermobacter defluvii ADZ, NR_028248
Methanothermobacter thermoflexus IDZ8, NR_028249
Methanothermobacter thermautotrophicus DeltaH, NC_000916
Methanothermobacter thermophilus M, X99048
Methanothermobacter thermaggregans OCM 141, AF095264
Methanothermobacter wolfeii DSM 2970, AB104858
Methanothermobacter marburgensis Marburg, X15364
Methanothermobacter crinale Tm2, HQ283273
Methanothermobacter tenebrarum RMAS, AB523785
Methanothermus fervidus DSM2088, M59145
Methanothermus sociabilis DSM3496, HE654004
Methanocaldococcus jannaschii DSM 2661, NC_000909
Methanocaldococcus fervens AG86, AF056938
Methanocaldococcus vulcanius M7, AF051404
Methanocaldococcus infernus ME, AF025822
Methanocaldococcus indicus SL43, AF547621
Methanotorris formicicus Mc–S–70, FJ766848
Methanotorris igneus DSM5666, M59125
Methanococcus maripaludis C6, CP000867
Methanococcus vannielii SB, AY196675
Methanococcus voltae PS, U38461
Methanothermococcus thermolithotrophicus DSM 2095, M59128
Methanococcus aeolicus Nankai–3, DQ195164
Methanothermococcus okinawensis IH1, AB057722

Methanopyrus kandleri AV19, AE009439

0.1

Fig. 1 (continued)

Within the order *Methanosarcinales*, the genera *Methanosarcina* and *Methanosaeta* are classified, members of which are known to contribute to the conversion of acetate to methane in various anaerobic environments. The rest of previously characterized genera in the *Methanosarcinales* other than *Methanosarcina* and *Methanosaeta* are known to metabolize relatively broad ranges of substrates, such as hydrogen, methanol, and methylamines [29]. Known members of the order *Methanomicrobiales* are all hydrogenotrophic methanogens, and some of them are often observed in anaerobic environments as important hydrogen scavengers [1]. The class *Methanobacteria* consists of the families *Methanobacteriaceae* and *Methanothermaceae*. They are recognized as important hydrogenotrophic methanogens in anaerobic ecosystems such as bioreactors [32, 33]. *Methanobacteriaceae* comprises four known genera, *Methanobacterium*, *Methanosphaera*, *Methanobrevibacter*, and *Methanothermobacter*, while *Methanothermaceae* has only one characterized genus *Methanothermus*. The class *Methanococci* includes the families *Methanococcaceae* and *Methanocaldococcaceae*, which are widely distributed in natural ecosystems, such as marine sediments and deep-sea geothermal sediments [1]. The class *Methanopyri* consists solely of the genus *Methanopyrus*, a hyperthermophilic, hydrogenotrophic methanogen isolated from the deep-sea hydrothermal field [34].

More recently, a methanogenic archaeon, *Methanomassiliicoccus luminyensis* strain B10, was isolated from human feces [35]. This strain is the first cultured methanogen of the class *Thermoplasmata*. A closely related strain has also been isolated from termite guts [36]. The other two *Thermoplasmata*-related methanogens *Ca. Methanomassiliicoccus intestinalis* and *Ca. Methanomethylophilus alvus* were also found in human feces [37, 38]. Iino et al. further reported that *Thermoplasmata*-related methanogen *Ca. Methanogranum caenicola* predominated in a methanogenic enrichment culture derived from the sludge of an anaerobic digestion process [39]. Together with previous findings on other *Thermoplasmata*-related methanogens, the family *Methanomassiliicoccaceae* and the order *Methanomassiliicoccales* were proposed for a methanogenic linage of the class *Thermoplasmata* [39]. *M. luminyensis* can produce methane by reducing methanol with hydrogen as electron donor [35]. The genomes of *M. luminyensis*, *Ca. M. intestinalis*, and *Ca. M. alvus* encode genes involved in the utilization of methanol, methylamines, and methylthiol [37, 40]. These observations indicate that *Methanomassiliicoccales*-related methanogens are methylotrophic.

Despite enormous effort in cultivating yet-to-be-cultured methanogens, there are still a vast number of uncultured archaeal taxa that may have similar metabolic functions as those of known methanogens. For example, members of WSA2 (or ArcI) group were frequently observed in methanogenic wastewater treatment

systems [41–43]. The WSA2 group is considered to be an archaeal taxon at the class level with no cultured representatives [44]. Chouari et al. have found that WSA2-related cells can be enriched using formate- or hydrogen-containing culture media, suggesting their capability of hydrogenotrophic methanogenesis [41]. Members of the Rice Cluster II (RC-II) group were also considered to be methanogens because 16S rRNA gene clones associated with this group were frequently observed in methanogenic enrichment cultures containing ethanol as an electron donor [45]. Based on the 16S rRNA phylogeny, RC-II group was found to be a lineage within the phylogenetic radiation of the orders *Methanosarcinales* and *Methanomicrobiales* (Fig. 1). In 2013, near-complete genomic sequence of a RC-II-related organism was reconstructed by metagenomic analysis of thawing permafrost ecosystem in northern Sweden, and the genome contained the gene sets necessary for hydrogenotrophic methanogenesis [27]. According to such genetic signatures, they proposed *Ca. Methanoflorens stordalenmirensis* of the family *Ca. Methanoflorentaceae* for the RC-II-related methanogen. Besides, uncultivated members of anaerobic methanotrophic archaea (ANME) are phylogenetically associated with the *Methanomicrobiales* and *Methanosarcinales* [46, 47]. According to biochemical and metagenomic data, ANME organisms take part in anaerobic oxidation of methane (AOM) by employing methanogenesis-related enzymes including mcr [48, 49].

Thus, there is no doubt that the actual biodiversity of methanogens will be much expanded in the future as the number of isolated and bioinformatically described methanogens continues to increase. In the present chapter, we mainly focus on the quantitative monitoring tools for cultured methanogens characterized to date.

3   Oligonucleotide Probes/Primers for 16S rRNA and Its Gene

16S rRNA and its gene are the most widely used biomarkers for the detection and quantification of methanogenic populations in environments. 16S rRNA gene-targeted probes/primers for methanogens are listed in Table 1, which is an updated version of a previous review [24]. The 16S rRNA gene-targeted primer pairs for a wide range of methanogen taxa, such as 146f/1324r [55], Met83F (or Met86F)/Met1340R [53], and 0357F/0691R [70], were developed to decipher entire methanogenic populations in ecosystems of interest. In addition, a large number of oligonucleotide probes/primers for specific and hierarchic detection of methanogens were designed to elucidate abundance of different methanogenic populations in anaerobic waste/wastewater treatment sludge [33, 39, 42, 83–86, 91, 96, 100, 112, 113], rumen [51, 76, 87, 106, 107], subseafloor sediments [92, 109], sediments [99], the human gut

Table 1
Oligonucleotide probes and primers targeting the 16S rRNA gene of methanogens

Target group	Probe name	Probe sequence (5′–3′)[a]	Application[b]	Probe length (mer)	Reference	Uncovered members[c]
Most methanogens	Arch21F (A2Fa)	TTCCGGTTGATCCYGCCGGA	PCR (forward)	20	[50]	
	Arch958R	YCCGGCGTTGAMTCCAATT	PCR (reverse)	19	[50]	
	Arch f2[d]	TTCYGGTTGATCCYGCCRGA	PCR (forward)	20	[51]	
	Arch r1386	GCGGTGTGTGCAAGGAGC	PCR (reverse)	18	[51]	
	A1f	TCYGKTTGATCCYGSCRGAG	PCR (forward), DGGE	20	[52]	
	A1100r	TGGGTCTCGCTCGTTG	PCR (forward), DGGE	16	[52]	
	Met83F	ACKGCTCAGTAACAC	PCR (forward)	15	[53]	
	Met86F	GCTCAGTAACACGTGG	PCR (forward)	16	[53]	
	Met448F	GGTGCCAGCCGCCGC	Sequencing	15	[53]	
	Met1027F	GTCAGGCAAGGAGCGAGACC	Sequencing	20	[53]	
	Met1340R	CGGTGTGTGCAAGGAG	PCR (reverse)	16	[53]	
	109f	ACKGCTCAGTAACACGT	PCR (forward)	17	[54]	
	146f	GGSATAACCYCGGGAAAC	PCR (forward)	18	[55]	
	1324r	GCGAGTTACAGCCCWCRA	PCR (reverse)	18	[55]	
	ARC344f	ACGGGGYGCAGCAGGCGCGA	PCR (forward), DGGE	20	[56, 57]	
	25f	CYGGTYGATYCTGCCRG	PCR (forward)	17	[58]	
	1391r	GACGGGCGGTGTGTRCA	PCR (reverse)	17	[59]	
	A24f	TCYGKTTGATCCYGSCRGA	PCR (forward), DGGE	19	[60]	
	A357f	CCCTACGGGGCGCAGCAG	PCR (forward), DGGE	18	[60]	
	A329r	TGTCTCAGGTTCCATCTCCG	PCR (reverse), DGGE	20	[60]	
	A348r	CCCCRTAGGGCCYGG	PCR (reverse), DGGE	15	[60]	
	A693r	GGATTACARGATTTC	PCR (reverse), DGGE	15	[60]	
	Met630F	GGATTAGATACCCSGGTAGT	qPCR (forward), DGGE	20	[61]	
	Met803R	GTTGARTCCAATTAAACCGCA	qPCR (reverse), DGGE	21	[61]	
	A1040f	GAGAGGWGGTGCATGGCC	PCR (forward), DGGE	18	[62]	

(continued)

Table 1
(continued)

Target group	Probe name	Probe sequence (5′–3′)[a]	Application[b]	Probe length (mer)	Reference	Uncovered members[c]
	ARC344	TCGGCGCCTGCTGCICCCGT	MH	20	[63]	
	ARC915	GTTGCTCCCCGCCAATTCCT	PCR (reverse), DGGE, MH, FISH	20	[57, 63]	
	MERI	GGGCACGGGTCTCGCT	PCR (reverse)	16	[64]	
	EURY514	GCGGCGGCTGGCACC	FISH	15	[65]	
	Arch349F	GYGCASCAGKCGMGAAW	qPCR (forward)	17	[66]	
	Arch806R	GGACTACVSGGGTATCTAAT	qPCR (reverse)	20	[66]	
	Arch516F	TGYCAGCCGCCGCGGT AAHACCVGC	qPCR (probe)	25	[66]	
	A571R	GCTACGGNYSCTTTARGC	PCR (reverse)	18	[67]	
	ARC787F	ATTAGATACCCSBGTAGTCC	PCR (forward)	20	[68]	
	ARC915F	AGGAATTGGCGGGGGAGCAC	PCR (probe)	20	[68]	
	ARC1059R	GCCATGCAACWCCTCT	PCR (reverse)	16	[68]	
	0915aR (Met915r)	GTGCTCCCCGCCAATTCCT	PCR (reverse)	20	[69]	
	0357F	CCCTACGGGGCGCAGCAG	PCR (forward), DGGE	18	[70]	
	0691R	GGATTACARGATTTCAC	PCR (reverse), DGGE	17	[70]	
	958arcF	AATTGGANTCAACGCGG	PCR (forward)	18	[71]	
	1048arcR-major	CGRCGGCCATGCACCWC	PCR (reverse)	17	[71]	
	1048arcR-minor	CGRCRGCCATGYACCWC	PCR (reverse)	17	[71]	
	Arc917	GUGCUCCCCGCCAAUUC	ANAEROCHIP	18	[72]	
	Ar109f	AHDGCTCAGTAACACRT	PCR (forward)	17	[47]	
	Ar912r	CCCCGCCAATTCCTTTAA	PCR (reverse)	19	[47]	
	340F	CCCTAYGGGGYGCASCAG	PCR (forward)	18	[73]	
	1000R	GAGARGWRGTGCATGGCC	PCR (reverse)	18	[73]	
	340Fmod	CCTAYGGGGYGCASCAG	PCR (forward)	17	[74]	
	Arch-340F	CCCTAHGGGGYGCASCA	PCR (forward)	18	[75]	
	Arch-915R	GWGCYCCCCGYCAATTC	PCR (reverse)	19	[75]	
	Nestmet-F[e]	AHGRWTCCAGGCCCTACGG	PCR (forward)	19	[76]	
	Nestmet-R	TGGCACCSGTCTTRCCC	PCR (reverse)	17	[76]	
	AR934F	AGGAATTGGCGGGGGAGCAC	qPCR (forward)	20	[77]	
	NOBI109f	ACTGCTCAGTAACACGT	qPCR (forward)	17	[78]	
	Forward	CCGGGTATCTAATCCGGTTC	qPCR (forward)	20	[79]	

Taxon	Primer/probe	Sequence	Application	Length	Ref.	Target organisms
Class *Methanomicrobia*	1068R	ATGCTTCACAGTACGAAC	PCR (reverse)	18	[80]	*Ca. Methanoflorens, Methanocella*
	CMSMM1068m	GGATGCTTCACAGTACGAAC	RNase H	20	[33]	*Ca. Methanoflorens, Methanocella*
	M(SA/MI)355	GTAAAGTTTTCGCG CCTG	MH	18	[81]	*Methanobalobium, Methanocella, Methanoregula, Methanocorpusculum,* and some *Methanohalophilus, Methanolobus, Methanococcoides, Methanosaeta, Methanofollis, Methanospirillum*
	EURY499	CGGTCTTGCCCGGCCCT	FISH	17	[65]	*Methanocorpusculum, Methanocalculus,* and some *Methanocella*
Order *Methanocellales* **Family** *Methanocellaceae* **Genus** *Methanocella*	Mcl282-F	ATCMGTACGGGTTGTGGG	qPCR (forward)	18	[16]	
	Mcl609-P	ATCCAGCGGCTTAACCGTTGGKCK	qPCR (probe)	24	[16]	
	Mcl832-R	CACCTAGCGRGCATCGTTTAC	qPCR (reverse)	21	[16]	
Species *M. paludicola*	SANAE1136	GTGTACTCGCCCTCCTCG	FISH	18	[82]	
Order *Methanomicrobiales*	MG1200	CGGATAATTCGGGGCATGCTG	MH, FISH	21	[63]	*Methanofollis, Methanoregula*
	MG1200m	CCGGATAATTCGGGGCATGCTG	RNase H	22	[33]	*Methanofollis, Methanoregula*
	MG1197fm	GTCAGCATGCCCGAATTAT	HOPE	20	[83]	*Methanofollis, Methanoregula*
	MMB282F	ATCGRTACGGGTTGTGGG	qPCR (forward)	18	[68]	*Methanolinea* and some *Methanocalculus*
	MMB749F	TYCGACAGTGAGGRACG AAAGCTG	qPCR (probe)	24	[68]	Some *Methanogenium, Methanofollis, Methanocorpusculum*
	MMB832R	CACCTAACGCRCATHGTTTAC	qPCR (reverse)	21	[68]	Some *Methanofollis, Methanospirillum, Methanocorpusculum*
	MG1200b	CCGGATAATTCGGGGCATGCTG	qPCR (reverse)	22	[77]	*Methanofollis, Methanoregula, Methanocorpusculum, Methanocalculus*
	EURY496	GTCTTGCCCGGCCCTTTC	FISH	18	[65]	
Family *Methanomicrobiaceae* **Genus** *Methanoculleus*	MMM309	TGTCTCAGATTCCATCTCCG	ANAEROCHIP	20	[72]	*Methanoculleus, Methanofollis,*
	Mcu-Taq	CGGTCTTGCCGGCCCTTTCT	qPCR (probe)	21	[42]	
	298F	GGAGCAAGAGCCCGGAGT	qPCR (forward)	18	[84]	
	586R	CCAAGAGACTTAACAACCCA	qPCR (reverse)	20	[84]	*M. chikugoensis*

Table 1
(continued)

Target group	Probe name	Probe sequence (5′–3′)[a]	Application[b]	Probe length (mer)	Reference	Uncovered members[c]
	F2SC668	TCCTACCCCGAAGTACCCCTC	RNase H	22	[33]	*M. chikugoensis*
	F2SC660fm	GGAGAGGTGAGGGGTACTTCG	HOPE	21	[83]	*M. chikugoensis*
	F2SC732	TCGAAGCCGTTCTGGTGAGGCG	RNase H	22	[33]	
	MCU1023TAQ	GAATGATTGCCGGGCTGAAGACTC	qPCR (probe)	24	[77]	
	Mcul574	AACCCAGCTACGAACGC	ANAEROCHIP	17	[72]	
	Mcul170	GCATCTGTAACCTATAGGGTATT	ANAEROCHIP	23	[72]	*M. boronobensis*, *M. hydrogenitrophicus*, *M. thermophilus*, *M. palmolei*, *M. receptaculi*
	Mcul302	TCCAACTCCGGGCTCTT	ANAEROCHIP	17	[72]	*M. receptaculi*
	Mcul393	TACAGGCACTCGAGGTTCC	ANAEROCHIP	19	[72]	*M. chikugoensis*
	Mcul669	CCCCGAAGTACCCCT	ANAEROCHIP	16	[72]	
	Mc-274F	GGAGCAAGAGCCCGGAGT	qPCR (forward)	18	[85]	
	Mc-361P	CGTGATAAGGGAACCTCGAGTGCCT	qPCR (probe)	25	[85]	*M. receptaculi*
	Mc-477R	CCAATAAAAGTGGCCACCACT	qPCR (reverse)	21	[85]	*M. bourgensis*
Species *M. thermophilus* and *M. hydrogenitrophicus*	Mc412f	CTGGGTGTCTAAAACACACCCAA	qPCR (forward)	23	[86]	
Species *M. thermophilus*	Mc578r	ATTGCCAGTATCTCTTAG	qPCR (reverse)	18	[86]	
	SMCUT1253	GCCTTTCGGCGTCGATACCC	RNase H	20	[33]	
Genus *Methanofollis*	F3SC984	CATATGCTGTCCTACCCGG	RNase H	20	[33]	*M. formosanus*
	F3SC979fm	CTCACCGGGTAGGACAGCGA	HOPE	20	[83]	
Genus *Methanogenium*	GMG1128	CGTTCCGAGAACAAGCTAG	RNase H	20	[33]	*M. frigidum*, *M. marinum*
	GMG266f	CCCAACAAGCCTGTAATCGA	HOPE	20	[83]	
	Mg229	CTAATCGACCGCAGATCC	ANAEROCHIP	18	[72]	
	Mg833	AGTGACTCGTGGTCACCAGT	ANAEROCHIP	20	[72]	*M. frigidum*, *M. marinum*
Species *M. cariaci*	Mg619	TAGACGCCCGTCGGTTA	ANAEROCHIP	17	[72]	
Genus	GMM829	CTCGTAGTTACAGGCACACC	FISH, RNase H	20	[87]	
Methanomicrobium	Mmic183	AGGAGACATTCCAGTACTCCT	ANAEROCHIP	21	[72]	
	Mmic615	ACGCCTCACAGTTAAGCC	ANAEROCHIP	18	[72]	
	Mmic829	AACTCGTAGTTACAGGCACAC	ANAEROCHIP	21	[72]	
	Mmic854	TCACGTTTTCACTTCGGC	ANAEROCHIP	18	[72]	

Taxon	Probe	Sequence	Method	Length	Ref.	Notes
Genus *Methanoplanus*						
Species *M. limicola* and *M. endosymbiosus*	SMPL623[f]	TTCTCTTAAAACGCCTGCAGG	RNase H	20	[33]	
Species *M. petrolearius*	SMPP1252[g]	CTTCTCAGTGTCGTTGCTCA	RNase H	20	[33]	
Genus *Methanolacinia*	SMPP577fm[g]	TGAGCAACGACACTGAGAAG	HOPE	20	[83]	
	SMPP1252[g]	CTTCTCAGTGTCGTTGCTCA	RNase H	20	[33]	*M. lacunae*
	SMPP577fm[g]	TGAGCAACGACACTGAGAAG	HOPE	20	[83]	*M. lacunae*
Family *Methanospirillaceae*						
Genus *Methanospirillum*	MSP1025TAQ	GAATGATAGTC GGGATGAAGACTCTA	qPCR (probe)	26	[88]	
	Mspi406	AGCCGTGCTGCTGG	ANAEROCHIP	16	[72]	
	Mspi422	TGGTGGACAGCCAGCC	ANAEROCHIP	16	[72]	
	F7SC1260	TATCCTCACCTCTGGTGTC	RNase H	20	[33]	
	F7SC1257f	TCGACACCGAGAGGTGAGG	HOPE	19	[83]	
Species *M. hungatei*	Mspi165	ACGAACTATGGAGTATTACCCC	ANAEROCHIP	22	[72]	
	MspiH174	AGTCAGCACGAACTATGGAG	ANAEROCHIP	20	[72]	
	MspiH193	CGGATCTTTCGTTCGCA	ANAEROCHIP	17	[72]	
	MspiH620	CCTGAACGCCCACCG	ANAEROCHIP	15	[72]	
Family *Methanoregulaceae*						
Genus *Methanoregula*						
Species *M. boonei*	6A8 644	TCTTCCGGTCCCTAGCCTGCCA	FISH	22	[89]	
Species *M. formicica*	SMSP129	TATCCCCTTCCATAGGGTAGATT	FISH	23	[90]	
Genus *Methanosphaerula*						
Genus *Methanolinea*	ML398fm	CCCGAGTGCCCGTAAATTC	HOPE	19	[91]	
Species *M. tarda*	NOBI633	GATTGCCAGTTTCTCCTG	qPCR (reverse), FISH	18	[78]	
Family *Methanocorpusculaceae*						
Genus *Methanocorpusculum*	F6SC393[h]	GACAGGCACTCAGGGTTTCC	RNase H	20	[33]	
	GMCP489	GCCCTGCCCTTTCTTCACAT	RNase H	20	[33]	
	GMCP490fm	TGTGAAGAAAGGGCAGGG	HOPE	18	[83]	
	Msco183	CCATTCCAGGACATATCCA	ANAEROCHIP	19	[72]	
	Msco583	CAAGAGACCTAACAGTCAAGC	ANAEROCHIP	21	[72]	
	Msco832	TAACACGTGGTTACCGACA	ANAEROCHIP	19	[72]	
	Msco843	TCGGCGCATCAGTAACAC	ANAEROCHIP	18	[72]	

Table 1
(continued)

Target group	Probe name	Probe sequence (5′–3′)[a]	Application[b]	Probe length (mer)	Reference	Uncovered members[c]
Family *Methanocalculaceae*						
Genus *Methanocalculus*	F6SC393[h]	GACAGGCACTCAGGGTTTCC	RNase H	20	[33]	
	GMCL488	CCCCGCCCTTTCTCCTGGTG	RNase H	20	[33]	
	Mcal465	CCCTTTCTCCTGGTGTGTGT	ANAEROCHIP	20	[72]	*M. natronophilus, M. chunghsingensis*
	Mcal585	CCCTTTCTCCTGGTGTGTGT	ANAEROCHIP	20	[72]	*M. halotolerans, M. natronophilus*
	Mcal600	GCCAAAAGATTTCCCCAG	ANAEROCHIP	18	[72]	*M. natronophilus*
	Mcal840	CACCTCAGGCGACTCGTGG	ANAEROCHIP	18	[72]	*M. natronophilus, M. chunghsingensis*
	Mcal422	ACACCAGAACAGCCTGCA	ANAEROCHIP	18	[72]	*M. pumilus*
	Mcal707	TCGGTCGTCCCTCGG	ANAEROCHIP	15	[72]	
Order *Methanosarcinales*	MSMX860	GGCTCGCTTCACGGCTTCCCT	MH	21	[63]	*Methanococcoides*
	MSMX859f	AGGGAAGCCGTGAAGCGAGC	HOPE	20	[83]	*Methanococcoides*
	MSSH859	TCGCTTCACGGCTTCCCT	FISH	18	[92]	
	MSMX860m	GCTCGCTTCACGGCTTCCCT	RNase H	20	[33]	*Methanococcoides*
	MSL812F	GTAAACGATRYTCGCTAGGT	qPCR (forward)	20	[68]	*Methanosalsum, Methanomethylovorans*
	MSL860F	AGGGAAGCCGTGAAGCGARCC	qPCR (probe)	21	[68]	Some *Methanosarcina, Methanosaeta*
	MSL1159R	GGTCCCCACAGWGTACC	qPCR (reverse)	17	[68]	*Methanosalsum, Methanobalobium, Methanosarcina, Methanimicrococcus,* and some *Methanolobus, Methanococcoides, Methanomethylovorans, Methanosarcina, Methanosaeta*
	EelMS240[i]	CTATCAGGTTGTAGTGGG	FISH	18	[92]	
	S-G-Msar-0450-S-a-19	TAGCAAGGGCCGGGCAAGA	qPCR (forward)	19	[93]	*Methermicoccus* and some *Methanohalophilus, Methanosaeta*
	886F-LCMS	GAAGTACGGCCGCAAGGC	PCR (forward)	18	[94]	*Methanomethylovorans,* some *Methanosaeta*

	Name	Sequence (5'–3')	Application	Length	Ref.	Target
Family *Methanosaetaceae*						
Genus *Methanosaeta*	MX1361	ACGTATTCACCGCGTTCTGT	FISH	20	[95]	
	S-G-Msae-0332-a-A-22[j]	TGCACCCXGTAGGGCCTGGATT	MH, FISH	22	[96]	
	S-F-Msaet-0540-A-a-31	AGACCCAATAAHARCGG TTACCACTCGRGCC	qPCR (probe)	31	[97]	
	S-F-Msaet-0573-A-a-17	GGCCGRCTACAGACCCT	qPCR (reverse)	17	[97]	
Species *M. concilii* and *M. thermophila*	MX825cm	GCTAGGTGTCRGYCACGGTGCGA	qPCR (forward)	23	[98]	
	Msae827	ACCGTGGCCGACACCT	ANAEROCHIP	16	[72]	
	MX825	TCGCACCGTGGCCGACACCTAGC	MH, FISH	23	[63]	
	MX825mix	TCGCACCGTGGCYGACACCTAGC	RNase H	23	[33]	
	Mst702F	TAATCCTYGARGGACCACCA	qPCR (forward)	20	[68]	
	Mst753F	ACGGC AAGGG ACGAA AGCTA GG	qPCR (probe)	22	[68]	
	Mst862R	CCTAC GGCAC CRACM AC	qPCR (reverse)	17	[68]	
	MX802f	GTCCTAGCCGTAAACGATA	HOPE	19	[83]	
Species *M. concilii*	MS1b	CCGGCCGGATAAGTCTCTTGA	qPCR (forward)	21	[77]	
	SAE761TAQ	ACCAGAACGGACCTGACGGCAAGG	qPCR (probe)	24	[77]	
	SAE835R	GACAACGGTGCACCGTGGCC	qPCR (reverse)	21	[77]	
	S-F-Msaet-0387-S-a-21[k]	GATAAGGGRAYCTCGAGTGCY	qPCR (forward)	21	[97]	
	Rotcl1	CTCCCGGCCTCGAGCCAGAC	FISH	20	[99]	
	MS1	CCGGATAAGTCTCTTGA	MH	17	[100]	
	MS2	CTGAATGAGAGGCGTTTCTTT	MH	21	[100]	
	MS5	GGCCACGGTGCGACCGTTGTCG	MH, FISH	22	[100]	
	MMX1273	GGTTTTAGGAGATTCCCGTC	RNase H	20	[33]	
	Msae406	AGCCAGATTTGTAACCTGG	ANAEROCHIP	19	[72]	
	Msae841	CACCGACAACGGTCGC	ANAEROCHIP	16	[72]	
	GTMS393m	ACCCAGCACTCGAGGTCCCC	RNase H	20	[33]	
Species *M. thermophila*	TMX745	CCCTTGCCGTCGGATCCGTT	RNase H	20	[33]	
Family *Methanosarcinaceae*	MS1414	CTCACCCATACCTCACTCGGG	MH, FISH	21	[63]	*Methanohalobium* and some *Methanohalophilus*, *Methanosarcina*
	Msc380F	GAAACCGYGATAAGGGGA	qPCR (forward)	18	[68]	*Methanimicrococcus* and some *Methanosarcina*, *Methanolobus*
	Msc492F	TTAGCAAGGGCCGGGCAA	qPCR (probe)	18	[68]	*Methanosalsum*, *Methanohalobium*, *Methanimicrococcus*, and some *Methanococcoides*, *Methanohalophilus*, *Methanolobus*, *Methanomethylovorans*, *Methanosarcina*

Table 1
(continued)

Target group	Probe name	Probe sequence (5′–3′)[a]	Application[b]	Probe length (mer)	Reference	Uncovered members[c]
	Msc828R R15F[l]	TAGCGARCATCGTTACG GCTACACGCGGGCTACAATGA	qPCR (reverse) qPCR (forward)	18 21	[68] [101]	*Methanomethylovorans*, *Methanococcoides*, *Methanobalophilus*, *Methanobalobium*, *Methanimicrococcus*, and some *Methanolobus*, *Methanosarcina*
	FMSC394	ATGCTGGCACTCGGTGTCCC	RNase H	20	[33]	*Methanolobus*, *Methanobalobium*, *Methanimicrococcus*, Some *Methanococcoides*, *Methanosarcina*
	MS821	CGCCATGCCTGACACCTAGCGAGC	MH, FISH	24	[63]	*Methanococcoides*, *Methanobalophilus*, *Methanolobus*, *Methanosalsum*, *Methanobalobium*, *Methanomethylovorans*, Some *Methanosarcina*
	MS821m[m]	GCCATGCCTGACACCTAGCG	RNase H	20	[33]	*Methanococcoides*, *Methanobalophilus*, *Methanolobus*, *Methanosalsum*, *Methanobalobium*, *Methanomethylovorans*, Some *Methanosarcina*
	MSr r859	TCGCTTCACGGCTTCCCTG	PCR (reverse)	19	[51]	*Methanococcoides*, *Methanosalsum*, *Methanobalobium*, *Methanimicrococcus*, and some *Methanomethylovorans*, *Methanosarcina*
	MIM594	CAGATTTCCCGGAAGACTG	ANAEROCHIP	19	[72]	*Methanococcoides*, *Methanosalsum*, *Methanobalobium*, *Methanimicrococcus*, *Methanosarcina*,

Genus					
Methanimicrococcus					
Genus *Methanosarcina*	GMIB1254	CACCTTTCGGTGTAGTTGCC	RNase H	20	[33]
	GMIB1225fm	CAACTACACCGAAAGGTGA	HOPE	19	[83]
	SARCI551	GACCCAATAATCACGATCAC	FISH	20	[102]
	SARCI645	TCCCGGTTCCAAGTCTGGC	FISH	19	[102]
	MB1	TTTGGTCAGTCCTCCGG	MH	17	[100]
	MB3	CCAGACTTGGAACCG	MH	15	[100]
	MB4	TTTATGCGTAAAATGGATT	MH, FISH	19	[100]
	240F	CCTATCAGGTAGTAGTGGGTGTAAT	qPCR (forward)	25	[84]
	589R	CCCGGAGGACTGACCAAA	qPCR (reverse)	18	[84]
	MB1b	CGGTTTGGTCAGTCCTCCGG	qPCR (forward)	20	[77]
	SAR761TAQ	ACCAGAACGGGTTCGACGGTGAGG	qPCR (probe)	24	[77]
	SAR835R	AGACACGGGTCGCGCCATGCCT	qPCR (reverse)	21	[77]
	S-P-Msar-0540-A-a-31	AGACCCAATAATCACGATCACCACTCGGGCC	qPCR (probe)	31	[93]
	S-G-Msar-0589-S-a-20[n]	TTCCCGGAGGACTGACCAAA	qPCR (reverse)	20	[93]
	MS537fm	GGCGGCCGAGTGGTGATCGT	HOPE	21	[83]
	Msar184	GACGCATAAAGCATTCCAG	ANAEROCHIP	19	[72]
	Msar197	CTTGGGCAGACGAATCC	ANAEROCHIP	17	[72]
	Msar549	CCCAATAATCACGATCACC	ANAEROCHIP	19	[72]
	Msar587	CCGGAGGACTGACCAAAC	ANAEROCHIP	18	[72]
	Msar416	CTGGACAGCCAGCATATGA	ANAEROCHIP	19	[72]
	Msar601	AGCTATCAGATTTCCCGG	ANAEROCHIP	18	[72]
Species					
M. thermophila	Ms413f	CAGATGTGTAAAATACATCTGTT	qPCR (forward)	23	[86]
	Ms578r	TCTGGCAGTATCCACCGA	qPCR (reverse)	18	[86]
Genus *Methanococcoides*	GMCO441	ACATGCCGTTTACACATGTG	RNase H	20	[33]
	GMCO437f	GTCCACATGTGTAAACG	HOPE	17	[83]
	Mcoc139	CGGAGTTATGCGGAACCT	ANAEROCHIP	18	[72]
	Mcoc194	CTTAGGCAACCGAAGTCTTT	ANAEROCHIP	20	[72]

Target species (right column):

- GMIB1254 — *M. semesiae, M. baltica*
- GMIB1225fm — *M. lacustris, M. baltica*
- SARCI551 — *M. baltica*
- SARCI645 — *M. lacustris, M. baltica*
- MB1 — *M. acctivorans, M. mazei, M. soligelidi, M. siciliae, M. lacustris, M. semesiae, M. baltica*
- MB4 — *M. acctivorans, M. semesiae, M. baltica*
- 240F — *M. baltica*
- 589R — *M. baltica*
- MB1b — *M. semesiae, M. baltica*
- SAR761TAQ — *M. semesiae, M. baltica*
- SAR835R — *M. semesiae, M. baltica*
- MS537fm — *M. barkeri, M. vacuolata, M. spelaei, M. boronobensis, M. acctivorans, M. thermophila, M. lacustris, M. baltica*
- Msar184 — *M. barkeri, M. semesiae, M. baltica*
- Msar197 — *M. semesiae, M. baltica*
- Msar549 — *M. baltica*
- Msar587 — *M. spelaei, M. boronobensis, M. acctivorans, M. siciliae, M. thermophila, M. mazei, M. soligelidi, M. lacustris, M. semesiae, M. baltica*
- Msar601 — *M. vacuolata, M. acctivorans, M. thermophila, M. lacustris, M. semesiae, M. baltica*
- Mcoc139 — *M. burtonii, M. vulcani*
- Mcoc194 — *M. alaskense*

(continued)

Table 1
(continued)

Target group	Probe name	Probe sequence (5′–3′)ᵃ	Applicationᵇ	Probe length (mer)	Reference	Uncovered membersᶜ
	Mcoc438	TGCCGTTTACACATGTGGA	ANAEROCHIP	19	[72]	
	Mcoc465	CTTGCTAACACATGCCGTT	ANAEROCHIP	19	[72]	M. alaskense, M. burtonii
	Mcoc584	GAAGAACTGATCAAAACGAG	ANAEROCHIP	19	[72]	
	MCOCID442	ACACATGCCGTTTACACATG	FISH	20	[103]	
Genus Methanolobium	GMHB842	TCGGCACTAGGAACGGCCGT	RNase H	20	[33]	
	Mhal183	ACCACAGAGCCTTCCAGC	ANAEROCHIP	18	[72]	
	Mhal236	CACTACCACCTGATAGACCG	ANAEROCHIP	20	[72]	
	Mhal598	CACCAGATTTCCCAATGG	ANAEROCHIP	18	[72]	
	Mhal829	GCCGTACCATCCCTAACAC	ANAEROCHIP	19	[72]	
Genus Methanolophilus	GMHP1258	CCGTCACTTTTCAGTGTAGG	RNase H	20	[33]	M. levihalophilus
Genus Methanolobus	GMLB834	TGAAACGGTCGCACCGTCCCAG	RNase H	22	[33]	M. taylorii, M. oregonensis, M. zinderi
	GMLB834rm	TGAAACGGTCGCACCGTCCCA	HOPE	21	[83]	M. taylorii, M. oregonensis, M. zinderi
	Mlob828	CGCACCRTCCCAGACACC	FISH	18	[104]	M. tindarius, M. vulcani, M. oregonensis, M. zinderi
	Mlob133	TATGCCTGACCTTAGGGC	ANAEROCHIP	18	[72]	
	Mlob183	CACAGAACATTCCAGTATCTATGA	ANAEROCHIP	24	[72]	M. tindarius, M. profundi, M. taylorii, M. oregonensis, M. zinderi
	Mlob389	GGCACTCAGTATCCCTTA	ANAEROCHIP	19	[72]	M. taylorii, M. oregonensis, M. zinderi
	Mlob821	GTCCCAGACACCTAGCGAG	ANAEROCHIP	19	[72]	M. taylorii, M. oregonensis, M. zinderi
Genus Methanomethylovorans	MML419r	ATGGACAGCCAACATAGG	HOPE	18	[83]	M. thermophila
Genus Methanosalsum	GMSS261	GTCGGCTAGCAGGTACCTTG	RNase H	20	[33]	
Family Methermicoccaceae Genus Methermicoccus						
WSA2	ArcI-F	GCTCATGCATTGCATGG	qPCR (forward)	17	[42]	
	ArcI-Taq	GTAATACCGGCAGCTCGAGTGG	qPCR (probe)	22	[42]	
	ArcI-R	TATCCGGCTACGAACGTT	qPCR (reverse)	18	[42]	
	Arc864	CCCTACAGCACAGGGCCA	FISH	18	[41]	

	Sequence	Application	Length	Reference	Target
Class *Methanobacteria*					
Order *Methanobacteriales*					
MBT929F	AGCACCACAACGCGTGGA	qPCR (probe)	18	[68]	Some *Methanobrevibacter*, *Methanobacterium*
MBT-NR°	CGGCGTTGAATCCAATTAAAC	qPCR (reverse)	21	[105]	Some *Methanobrevibacter*, *Methanobacterium*
MBT-N-probe	AGCACCACAACGCGTGGAGCC	qPCR (probe)	21	[105]	Some *Methanobrevibacter*, *Methanobacterium*, *Methanothermobacter*
Family *Methanobacteriaceae*					
MB310	CTTGTCTCAGGTTCCATCTCCG	MH	22	[63]	Some *Methanobrevibacter*, *Methanobacterium*
MB311	ACCTTGTCTCAGGTTCCATCTCC	FISH	23	[95]	Some *Methanobacterium*, *Methanothermobacter*
Mbac f331	CTTGTCTCAGGTTCCATCTC	PCR	20	[51]	Some *Methanobacterium*
MB1174	TACCGTCGTCCACTCCTTCCTC	MH, FISH	22	[63]	Some *Methanothermobacter*
MB1167fm	TAAAYWGGAGGAAGGAGTGGA	HOPE	21	[83]	Some *Methanothermobacter*
MBT857F	CGWAGGGAAGCTGTTAAGT	qPCR (forward)	19	[68]	Some *Methanothermobacter*
MBT1196R	TACCGTCGTCCACTCCTT	qPCR (reverse)	18	[68]	Some *Methanothermobacter*
1401R	KTTTGGGTGGYGTGACGGGC	PCR (reverse)	20	[80]	*Methanosphaera* and some *Methanobacterium*, *Methanobrevibacter*
MB1175n	CCGTCGTCCACTCCTTCCTC	RNase H	20	[33]	Some *Methanothermobacter*
MEB859	AGGGAAGCTGTTAAGTCC	FISH	18	[92]	Some *Methanothermobacter*
uniMet1-F	CCGGAGATGGAACCTGAGAC	qPCR (forward)	20	[106]	Some *Methanobrevibacter*
uniMet1-R	CGGTCTTGCCCAGCTCTTATTC	qPCR (reverse)	22	[106]	*Methanothermobacter* and some *Methanobrevibacter*, *Methanobacterium*
MBT-NF	TCGCAAGACTGAAACTTAAAGGAA	qPCR (forward)	24	[105]	*Methanothermobacter* and some *Methanobrevibacter*, *Methanobacterium*
Mbac855	CTTAACAGCTTCCCTTCGG	ANAEROCHIP	19	[72]	Some *Methanothermobacter*
Mbac313	ACCTTGTCTCAGGTTCCATC	ANAEROCHIP	20	[72]	Some *Methanobacterium*
MMMM817	CAACACCAAGTCCACATCG	ANAEROCHIP	19	[72]	Some *Methanobrevibacter*, *Methanobacterium*, *Methanothermobacter*
MbM405	ACCCCGTTAAGAGTGGC	ANAEROCHIP	17	[72]	*Methanobrevibacter*, *Methanosphaera*, some *Methanobacterium*, *Methanothermobacter*

Table 1
(continued)

Target group	Probe name	Probe sequence (5′–3′)[a]	Application[b]	Probe length (mer)	Reference	Uncovered members[c]
Genus *Methanobrevibacter*	fMbb1	CTCCGCAATGTGAGAAATCG	PCR	20	[51]	*M. boviskoreani, M. acididurans, M. curvatus*
	GMB406	GCCATCCCGTTAAGAATGGC	RNase H	20	[33]	*M. gottschalkii, M. wolinii, M. curvatus*
	Msmithii_reverse	CTCCCAGGGTAGAGGTGAAA	qPCR (reverse)	20	[79]	*M. wolinii, M. boviskoreani, M. acididurans, M. cuticularis, M. arboriphilus, M. filiformis, M. curvatus*
	GMB376rm	TGAGAAATCGCGACGGGGGG	HOPE	20	[83]	*M. boviskoreani, M. acididurans, M. curvatus*
	AbM4-F	TTTAATAAGTCTCTGGTGAAATC	qPCR (forward)	23	[106]	*M. woesei, M. wolinii, M. acididurans, M. olleyae, M. ruminantium, M. arboriphilus, M. filiformis, M. curvatus*
	Rum16S 740F	TCCCAGGGTAGAGGTGAAA	qPCR (forward)	19	[107]	*M. wolinii, M. boviskoreani, M. acididurans, M. cuticularis, M. arboriphilus, M. filiformis, M. curvatus*
	Rum16S FAM	CCGTCAGGTTCGTTCCAGTTAG	qPCR (probe)	22	[107]	*M. thaueri, M. millerae, M. gottschalkii, M. smithii, M. woesei, M. wolinii, M. boviskoreani*
	NestMbb-F	TGGGAATTGCTGGWGATACTRTT	qPCR (forward)	23	[76]	*M. woesei, M. acididurans, M. cuticularis, M. arboriphilus, M. filiformis*
	NestMbb-R	GGAGCRGCTCAAAGCCA	qPCR (reverse)	17	[76]	*M. gottschalkii, M. smithii, M. wolinii, M. wolinii, M. boviskoreani, M. acididurans, M. arboriphilus, M. filiformis, M. curvatus*
	Mbre364	TTCTCACATTGCGGAGGT	ANAEROCHIP	18	[72]	*M. boviskoreani, M. acididurans, M. curvatus*
	Mbre377	CCCGTCGCGATTTCTC	ANAEROCHIP	16	[72]	*M. boviskoreani, M. acididurans, M. curvatus*
	Mbre406	CCATCCCGTTAAGAATGG	ANAEROCHIP	18	[72]	*M. gottschalkii, M. wolinii, M. curvatus*
	Acrh21f-Mvb	TTCTGTTTGATCCTGGCAGA	PCR (forward)	20	[103]	*M. gottschalkii, M. wolinii, M. curvatus*

	Name	Sequence	Method	Length	Ref.	Target species
Species *M. boviskoreani*	AbM4-R	AGATTCGTTCTAGTTAGACGC	qPCR (reverse)	21	[106]	
Species *M. curvatus*	Mbre846	CCCTTCGGCACTGAAAC	ANAEROCHIP	17	[72]	
Species *M. ruminantium* and *M. olleyae*	MBR1001	TCAGCCTGGTAATCATACA	FISH	19	[87]	
Species *M. smithii*	Msmithii_probe	CCGTCAGAATCGTTCCAGTCAG	qPCR (probe)	22	[79]	
	MbreS840	ACTGGGACAGCTCAAAGC	ANAEROCHIP	18	[72]	
	MbreS731	AGAATCGTTCCAGTCAGACG	ANAEROCHIP	20	[72]	
	MbreS178	CATTACAGGAATAATTGCCTAT	ANAEROCHIP	22	[72]	
	Rum16S 862R	CGTCAGAATCGTTCCAGTCA	qPCR (reverse)	20	[107]	
Genus *Methanobacterium*	fMbium	CGTTCGTAGCCGGGCYTGA	PCR	18	[51]	*M. movens, M. alcaliphilum, M. flexile, M. lacus, M. palustre*
	GMBA755	TGGCTTTCGTTACTCACC	RNase H	18	[33]	*M. movens, M. alcaliphilum, M. flexile, M. aarhusense* Also cover some *Methanothermobacter*
	S-F-Mbac-0398-S-a-20[p]	CCCAAGTGCCACTCTTAACG	qPCR (forward)	20	[93]	*M. arcticum*
	S-G-Mbac-0526-A-a-33	AAYGGCCACCACTTGA GCTGCCGGTGTTACCGC	qPCR (probe)	33	[93]	*M. movens, M. alcaliphilum, M. flexile, M. espanolae, M. movilense, M. beijingense*
	S-G-Mbac-0578-A-a-22	AGACTTATCAARCCGGCTACGA	qPCR (reverse)	22	[93]	*M. oryzae, M. aarhusense, M. movilense, M. paludis, M. beijingense*
	Mbt-210F	CCAAGCCWKTRATCTGTACG	qPCR (forward)	20	[42]	*M. movens, M. alcaliphilum, M. flexile, M. oryzae, M. aarhusense, M. movilense, M. congolense, M. paludis, M. petrolearium, M. ferruginis*
	Mbt-341Taq[q]	CGCGAAACCTCCGCAATGC	qPCR (probe)	19	[42]	*M. beijingense*
	Mbt-359R[r]	CGTTAAGAGTGGCACTTGGG	qPCR (reverse)	20	[42]	*M. arcticum*
	Mbc263	AUCUCCAGCAAUUCCACAC	ANAEROCHIP	19	[72]	*M. movens, M. alcaliphilum, M. flexile, M. ivanovii, M. espanolae, M. bryantii, M. arcticum, M. veterum, M. oryzae, M. aarhusense, M. movilense, M. congolense, M. paludis, M. beijingense, M. lacus, M. petrolearium, M. ferruginis*

Table 1
(continued)

Target group	Probe name	Probe sequence (5'–3')[a]	Application[b]	Probe length (mer)	Reference	Uncovered members[c]
	Mbc542	AAAATGGCCACCACTTGAG	ANAEROCHIP	19	[72]	*M. movens, M. alcaliphilum, M. flexile, M. oryzae, M. aarhusense, M. movilense, M. beijingense, M. petrolearium, M. ferruginis*
	Mbc644	CCGGCCTCAAGCCTAAT	ANAEROCHIP	17	[72]	*M. oryzae, M. aarhusense, M. movilense, M. congolense, M. paludis, M. beijingense, M. lacus, M. kanagiense, M. petrolearium, M. ferruginis*
	Mbc744	TACTCACCGTCAGGTCCG	ANAEROCHIP	18	[72]	*M. oryzae, M. aarhusense, M. movilense, M. congolense, M. paludis, M. beijingense, M. lacus, M. formicicum, M. kanagiense*
Species *M. ferruginis*	MMbac183	TAGGGAACCAATTCCAGG	ANAEROCHIP	18	[72]	
Species *M. formicicum*	MbcF733	GGCCCGTTCCAGTTAGC	ANAEROCHIP	17	[72]	
	MbcF745	TTACTCACCGTCAGGCCC	ANAEROCHIP	18	[72]	
Genus *Methanosphaera*	Stad-R	TTCGTTACTCACCGTCAAGATC	qPCR (reverse)	22	[106]	
	Stadt_16SF	AGGAGCGACAGCAGAATGAT	qPCR (forward)	20	[108]	
	Msph598	GCTACAAGCTTTCACCAAAG	ANAEROCHIP	20	[72]	
	Msph743	TTACTCACCGTCAAGATCGT	ANAEROCHIP	20	[72]	
	Msph673	CCCCTACCCCGGTAGTAC	ANAEROCHIP	18	[72]	
Species	GMSP838	CCGGAACAACTCGAGGCCAT	RNase H	20	[33]	
M. stadtmanae	GMSP1129fm	AACACAATCTCTGGATTGG	HOPE	19	[83]	
	Stadt_16SR	CAGGACGCTTCACAGTACGA	qPCR (reverse)	20	[108]	
	MsphS193	CTTAGGCGAAAATACATTTC	ANAEROCHIP	21	[72]	
	MsphS838	GGAACAACTCGAGGCCA	ANAEROCHIP	17	[72]	
	MsphS174	CAGGCATAACCTAATATCCAG	ANAEROCHIP	21	[72]	
Species *M. cuniculi*	Stad-F	CTTAACTATAAGAATTGCTGGAG	qPCR (forward)	23	[106]	
	Msph125	CAGTCCTAAGGTAAGTTATCC	ANAEROCHIP	22	[72]	
	Msph841	GGCACTGGAACAACTCGA	ANAEROCHIP	18	[72]	

		Sequence	Method		Ref	Target species
Genus *Methanothermobacter*	Mt392f[t]	ACTCTTAACGGGGTGGCTTTT	qPCR (forward)	21	[86]	*M. thermophilus*
	Mt578r	TCATGATAGTATCTCCAGC	qPCR (reverse)	19	[86]	*M. crinale, M. tenebrarum*
	410F[t]	CTCTTAACGGGGTGGCTTTT	qPCR (forward)	20	[84]	*M. thermophilus*
	667R	CCCTGGGAGTACCTCCAGC	qPCR (reverse)	19	[84]	*M. crinale, M. tenebrarum*
	GMTB541	AAAAGCGGCTACCACTTGAGCT	RNase H	22	[33]	*M. crinale, M. tenebrarum*
	GMTB645f[t]	TCAGACTCGAGGTCGG	HOPE	17	[83]	*M. crinale, M. tenebrarum*
	S-F-Mbac-0398-S-a-20[p]	CCCAAGTGCCACTCTTAACG	qPCR (forward)	20	[93]	*M. crinale, M. tenebrarum*
	S-G-Mthb-0549-S-a-32	CGGACGCTTTAGGCCCAA TAAAAGCGGCTACC	qPCR (probe)	32	[93]	*M. crinale, M. tenebrarum*
	S-G-Mthb-0589-A-a-25	GGGATTTCACCAGAGACTTATCAG	qPCR (reverse)	25	[93]	*M. crinale, M. tenebrarum*
	Mthe167	TCACCTATCCGGGTTTATC	ANAEROCHIP	19	[72]	*M. defluvii, M. marburgensis, M. crinale, M. tenebrarum*
	Mthe190	GTGTTTCGGTGAAGAACCA	ANAEROCHIP	19	[72]	*M. wolfeii, M. marburgensis, M. crinale, M. tenebrarum*
	Mthe426	CACTTCTGAAAAGCCACCC	ANAEROCHIP	19	[72]	*M. crinale, M. tenebrarum*
	Mthe643[v]	CCGACCTCGAGTCAGTATA	ANAEROCHIP	19	[72]	*M. crinale, M. tenebrarum*
	MtheT193	GAAGGTGTTTCGGTGAAGA	ANAEROCHIP	19	[72]	*M. defluvii, M. thermoflexus, M. wolfeii, M. marburgensis, M. crinale, M. tenebrarum*
	MtheT176	AGGCAGCATCACCTATCC	ANAEROCHIP	18	[72]	*M. thermaggregans, M. wolfeii, M. marburgensis, M. crinale, M. tenebrarum*
Family *Methanothermaceae* Genus *Methanothermus*	FMTH1183	TACGGACCTACCGTGCCCGCA	RNase H	22	[33]	
Class *Methanococci* Order *Methanococcales*	MCC495F	TAAGGGCTGGGCAAGT	qPCR (forward)	16	[68]	*Methanotorris* and some *Methanocaldococcus*
	MCC686F	TAGCGGTGRAATGYGTTGATCC	qPCR (probe)	22	[68]	
	MCC832R	CACCTAGTYCGCARAGTTTA	qPCR (reverse)	20	[68]	
	1202R	CCAGGRGATTCGGGGCATGC	PCR (reverse)	20	[80]	Some *Methanocaldococcus, Methanococcus*
	MC504	GGCTGCTGGCACCGGACTTGCCCA	FISH	24	[95]	
	MCMT1008fm	AGGTTGACGACCTTGCCWGAMGC	HOPE	23	[83]	Some *Methanocaldococcus, Methanotorris, Methanococcus, Methanothermococcus*

Table 1
(continued)

Target group	Probe name	Probe sequence (5'–3')[a]	Application[b]	Probe length (mer)	Reference	Uncovered members[c]
Family *Methanocaldococcaceae*	S-F-Mcc-1109-b-A-20	GCAACATGGGGCRCGGGTCT	MH	20	[109]	Some *Methanotorris*
	FMCMT1044[w]	GTCAACCTGGCCTTCATCCTGC	RNase H	22	[33]	
	Mcald444	CCCGGAGCTGTTTACACT	ANAEROCHIP	18	[72]	Some *Methanocaldococcus*
Genus *Methanocaldococcus*	GMCC829f	GTGTCGCGTGGGCTTCGGG	HOPE	19	[83]	*M. infernus, M. indicus, M. villosus*
	Mcald179	TCCAGACCTCCTCCCCTA	ANAEROCHIP	18	[72]	
	Mcald276	GCCCGTACGGATCGTAG	ANAEROCHIP	17	[72]	*M. vulcanius*
	Mcald822	CGACGCGACACCTAGTCC	ANAEROCHIP	18	[72]	*M. infernus, M. indicus, M. villosus*
Genus *Methanotorris*						
Family *Methanococcaceae*	MC1109	GCAACATAGGGCACGGGTCT	MH	20	[63]	Some *Methanococcus,* *Methanothermococcus*
	M(CO/BA)377	CCCCGTTCGCACTTKCGTG	MH	19	[81]	
Genus *Methanococcus*	Mcc r	WASTVGCAACATAGGGCACGG	PCR (reverse)	21	[51]	
	GMC728	ACCGTTCCAGACAAGTGCCTT	RNase H	22	[33]	*M. aeolicus*
	GMC231	ACTACCTAATCGAGGCAGTCC	RNase H	22	[33]	*M. aeolicus*
	GMC416	TTGATAAAAGCCCATGCTGTGC	RNase H	22	[33]	*M. aeolicus, M. voltae*
	GMC735m	TCACCGTCGGACCCGTTCCAGACA	HOPE	24	[83]	*M. aeolicus*
Genus *Methanothermococcus* Species *M. thermolithotrophicus*	GMTL416	TAGAAAAGCCTACGCAGTGC	RNase H	20	[33]	
Class *Methanopyri* Order *Methanopyrales* Family *Methanopyraceae* Genus *Methanopyrus*	FMCMT1044[w]	GTCAACCTGGCCTTCATCCTGC	RNase H	22	[33]	
	S-G-Mp-0431-a-A-20	TTACACCCCGGTACAGCCGC	MH	20	[109]	
	GMPK1331	GGTTACTACCGATTCCACCTTC	RNase H	22	[33]	
Class *Thermoplasmata* Order *Methanomassilii-coccales* Family *Methanomassilii-coccaceae*						

		Sequence	Method		Ref
Genus *Methanomassiliicoccus*	RC-III131	CATGCCCTAAGGTGGGGG	FISH	18	[110]
	B10-dir	GTTCGGATTGGGGGGCTGTAA	qPCR (forward)	20	[111]
	B10-VIC	TCACCGCTTGATGTT GACAAGCGATT	qPCR (probe)	26	[111]
	B10-rev	CCCCAATAAGAAACATCCTC	qPCR (reverse)	20	[111]
Species *M. luminyensis* Genus *Ca. Methanogranum*	RC281r2[x]	AAGGCCCATACCCGTCATA	FISH	19	[39]
Genera *Ca. Methanomethylophilus* and *Ca. Methanogranum*	UncM283	CTCTCAAGGCCCATACCC	ANAEROCHIP	18	[72]
	UncM301	CCAACTCTGGGCTCCCT	ANAEROCHIP	17	[72]
	UncM385	CTAGGAATTCCCTCATCGG	ANAEROCHIP	19	[72]
	UncM869	TCCCCAAGTAGCAGACTTAA	ANAEROCHIP	20	[72]

[a] IUPAC Ambiguity Codes: Y = C or T, R = A or G, K = G or T, S = C or G, W = A or T, M = A or C, H = A or C or T, V = A or C or G, D = A or G or T, B = C or G or T, N = G or A or T or C. X = 5-nitroindole

[b] *MH* membrane hybridization, *FISH* fluorescence in situ hybridization, *DGGE* denaturing gradient gel electrophoresis, *T-RFLP* terminal restriction fragment length polymorphism, *qPCR* quantitative PCR, *HOPE* hierarchical oligonucleotide primer extension, *RNase H* sequence-specific cleavage of 16S rRNA with ribonuclease H method

[c] Uncovered members were described at species level for genus-specific probes/primers and at genus level for class-, order-, and family-specific probes/primers

[d] Arch f2 probe covers members of the orders *Methanomicrobiales*, *Methanosarcinales*, and *Methanococcales*

[e] The original sequence of Nestmet-F is AWGWTCCAGGCCCTACGG; however, possible mistakes are found according to our evaluation based on ARB program [30]

[f] SMPL623 probe covers members of the *Methanoplanus limicola* and *M. endosymbiosus*

[g] SMPP1252 and SMPP577fm probes cover members of the *Methanoplanus petrolearius* and *Methanolacinia*

[h] F6SC393 probe covers members of the genera *Methanocorpusculum* and *Methanocalculus*

[i] EelMS240 probe targets for members of the genera *Methanolobus*, *Methanohalophilus*, *Methanococcoides*, and *Methanomethylovorans*

[j] S-G-Msae-0332-a-A-22 probe covers *Methermicoccus*

[k] S-F-Msaet-0387-S-a-21 probe covers *Methermicoccus* and some *Methanoculleus*

[l] R15F probe covers members of the genera *Methanomethylovorans* and *Methanosarcina* and *Methanolobus psychrophilus*

[m] MS821m probe covers members of the genera *Methanimicrococcus* and *Methanosarcina*

[n] The original sequence of S-G-Msar-0589-S-a-20 is ATCCCGGAGGACTGACCAAA; however, possible mistakes are found according to our evaluation based on ARB program [30]

[o] MBT-NR probe covers some *Methanococci* and *Methanomicrobia*

[p] S-F-Mbac-0398-S-a-20 probe covers *Methanobacterium* and *Methanosphaera*

[q] Mbt-341Ttaq probe covers *Methanothermobacter*, *Methanopyrus*, and some *Methanococcales*

[r] Mbt-359R probe covers *Methanothermobacter*

[s] Mt392f probe covers some *Methanobacterium*

[t] 410F probe covers some *Methanobacterium*

[u] The original sequence of GMTB645f is TCATGACTCGAGGTCGG; however, possible mistakes are found according to our evaluation based on ARB program [30]

[v] The original sequence of Mthe643 is CCGACCTCGAGTCATGATA; however, possible mistakes are found according to our evaluation based on ARB program [30]

[w] FMCMT1044 probe covers members of the family *Methanocaldococcaceae* and genus *Methanopyrus*

[x] The original sequence of RC281r2 is AAGGCCCATACCCGTCATC; however, possible mistakes are found according to our evaluation based on ARB program [30]

[79, 105, 108, 111], wetlands [101, 114, 115], lake [65], rice paddy field [70], soil [16, 110], and natural gas field [104] (Table 1). To date, almost all of the known culturable methanogens can be detected using these probes/primers at the class, order, family genus, and even species levels. The targeted regions of probes/primers frequently used for detection of 16S rRNA/rRNA gene are shown in Fig. 2. At the genus level, the probes/primers targeting the genera *Methermicoccus*, *Methanoregula*, *Methanosphaerula*, and *Methanotorris* are lacking.

4   Oligonucleotide Probes/Primers for *mcrA* Gene

Methyl-coenzyme M reductase (mcr) is the terminal enzyme involved in methanogenesis, which catalyzes the reduction of the methyl group bond of methyl-coenzyme M to form methane [28]. It has been noted that the phylogeny of methanogens determined based on methyl-coenzyme M reductase gene (*mcrA*) and its iso-enzyme gene (*mrtA*) (or their translated amino acid) sequences is in good accordance with those determined based on 16S rRNA gene sequences (Fig. 3) [118]. Since the *mcrA* and *mrtA* are highly conserved among known methanogens, *mcrA/mrtA*-targeted oligonucleotide probes/primes have been developed and are widely used for the detection of methanogens. The targeted regions of the forward primers of these sets are considerably different, whereas those of the reverse primers are almost the same with an exception for the METH-r primer (Fig. 4). Previously reported, frequently used probes/primers for *mcrA/mrtA* are categorized into at least seven groups, namely, MCR [119], ME [64], ML [118], MR1/ME2 [122], METH [123], ME3/ME2 [124], and mlas/mcrA-rev [125] (Table 2). The MCR set was originally designed to determine the phylogeny of the family *Methanosarcinaceae* [119]. The ME set was designed to analyze methanogenic populations in wetlands [64], but the difficulty in amplifying *mcrA/mrtA* relevant to *Methanosarcinaceae* and *Methanobacteriaceae* using the primer set has also been noted [132, 133]. The ML set was developed on the basis of the *mcrA* sequences obtained from five orders *Methanosarcinales*, *Methanomicrobiales*, *Methanobacteriales*, *Methanococcales*, and *Methanopyrales* [118]. The MR1/ME2-type primer set could be used to obtain near full length of *mcrA* sequences and was successfully applied for the amplification of *mcrA* gene from pure cultures of the *Methanocorpusculum* [123] and *Methanosaeta pelagica* [98]. The METH and ME3/ME2 sets were both designed for the detection of methanogens and ANME archaea populations in deep-sea sediments [122, 124]. The mlas forward primer is a short version of MLf primer (truncated 9 bases of the 3′-end of MLf primer) with two additional degenerate positions to improve the coverage of methanogens, while mcrA-rev reverse primer has the
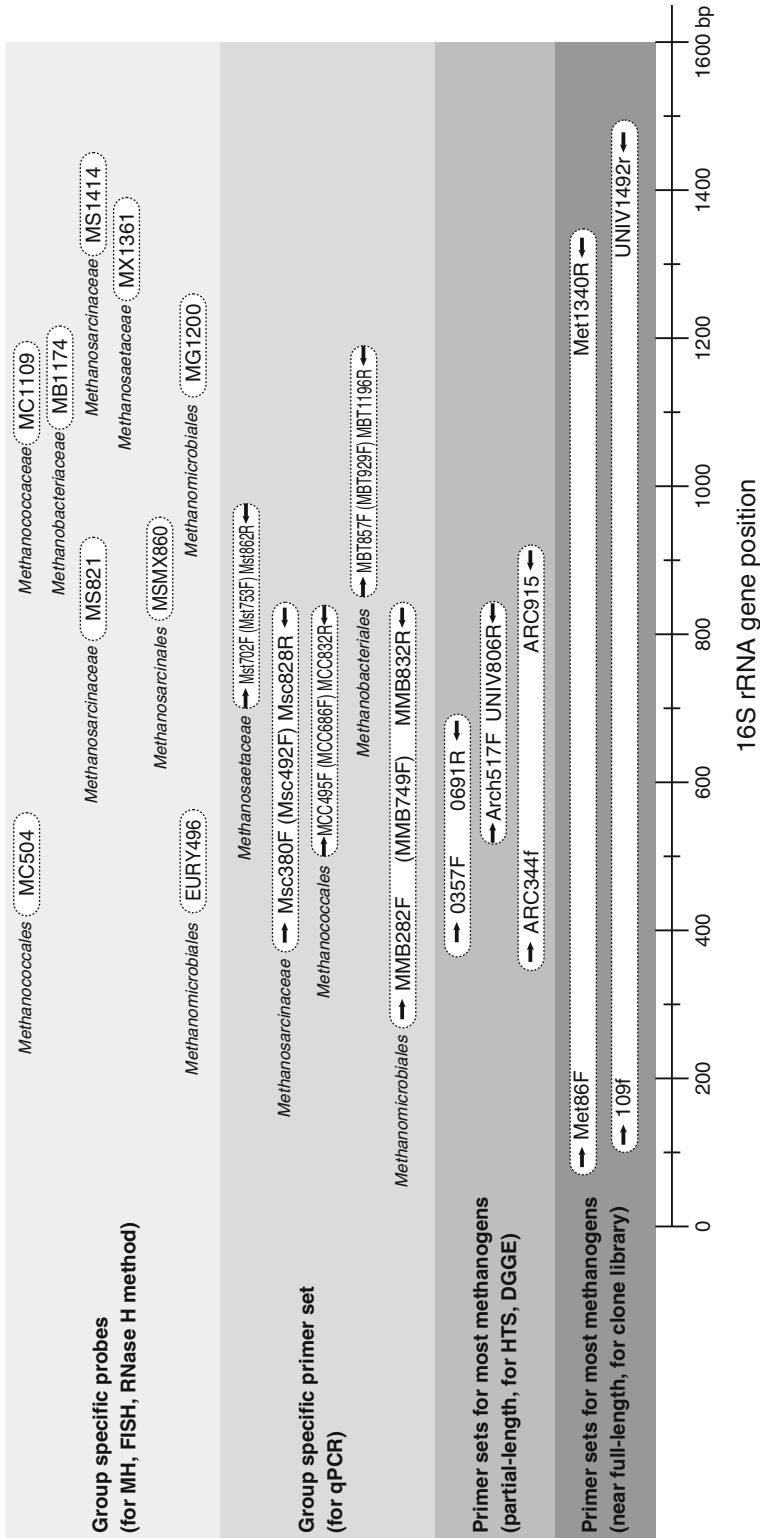
Fig. 2 The coverage of 16S rRNA with different types of probes/primers listed in Table 1. The position (bp) is according to *Escherichia coli*. *MH* membrane hybridization, *FISH* fluorescence in situ hybridization, *qPCR* quantitative PCR, *HTS* high-throughput sequencing, *DGGE* denaturing gradient gel electrophoresis
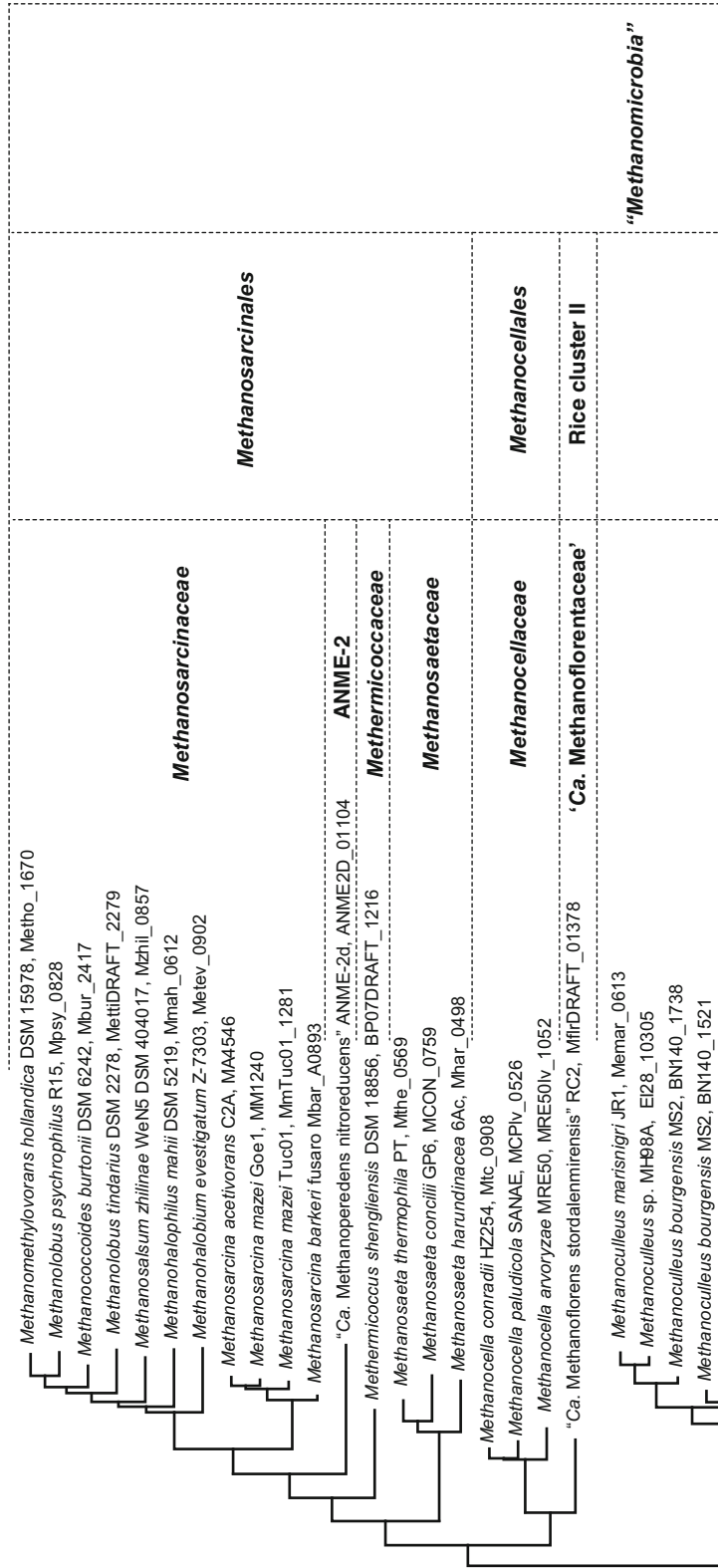
Fig. 3 *mcrA* gene-based phylogeny of methanogens. The maximum likelihood tree was constructed on the basis of *mcrA* gene sequences using the MEGA package [116] with known amino acid sequences of Mcr obtained from integrated microbial genomes (IMG) database [117]

Fig. 3 (continued)

Methanobacteria

Methanobacteriales

Methanobacteria mcrI

Methanobacteria mcrII

Methanobacterium sp. Maddingley, Mmad_01094
Methanobacterium formicicum DSM 3637, A994_09406
Methanobacterium paludis SWAN-1, MSWAN_2056
Methanothermobacter thermautotrophicus Delta H, MTH1164
Methanothermobacter marburgensis Marburg, MTBMA_c15480
Methanobacterium veterum MK4 DSM 19849, EJ01DRAFT_2878
Methanobacterium arcticum M2 DSM 19844, EI99DRAFT_02819
Methanobrevibacter smithii DSM 2375, METSMIALI_00940
Methanobrevibacter smithii ATCC 35061, Msm_1015
Methanobrevibacter smithii DSM 2374, METSMIF1 00790
Methanobrevibacter ruminantium M1, mru_1924
Methanobrevibacter wolinii SH, T523DRAFT_00656
Methanothermus fervidus DSM 2088, Mfer_0784
Methanobacterium veterum MK4 DSM 19849, EJ01DRAFT 0351
Methanobacterium arcticum M2 DSM 19844, EI99DRAFT 01149
Methanobacterium lacus AL-21, Metbo 2281
Methanobacterium formicicum DSM 3637, A994_00370
Methanobacterium sp. Maddingley, Mmad_00646
Methanothermus fervidus DSM 2088, Mfer_0734
Methanothermobacter thermautotrophicus Delta H, MTH1129
Methanothermobacter marburgensis Marburg, MTBMA_c15120
Methanosphaera stadtmanae DSM 3091, Msp_0321
Methanobrevibacter smithii ATCC 35061, Msm_0902
Methanocaldococcus sp. FS406-22, MFS40622_0424
Methanocaldococcus jannaschii DSM 2661, MJ0083

Fig. 3 (continued)

Fig. 4 The coverage of *mcrA* with different types of PCR primer sets listed in Table 2. The position (bp) is according to *Methanothermobacter thermautotrophicus* strain delta H *mcrA* gene (accession number U10036) following Steinberg and Regan [125] and Angel et al. [16]

consensus sequence of MCRr, ME2, and MLr primers [125]. Two other taxon-specific primer sets for *Methanosphaera stadtmanae* [128] and *Methanomassiliicoccales* [127] have also been reported (Table 2). In addition, several family-/genus-specific probes were developed for qPCR and FISH (Table 2).

5   PCR-Based Methods

5.1   Clone Library   Clone library analysis has long been used to elucidate entire community composition and diversity of methanogens in environments. Based on the frequency of retrieval of phylotypes in the library, relative abundance of phylotypes of interest can be inferred. Several sets of primers for 16S rRNA genes have been used for the detection and identification by PCR to explore the biodiversity of methanogens in a wide range of environment (Table 3). For example, the 109f/UNIV1492r primer set for virtually all known methanogens was designed for the 16S rRNA gene clone library analysis of anaerobic wastewater treatment process [112, 113, 134]. In addition, the Met86F/Met1340R primer set was used for the same purpose in freshwater sediments [184], rabbit cecum [165], and rumen [168] samples.

Similarly, the primers for *mcrA* have also been used for the construction of clone library to exclusively explore the diversity of methanogens in samples. For example, the MCR set was used to elucidate the diversity of methanogens in various environments with PCR-based cloning [176, 193, 195]. The ME primer set was mainly used for clone library analysis of wetland-related environments [64, 154]. The ML primer set was also used for cloning analysis in various environmental samples [118, 156, 167, 177, 194, 201]. Comparative studies using these three primer sets have indicated that the ML primer set is more efficient for retrieving phylogenetically diverse methanogens in the wetland than others [132, 202]. Besides, the ME3MF-ME3MF-e/ME2r′ primer set

Table 2
Oligonucleotide PCR primers and probes targeting the *mcrA* gene

Target group	Set type	Name	Direction/Application[a]	Probe sequence (5′–3′)	Probe length (m)	Reference
PCR primer						
Most methanogens	MCR	MCRf	Forward	TAYGAYCARATHTGGYT	17	[119]
		MCRr	Reverse	ACRTTCATNGCRTARTT	17	
	ME1/ME2	ME1	Forward	GCMATGCARATHGGWATGTC	20	[64]
		ME2	Reverse	TCATKGCRTAGTTDGGRTAGT	21	
		ME1	Forward	GCMATGCARATHGGWATGTC	20	[120]
		ME2b	Reverse	TCCTGSAGGTCGWARCCGAAGAA	23	
	ML	MLf	Forward	GGTGGTGTMGGATTCACACARTAY GCWACAGC	32	[118]
		MLr	Reverse	TTCATTGCRTAGTTWGGRTAGTT	23	
		mcrAfornew	Forward	GGTGTMGGDTTCACHCARTAYGC	23	[121]
		mcrArevnew	Reverse	TTCATNGCRTAGTTHGGRTAGTT	23	
	MR1/ME2	MR1	Forward	GACCTCCACTWCGTVAACAACGC	23	[122]
		ME2	Reverse	TCATKGCRTAGTTDGGRTAGT	21	
		MR1mod	Forward	GACCTSCACTWCGTVAACAAC	21	[98]
		ME2mod	Reverse	TCATBGCRTAGTTNGGRTAGT	21	
	METH	METH-f	Forward	RTRYTMTWYGACCARATMTG	20	[123]
		METH-r	Reverse	YTGDGAWCCWCCRAAGTG	18	
	ME3/ME2	ME3MF	Forward	ATGTCNGGTGGHGTMGGSTTYAC	23	[124]
		ME3MF-e	Forward	ATGAGCGGTGGTGTCGGTTTCAC	23	
		ME2r′	Reverse	TCATBGCRTAGTTDGGRTAGT	21	
	mlas/mcrA-rev	mlas	Forward	GGTGGTGTMGGDTTCACMCARTA	24	[125]
		mcrA-rev	Reverse	CGTTCATBGCGTAGTTVGGRTAGT	24	
		mlas-mod	Forward	GGYGGTGTMGGDTTCACMCARTA	23	[126]
		mcrA-rev	Reverse	CGTTCATBGCGTAGTTVGGRTAGT	24	
		mlas-mod2	Forward	GGTGGTGTMGGDTTYACHCARTA	23	[127]
		mcrA-rev	Reverse	CGTTCATBGCGTAGTTVGGRTAGT	24	
Methanosphaera stadtmanae	MrtA	MrtA_for	Forward	AAACAATCAACCACGCACTC	20	[128]
		MrtA_rev	Reverse	GTGAGCCCAATCGAAGGA	18	
Methanomassilii-coccales	Tp-mcrA	Tp-mcrA-F	Forward	GAYRACATCCTBGARGAYTA	20	[127]
		Tp-mcrA-R	Reverse	RTCGWAWCCRTAGAATCCGAG	21	

(continued)

Table 2
(continued)

Target group	Set type	Name	Direction/Application[a]	Probe sequence (5′–3′)	Probe length (m)	Reference
Probe						
Most of methanogens		ME3	Clone screening	GGTGGHGTMGGWTTCACACA	20	[64]
Genus *Methanosaeta*		SAE716TAQ	TaqMan probe	AGGCCTTCCCCACTCTGCTTGAGGAT	26	[120]
Genus *Methanosarcina*		SAR716TAQ	TaqMan probe	AGAAATTCCCAACAGCCCTTGAAGAC	26	[120]
Genus *Methanoculleus*		MCU716TAQ	TaqMan probe	AGCAGTACCGACCATGATGGAGGAC	26	[120]
Family *Methanobacteriaceae*		mbac-mcrA	TaqMan probe	ARGCACCKAACAMCATGGACACWGT	25	[129]
Family *Methanobacteriaceae*		mrtA	TaqMan probe	CCAACTCYCTCTCMATCAGRAGCG	24	[129]
Family *Methanocorpu-sculaceae*		mcp	TaqMan probe	AGCCGAAGAAACCAAGTCTGGACC	24	[129]
Family *Methanospirillaceae*		msp	TaqMan probe	TGGTWCMACCAACTCACTCTCTGTC	25	[129]
Genus *Methanoregula*		Fen	TaqMan probe	AAVCACGGYGGYMTCGGMAAG	21	[129]
Family *Methanosaetaceae*		msa	TaqMan probe	CCTTGGCRAATCCKCCGWACTTG	23	[129]
Genus *Methanosarcina*		msar	TaqMan probe	TCTCTCWGGCTGGTAYCTCTCC ATGTAC	28	[129]
Genus *Methanocella*		mcel/rc-I	TaqMan probe	CTVGGMTTCTWCGGBTACGACYT	23	[126]
Methanococcus vannielii		McvME0	FISH	GGAAAAATTCGAAGAAGATC	20	[130]
Methanococcus vannielii		McvME3r	FISH	TGTGTGAAACCTACGCCACC	20	[130]
Methanococcus vannielii		McvME1r	FISH	GACATTCCAATCTGCATTGC	20	[130]
Methanococcus maripaludis		MES2_150	Two-pass TSA-FISH	CAAATCTTACACAAAGAATACCAC	24	[131]

[a]*FISH* fluorescence in situ hybridization, *TSA-FISH* tyramide signal amplification FISH

Table 3

Examples of oligonucleotide primer sets for PCR-based analyses for methanogens

Type of sample	Target gene	Application	Target group	Probe set (forward/reverse/probe)[a]	Reference
Anaerobic process	16S rRNA	PCR-cloning	Most methanogens	109f/UNIV1492r[b]	[112, 113, 134]
			Most methanogens	25f/1391r	[112]
			Most methanogens	25f/UNIV1492r[b]	[112]
			Most methanogens	Arch21F + Acrh21f-Mvb/Ar912r	[103]
		PCR-DGGE	Most methanogens	ARC787F/ARC1059R	[135]
		PCR-pyrotag	Most methanogens	ARC344f/ARC915	[136]
			Most methanogens	Arch-340F/Arch-915R	[137]
		PCR-MiSeq	Most methanogens	Arch516F/UNIV806R	[138]
		qPCR	Most methanogens	ARC787F/ARC915F/ARC1059R	[68]
			Methanomicrobiales	MMB282F/MMB832R/MMB749F	[68, 139–147]
			Methanosarcinales	MSL812F/MSL1159R/MSL860F	[68, 142]
			Methanobacteriales	MBT857F/MBT1196R/MBT929F	[68, 139–147]
			Methanococcales	MCC495F/MCC832R/MCC686F	[68, 140, 142–145, 147]
			Methanosarcinaceae	Msc380F/Msc828R/Msc492F	[68, 139–141, 143–147]
			Methanosaetaceae	Mst702F/Mst862R/Mst753F	[68, 139–141, 143–147]
			Methanoculleus	298F/586R	[84]
			Methanosarcina	240F/589R	[84]
			Methanothermobacter	410F/667R	[84]
			Methanoculleus thermophilus	Mc412f/Mc578r	[86]
			Methanosaeta thermophila	Ms413f/Ms578r	[86]
			Methanothermobacter	Mt392f/Mt578r	[86]
			Methanosaeta	MS1b/SAE835R/SAE761TAQ	[77]
			Methanosarcina	MB1b/SAR835R/SAR761TAQ	[77]
			Methanoculleus	AR934F/MG1200b/MCU1023TAQ	[77]
			Methanosaeta	S-F-Msaet-0387-S-a-21/S-F-Msaet-0540-A-a-31/S-F-Msaet-0573-A-a-17	[97]
			Methanosarcina	S-G-Msar-0450-S-a-19/S-P-Msar-0540-A-a-31/S-G-Msar-0589-S-a-20	[93]
			Methanobacterium	S-F-Mbac-0398-S-a-20/S-G-Mbac-0526-A-a-33/S-G-Mbac-0578-A-a-22	[93]
			Methanothermobacter	S-F-Mbac-0398-S-a-20/S-G-Mthb-0549-S-a-32/S-G-Mthb-0589-A-a-25	[93]
			Methanospirillum	AR934F/MG1200b/MSP1025TAQ	[88]
			Methanolinea	NOBI109f/NOBI633	[78]

(continued)

Table 3
(continued)

Type of sample	Target gene	Application	Target group	Probe set (forward/reverse/probe)[a]	Reference
			WSA2	ArcI-F/ArcI-R/ArcI-Taq	[42]
			Methanobacterium	Mbt-210F/Mbt-359R/Mbt-341Taq	[42]
			Methanoculleus	298F/586R/Mcu-Taq	[42]
			Methanosarcina	MB1b/SAR835R/SAR761TAQ	[42]
			Methanosaeta	MS1b/SAE835R/SAE761TAQ	[42]
			Methanoculleus	Mc-274F/Mc-477R/Mc-361P	[85]
			Methanosaeta	MS1b 585F/Sae 835R/SAE761TAQ	[148]
			Methanosarcina	Mb1b 586F/Sar 835R/SAR761TAQ	[148]
			Methanosaeta	MS1b/SAE835R/SAE761TAQ	[149]
			Methanosarcina	MB1b/SAR835R/SAR761TAQ	[149]
	mcrA	PCR-cloning	Most methanogens	MLf/MLr	[103]
		PCR-DGGE	Most methanogens	MLf/MLr	[150]
		LH-PCR	Most methanogens	mcrAfornew/mcrArevnew	[121]
		qPCR	*Methanosaeta*	ME1/ME2b/SAE716TAQ	[120]
			Methanosarcina	ME1/ME2b/SAR716TAQ	[120]
			Methanoculleus	ME1/ME2b/MCU716TAQ	[120]
			Methanocorpusculaceae	mlas/mcrA-rev/mcp	[151]
			Methanosaetaceae	mlas/mcrA-rev/msa	[151]
			Methanobacteriaceae	mlas/mcrA-rev/mrtA	[151]
			Methanosarcina	mlas/mcrA-rev/msar	[151]
			Most methanogens	mlas/mcrA-rev	[152]
			Most methanogens	MLf/MLr	[153]
Anaerobic process, peat soil	*mcrA*	PCR-cloning	Most methanogens	mlas/mcrA-rev	[125]
		qPCR	*Methanocorpusculaceae*	mlas/mcrA-rev/mcp	[129]
			Methanospirillaceae	mlas/mcrA-rev/msp	[129]
			Methanosaetaceae	mlas/mcrA-rev/msa	[129]
			Methanobacteriaceae	mlas/mcrA-rev/mbac-mcrA	[129]
			Methanobacteriaceae	mlas/mcrA-rev/mrtA	[129]
			Methanoregula	mlas/mcrA-rev/Fen	[129]
			Methanosarcina	mlas/mcrA-rev/msar	[129]
Wetland, fen, peat soil	*mcrA*	PCR-cloning	Most methanogens	ME1/ME2	[64, 154]
			Most methanogens	ME3MF and ME3MF-e/ME2r′	[155]
			Most methanogens	MLf/MLr	[156]
		PCR-cloning/T-RFLP/DGGE	Most methanogens	ME1/ME2	[157, 158]
		qPCR	Most methanogens	MLf/MLr	[159, 160]

Source	Target gene	Method	Specificity	Primers	References
Human gut	16S rRNA	qPCR	*Methanobrevibacter smithii*	Msmithii_forward/reverse/probe	[108]
			Methanosphaera stadtmanae	Stadt_16SF/Stadt_16SR/Stadt_16SFAM	[108]
			Methanomassiliicoccus luminyensis	B10-dir/B10-rev/B10-VIC	[111]
			Methanomicrobiales	MMB282F/MMB832R/MMB749F	[161]
			Methanobacteriales	MBT857F/MBT1196R/MBT929F	[161]
			Methanosaetaceae	Mst702F/Mst862R/Mst753F	[161]
			Methanobacteriales	MBT-NF/MBT-NR/MBT-N-probe	[105]
	mcrA	PCR-cloning	*Methanosphaera stadtmanae*	MrtA_for/MrtA_rev	[128]
Human subgingival plaque	*mcrA*	PCR-cloning/T-RFLP	Most methanogens	MEI/MLr	[162]
		qPCR	Most methanogens	MLf/MLr	[163]
Insect gut	16S rRNA	PCR-DGGE	Most methanogens	0357F/0691R	[164]
Rabbit cecum	16S rRNA	PCR-cloning	Most methanogens	Met86F/Met1340R	[165]
	16S rRNA	PCR-DGGE	Most methanogens	0357F/0691R	[165]
Pig slurry, swine feces	16S rRNA	PCR-pyrotag	Most methanogens	ARC344f/ARC915	[166]
	mcrA	PCR-cloning	Most methanogens	MLf/MLr	[167]
		PCR-cloning/T-RFLP	Most methanogens	mlas-mod2/mcrA-rev	[127]
		qPCR	*Thermoplasmata*-related	Tp-mcrA-F/Tp-mcrA-R	[127]
Rumen	16S rRNA	PCR-cloning	Most methanogens	Met86F/Met1340R	[168]
		PCR-cloning/T-RFLP	Most methanogens	Met86F/0915aR	[169]
		PCR-DGGE	Most methanogens	Met86F/0915aR	[170]
			Most methanogens	A357f/A693r	[60]
			Most methanogens	A24f/A329r	[60]
			Most methanogens	A24f/A348r	[60]
		PCR-typing	*Methanosarcinales*	Arch f2/Arch r1386	[51]
			Methanobacteriales	Arch f2/MSr r859	[51]
			Methanobacterium	Mbac f331/Arch r1386	[51]
			Methanococcales	fMbium/Arch r1386	[51]
			Methanobrevibacter	Arch f2/Mcc r	[51]
			Most methanogens	fMb1/Arch r1386	[51]
		DGGE, qPCR	*Methanobrevibacter smithii*	Met630F/Met803R	[61]
		qPCR	Most methanogens	Msmithii_forward/reverse/probe	[79]
			Methanosphaera	Met630F/Met803R	[107]
				Stad-F/Stad-R	[107]

(continued)

Table 3
(continued)

Type of sample	Target gene	Application	Target group	Probe set (forward/reverse/probe)[a]	Reference
			Methanobrevibacter smithii	Forward/reverse/probe	[107]
			Methanobrevibacter ruminantium	Rum16S 740F/Rum16S 862R/Rum16S FAM	[107]
			Methanosphaera	Stad-F/Stad-R	[106]
			Methanobrevibacter sp. AbM4	AbM4-F/AbM4-R	[106]
	mcrA		*Methanobacteriaceae*	uniMet1-F/uniMet1-R	[106]
			Methanobacteriales	MBT857F/MBT1196R	[169]
			Methanomicrobiales	MMB282F/MMB832R	[169]
			Methanobacteriaceae	uniMet1-F/uniMet1-R	[169]
			Methanobacteriaceae	uniMet1-F/uniMet1-R	[170]
		PCR-cloning	Most methanogens	ME1/ME2	[171]
		qPCR	Most methanogens	MLf/MLr	[172]
			Most methanogens	MLf/MLr	[107]
			Most methanogens	MLf/MLr	[168]
			Most methanogens	MLf/MLr	[173]
Rumen, protozoa associated	16S rRNA	PCR-cloning, qPCR	Most methanogens	Met86F/Met915R	[76]
		qPCR	Most methanogens	Nestmet-F/Nestmet-R	[76]
			Methanobrevibacter	NestMbb-F/NestMbb-R	[76]
	mcrA	PCR-cloning	Most methanogens	mlas/mcrA-rev	[76]
Zoanthid associated	16S rRNA	PCR-pyrotag	Most methanogens	ARC344f/ARC915	[174]
Deep-sea water/ sediments/ hydrothermal vents	16S rRNA	PCR-cloning	Most methanogens	146f/1324r	[55]
		PCR-pyrotag	Most methanogens	958arcF/1048arcR-major, 1048arcR-minor	[71, 94, 175]
		PCR-pyrotag	Most methanogens	340Fmod/1000R	[74]
	mcrA	PCR-cloning	Most methanogens	MCRf/MCRr	[176]
			Most methanogens	MLf/MLr	[177]
		qPCR	Most methanogens	METH-f/METH-r	[123]
			Most methanogens	ME3MF + ME3MF-e/ME2r'	[124, 178]
Marine thrombolitic mat	16S rRNA	PCR-pyrotag	Most methanogens	ARC344f/ARC915	[179]
Marine sediment	16S rRNA	PCR-pyrotag	Most methanogens	ARC344f/ARC915	[180]

Sample	Target gene	Method	Target group	Primers	References
Coastal water	16S rRNA	qPCR	*Methanosaeta*	MX825cm/Arc915	[98]
		PCR-pyrotag	Most methanogens	Arch349F/Arch806R	[181]
Mudflat sediments	*mcrA*	PCR-pyrotag	Most methanogens	mlas/mcrA-rev	[182]
		qPCR	Most methanogens	mlas/mcrA-rev	[182]
Lake sediment	16S rRNA	PCR-cloning	*Methanomicrobia*	355F[c]/1068R	[80]
			Methanobacteriales	109f/1401R	[80]
			Methanococcales	344F[d]/1202R	[80]
	mcrA	PCR-cloning, qPCR	*Methanomicrobia*	355F[c]/1068R	[183]
		PCR-cloning, qPCR	Most methanogens	MLf/MLr	[183]
Freshwater sediments	16S rRNA	PCR-cloning	Most methanogens	Met86F/Met1340R	[184]
Soda lake sediment	*mcrA*	qPCR	Most methanogens	ME1/ME2	[185]
Sulfurous lake	16S rRNA	PCR-DGGE	Most methanogens	ARC344f/ARC915	[56, 186]
Hot spring	16S rRNA	PCR-pyrotag	Most methanogens	Arch21F[e]/A571R[e]	[187]
		qPCR	Most methanogens	Arch349F/Arch806R	[188]
Landfill	*mcrA*	PCR-cloning	Most methanogens	MLf/MLr	[118]
Ciliate endosymbiont	16S rRNA	PCR-DGGE	Most methanogens	A1f/A1100r	[52]
Rice paddy soil	16S rRNA	PCR-cloning/T-RFLP	Most methanogens	A109f/A912r	[189]
		PCR-DGGE	Most methanogens	109f/ARC915	[54]
		PCR, DGGE	Most methanogens	0357F/0691R	[70]
	mcrA	PCR-cloning/T-RFLP	Most methanogens	MCRf/MCRr	[189]
		qPCR	Most methanogens	MLf/MLr	[190]
Cold desert soil	*mcrA*	qPCR	Most methanogens	mlas-mod/mcrA-rev	[191]
Desert soil crust	*mcrA*	PCR-cloning/qPCR	Most methanogens	mlas-mod/mcrA-rev	[126]
		qPCR	*Methanosarcina*	mlas-mod/mcrA-rev/msar	[126]
		qPCR	*Methanocella*	mlas-mod/mcrA-rev/mcel-rcl	[126]
Permafrost soil	16S rRNA	PCR-pyrotag	Most methanogens	Arch349F/Arch806R	[192]

Table 3
(continued)

Type of sample	Target gene	Application	Target group	Probe set (forward/reverse/probe)[a]	Reference
Soil	16S rRNA	PCR-cloning/T-RFLP	Most methanogens	109f/ARC934b	[193]
	mcrA	qPCR	Methanocella	Mcl282-F/Mcl609-P/Mcl832-R	[16]
		PCR-cloning	Most methanogens	mlas-mod/mcrA-rev	[16]
		PCR-cloning	Most methanogens	MLf/MLr	[194]
		PCR-cloning/T-RFLP	Most methanogens	MCRf/MCRr	[193]
Mud volcanoes	mcrA	PCR-cloning	Most methanogens	Most methanogens	[195]
Compost	mcrA	qPCR	Most methanogens	mlas/mcrA-rev	[196]
Oil-degrading microcosm	16S rRNA	qPCR	Methanomicrobiales	MMB282F/MMB832R	[197, 198]
			Methanosaetaceae	Mst702F/Mst862R	[197]
			Methanosarcinaceae	Msc380F/Msc828R	[197]
Pure cultures	16S rRNA	PCR-ribotyping	Most methanogens	Met83F (or Met86F)/Met1340R	[53]
		qPCR	Methanosphaera stadtmanae	Stadt_16SF/Stadt_16SR/Stadt_16SFAM	[199]
	mcrA	PCR-cloning	Most methanogens	Most methanogens	[119]

[a]The primer sequences were shown in Tables 1 and 2

[b]UNIV1492r reverse primer was originally referred from Lane et al. [200] as a universal primer

[c]355F forward primer was originally referred as M(SA/MI)355 probe developed by Ovreås et al. [81] as shown in Table 1

[d]344F forward primer was originally referred as ARC344 probe developed by Raskin et al. [63] as shown in Table 1

[e]Arch21F was mixed with N3F primer for Nanoarchaeota (5′-TCCCGTTGATCCTGCG-3′)

[155], mlas/mcrA-rev set [125], and mlas-mod/mcrA-rev [16] were used for the construction of *mcrA* clone libraries of some methanogenic communities. It has been noted that these *mcrA*-targeted primer sets (ME-related primer set in particular) can also be used for the quantitative detection of ANME in methane seep sediments [124, 203, 204]. This is due to the fact that anaerobic methane oxidation performed by the ANME group is considered to proceed with mcr-type enzymes [205]. Detailed information about the *mcrA*-based qPCR for ANMEs is described in later section.

5.2 Fingerprinting

PCR-based fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), and length heterogeneity-PCR (LH-PCR), can be used for obtaining a big picture of microbial community structures in environmental samples in a technically simple manner, because relative abundance and phylogenetic affiliation of phylotypes can be evaluated on the basis of intensity and migration time of DNA bands or T-RF in electropherogram. Some of the 16S rRNA gene primers have been used for PCR to reveal methanogen populations by DGGE [56, 60, 61, 70, 135, 164, 165, 170, 186], T-RFLP [189, 193], and ribotyping [51, 53]. For *mcrA*-based fingerprinting, the MCR primer set was used to elucidate the diversity of methanogens in various environments with T-RFLP analyses [189, 193]. The ME primer set was used for the polyphasic molecular analyses of wetland-related environments (i.e., cloning, RFLP, and DGGE) [157, 158]. Gagnon et al. developed *mcrA*-based LH-PCR technique with a modified ML primer set (mcrAfornew/mcrArevnew) to identify the major methanogenic constituents in an anaerobic reactor treating swine manure [121]. In addition, some studies have conducted DGGE [150] and T-RFLP [127] analysis with ML and mlas-mod2/mcrA-rev primer sets, respectively [127, 150].

5.3 High-Throughput Sequencing

Massive parallel sequencing of PCR-amplified 16S rRNA genes using high-throughput DNA sequencers (such as the Roche/454 pyrosequencers and Illumina HiSeq/NextSeq/MiSeq) allows us to obtain a plenty of community sequence tags (e.g., ca. 10,000–100,000 16S rRNA gene reads for each sample) that the previous Sanger-based cloning method can never provide. The methodological advancements of high-throughput sequencing technologies include higher resolution (more sequence reads) for community structure analysis, analysis of multiple related samples, and use of metadata [206]. Due to these advancements, in combination with recent development of analytical tools for massive sequence data such as QIIME [207] and mothur [208], the method will undoubtedly be a standard means for characterizing the diversity of methanogens in ecosystems. So far, these techniques have been used for characterizing archaeal populations

(including methanogens) in marine-related environments [71, 74, 94, 174, 175, 179, 180], pig manure [166], permafrost soil [192], hot spring [187], and anaerobic bioreactors [136, 137] with archaea-specific primer sets (e.g., ARC344f/ARC915). The *mcrA*-based pyrotag analysis was also conducted mudflat sediments [182].

5.4   qPCR

Quantitative PCR (qPCR) has widely been used for the quantitative detection of methanogens with primer set targeting 16S rRNA and *mcrA* genes (Table 3). For example, the 16S rRNA gene primers Met630F/Met803R were developed for the SYBR green-based qPCR for virtually all known methanogens in ruminal environments [61, 107]. Tymensen et al. employed nested qPCR to analyze methanogenic populations in the rumen, where the primers Met86F/Met915R were used for the first PCR, and the Nestmet-F/Nestmet-R and NestMbb-F/NestMbb-R were further used for the second PCR to detect members of the entire methanogenic populations and those of the *Methanobrevibacter*, respectively [76]. Yu et al. designed TaqMan-based qPCR probes/primer sets (seven sets in total) for the majority of methanogens and each of the orders *Methanomicrobiales*, *Methanosarcinales*, *Methanobacteriales*, and *Methanococcales*, as well as the families *Methanosaetaceae* and *Methanosarcinaceae* [68]. These primer sets have frequently been used for the quantification of methanogens in anaerobic bioprocesses [139–147, 209], human gut [161], rumen [169], and oil-degrading microcosm [197, 198]. qPCR measurement methods for particular groups of methanogens of interest, such as *Methanoculleus* [42, 77, 84–86], *Methanolinea* [78], *Methanospirillum* [88], *Methanosaeta* [77, 86, 97], *Methanosarcina* [77, 84, 93], *Methanolobus* [101, 115], *Methanocella* [16], *Methanomassiliicoccus* [111, 127], *Methanobrevibacter* [79, 106, 107], *Methanosphaera* [106, 108], *Methanobacterium* [42, 93], *Methanothermobacter* [84, 86, 93], and WSA2 clade [42], have also been developed (Table 1).

For *mcrA* quantification with qPCR method, ME-type primer sets were used for methanogens and ANME populations in methane seep sediments [203, 204], soda lake sediment [185], and anaerobic reactors [120]. Nunoura et al. refined the ME primer series for the detection of methanogens and ANME organisms and showed that the mixture of the ME3MF and ME3MF-e forward primers and the ME2′ reverse primer can be successfully applied for the qPCR detection of the methanogens and ANME organisms in anaerobic digester sludge and methane seep sediments [124, 178]. The ML primer set was also used for the quantification of methanogenic populations in an anaerobic wastewater treatment reactor [153], peat soil [159, 160], rumen [107, 168, 172, 173], lake sediment [183], rice paddy soil [190], and human subgingival plaque [163]. Steinberg and Regan developed the mlas/mcrA-rev

primer set for the clone library construction and qPCR analyses of methanogens in oligotrophic fen and anaerobic digester sludge [125, 129]. They also developed the genus-specific TaqMan probes for the *mcrA*-based quantitative detection of the *Methanocorpusculaceae*, *Methanospirillaceae*, *Methanosaetaceae*, *Methanobacteriaceae*, *Methanobacteriaceae*, *Methanoregula*, and *Methanosarcina* in anaerobic digester sludge and peat soil samples [129]. The mlas/mcrA-rev primer set was also used for the qPCR of methanogens in anaerobic bioreactors [151, 152], rumen [76], liver sediments [182], and compost [196].

5.5 HOPE

The probes/primers for methanogens have also been used in the hierarchical oligonucleotide primer extension (HOPE) method that is quantitative, multiplex detection of targeted microbial genes among PCR-amplified genes [210]. Wu et al. developed a set of group-specific probes for the HOPE detection of methanogens and used for the quantitative detection of methanogens in a total of 19 reactors at different taxonomic levels [83].

In short, PCR-based methods using methanogen-specific probes/primers are widely used for the characterization of methanogen community in various types of environments. In particular, qPCR and HOPE methods provide sensitive, quantitative data of gene of interest with a sufficiently high dynamic range of quantification. These methods may be further used for quantitative monitoring of methanogen taxa of interest in microbial communities. It should be noted, however, that the results obtained by PCR-based method, multi-template PCR methods in particular, might have unavoidable biases involved in bulk cell lysis, DNA extraction, probe/primer selection, and the PCR amplification step [211], and hence researchers need to be careful about the experimental verification of the method in use, as well as about the interpretation of data from these analyses.

6 PCR-Independent Methods

6.1 Membrane Hybridization

RNA-based community analysis is known to indicate the in situ activity of individual groups in ecosystems because RNA synthesis is known, with some exceptions [212], to reflect the in situ growth rates of organisms [213, 214] and that the turnover of RNA is thought to be much faster than that of DNA. Quantitative membrane hybridization of labeled DNA probes to community rRNAs has been applied to the quantitative detection of active members of methanogens present in complex microbial communities [215, 216]. In 1994, Raskin and colleagues conducted the first studies on the quantitative detection of methanogens in anaerobic wastewater treatment sludge by using membrane hybridization technique [63, 215]. They developed a variety of group-

specific oligonucleotide probes targeting 16S rRNA of *Methanomicrobiales* (probes MG1200 and MSMX860), *Methanobacteriaceae* (probes MB310 and MB1174), and *Methanococcales* (probe MC1109). Because of the importance of acetoclastic methanogenesis in anaerobic bioreactors, specific probes for *Methanosarcinaceae* (probes MS1414 and MS821) and *Methanosaetaceae* (probe MX825) were also developed. These probes are still quite useful and have been successfully applied to the quantification of methanogens in laboratory- and full-scale anaerobic bioreactors [217–221].

Although membrane hybridization enables the sensitive quantification of individual species of rRNA molecules, this method requires several laborious experimental steps, often radioactively labeled DNA probes, and reference rRNA samples as external standards for each experiment. Thus, the method itself may be replaced by similar but more rapid and simple methods, such as reverse transcription (RT)-qPCR and RNase H methods.

6.2   FISH

Whole-cell fluorescence in situ hybridization (FISH) targeting 16S rRNA is now commonly used to detect specific groups of microbial cells and to quantify populations of interest in environments by direct counting under a microscope [213]. Basically, the sequences of the probes developed for membrane hybridization or reverse primers for PCR amplification of methanogen 16S rRNA genes can be directly used for probe synthesis in FISH studies. For example, the probes previously designed by Raskin et al. [63] have frequently been used for FISH-based detection of methanogens in various anaerobic ecosystems, such as the rumen [222], peat bog [223], aquifer [224], landfills [11], natural gas field [104], and anaerobic bioprocesses [103, 225–228]. Crocetti et al. refined the experimental conditions of the probes designed by Raskin et al. for FISH analysis to accurately and sensitively detect methanogen cells [95]. Besides, anaerobic ciliates often posses endosymbiotic methanogens within their cells, and the distribution of such methanogen cells in eukaryotic cells has been observed by the FISH method [52, 229, 230].

Although FISH is a powerful method for visualizing the cells of interest, there are some drawbacks in detecting cells; one of such problems is concerned with the penetration of oligonucleotide probes into the cells [213]. For methanogens, FISH staining is often difficult for some *Methanobacterium* and *Methanobrevibacter* cells, for which oligonucleotide probes do not penetrate into their cells [87, 228, 231]. To solve this problem, fixed cells were subjected to freeze-thaw cycles before hybridization, resulting in the improvement of probe penetration [228]. Another way to solve this problem is the use of recombinant pseudomurein endoisopeptidase, which increases the permeability of oligonucleotide probes into methanogen cells and allows better visualization of

methanogen cells in anaerobic granular sludge and endosymbiotic methanogen cells in the anaerobic ciliate *Trimyema compressum* [231]. An improved protocol of catalyzed reporter deposition (CARD)-FISH for methanogens with recombinant pseudomurein endoisopeptidase has also been reported, which can increase the fluorescence signal intensity in FISH for detecting cells with a low rRNA content [232]. In addition, *mcrA*-based in situ detection of methanogens has also been performed using the two-pass tyramide signal amplification (TSA)-FISH approach combined with locked nucleic acids [130, 131, 233]. These attempts were, at this point, only partially successful in detecting methanogen cells, because *mcrA* is generally present as a single-copy gene on their chromosome, which results in a low sensitivity of detection.

6.3   RNase H Method

Although the abovementioned quantitative methods such as membrane hybridization and qPCR with methanogen-specific probes/primers are becoming common approaches to determine the abundance of the population of interest in a complex microbial community, there is a need to develop more simple and rapid techniques that meet the needs for real-time monitoring of the population of interest in a complex community. Uyeno et al. developed a simple and rapid quantification method based on the sequence-specific cleavage of 16S rRNA with ribonuclease H (RNase H) and oligonucleotide (scissor) probes (namely, the RNase H method) [234]. RNAs from a complex community were first mixed with an oligonucleotide and subsequently digested with RNase H. Because RNase H specifically degrades the RNA strand of RNA/DNA hybrid heteroduplexes, the targeted rRNAs are cleaved at the hybridization site in a sequence-dependent manner and are consequently cut into two fragments. In contrast, nontargeted rRNAs remain intact under the same conditions. For the detection of cleaved rRNAs, the resulting RNA fragment patterns can be resolved by gel electrophoresis or capillary electrophoresis using RNA-staining dyes. The relative abundance of the targeted species of 16S rRNA fragments in total 16S rRNA can also be quantified by determining the signal intensity of individual 16S rRNA bands in an electropherogram (without the use of external standards). Because this method does not require an external RNA standard for each experiment, as is required in membrane hybridization, and because the present method is relatively easy to perform within a short time (i.e., within 2–3 h), this technique may provide direct, rapid, and easy means of the quantitative detection of particular groups of anaerobes based on their rRNA, such as those of methanogens as well.

In general, oligonucleotide probes used in FISH and membrane hybridization methods can directly be used as scissor probes in the RNase H method. This method has also been applied to the quantification of active methanogens in anaerobic sludge

[33, 234–236] and ruminal ecosystems [237]. Because of the reasons that this method is based on rRNA and that the RNA (rRNA) level is often dependent on the in situ activity of individual cells [213], this method may be used for real-time monitoring of active methanogens and other important bacteria in engineered ecosystems such as waste/wastewater treatment systems to better control such bioreactors [238]. Besides, cleavage reaction using RNase H is further applied for the capillary electrophoresis ribosomal RNA single-stranded conformation polymorphism [239], and the efficiency of the reaction can be improved by the fusion of RNase H with a zinc finger [240].

6.4 Microarray   DNA microarray platforms, such as ANAEROCHIP [72], GeoChip [241], and PhyloChip [242], are useful tools for parallel, high-throughput, and comprehensive detection of different microbial community members in ecosystems. Franke-Whittle et al. developed the ANAEROCHIP platform that consists a total of 98 methanogen-specific oligonucleotide probes [72], and it has been successfully applied for the comprehensive detection of methanogens in anaerobic bioprocesses [72, 243, 244]. The GeoChip platform is composed of a vast numbers of oligonucleotide probes for functional genes including *mcrA* [241]. The GeoChip-based profiling was conducted for the analysis of metabolic diversity in hydrothermal vent, and significant signals of *mcrA* along with other functional genes (e.g., aerobic and anaerobic methane oxidation, denitrification, and sulfate reduction) were detected [245]. Several researchers used the PhyloChip to the community analysis of contaminated subsurface sediments [246] and solar saltern [247], and they observed *Methanobacteria*- and *Methanomicrobia*-specific signals, implying their important role in these ecosystems.

7 SIP-Based Detection

Stable isotope probing (SIP) of DNA [248] and RNA [249] has been used in recent years in order to identify metabolically active populations in environments. In principle, SIP technology is based on the incorporation of ^{13}C-labeled substrates into the nucleic acids. The separation of isotopically labeled (active) fractions from unlabeled (inactive) fractions is generally performed with density gradient centrifugation. The substrate-assimilated microorganisms in the labeled fractions are identified by a set of PCR-based molecular techniques such as gene cloning, T-RFLP, and other methods. Therefore, for the purpose of identifying active methanogens that are responsible for particular metabolisms in environments, the probes/primers listed in Tables 1 and 2 can potentially be used. As examples, Conrad and colleagues have intensively analyzed active methanogen populations in rice paddy soil by using SIP

followed by fingerprinting [250–253]. The active methanogenic populations in enrichment culture of municipal solid waste digester residues [254], fen [255], rice rhizospheres [256], rice paddy soil [257–259], swine manure storage tank [260], and coal mine [261] were also determined by SIP-based molecular analysis.

8   Best Probes/Primers to Detect Methanogens in the Environment: Anaerobic Sludge as a Case Study

In the previous sections, we comprehensively address the current status of oligonucleotide probes/primers targeting 16S rRNA and *mcrA* genes and molecular methods to detect methanogens in the environments. Because these molecular methods have their own pros and cons, researchers need to think carefully about appropriate combinations of methods and probes/primers depending on what the researchers need to know. For details, recent reviews may be helpful for the selection of molecular techniques to be employed [21, 22, 24, 262]. In a practical way, polyphasic analyses using multiple molecular techniques are best to gain a reliable picture of methanogen populations in environments. In this section, we propose the best possible combination of the methods and corresponding probes/primers to detect and quantify methanogens in sludge samples from anaerobic wastewater treatment process as a case study.

At the beginning of study, we may need to understand what types of methanogens are present in the sludge. For this purpose, clone library analysis with a primer set that covers near full length of 16S rRNA (ca. 1,300–1,400 bp) and targets most methanogens (e.g., 109f/UNIV1492r [113] and Arch21F/UNIV1492R [103]) is a good first choice. Narihiro et al. constructed clone libraries with the 109f/UNIV1492r primer set to analyze archaeal populations in 12 sludge samples from full-scale anaerobic upflow anaerobic sludge blanket (UASB) reactors treating various food-processing wastewater, and they revealed that *Methanosaeta*- and *Methanobacterium*-related phylotypes were the major acetoclastic and hydrogenotrophic methanogens in the processes, respectively [113]. Instead of clone library construction, high-throughput sequencing using a primer set that covers partial 16S rRNA (ca. 300–500 bp) of members of the domain *Archaea* (e.g., ARC344f/ARC915 [136] and Arch516F/UNIV806R [138]) is an alternative (and more powerful) way to obtain entire picture of methanogen populations. Kuroda et al. employed Illumina MiSeq sequencing with the Arch516F/UNIV806R primer set to analyze archaeal communities in 12 anaerobic (or anoxic) sludge samples. The results showed that known methanogens (e.g., *Methanosaeta*, *Methanobacterium*, *Methanothermobacter*, and *Methanolinea*) dominated in all of the samples, while a relatively low percentage (0.4–6.8%) of

WSA2-related methanogen was found in 11 out of 12 sludge samples [138].

Once you have obtained basic information about methanogen populations in the sludge, quantification of dominant or specific members of interest can be the next step. qPCR-based group-specific quantification is one of the best ways to perform temporal monitoring of methanogen populations in the process. For example, TaqMan-based 16S rRNA-targeted qPCR probes/primer sets developed by Yu et al. (e.g., MMB282F/MMB832R/MMB749F for *Methanomicrobiales*, MBT857F/MBT1196R/MBT929F for *Methanobacteriales*, MCC495F/MCC832R/MCC686F for *Methanococcales*, Msc380F/Msc828R/Msc492F for *Methanosarcinaceae*, and Mst702F/Mst862R/Mst753F for *Methanosaetaceae*) are widely used for this purpose [68]. Bialek et al. reported the methanogenic community dynamics of two types of anaerobic bioreactors (inverted fluidized bed and expanded granular sludge bed) treating synthetic daily wastewater during operating temperature transition from 37 to 15°C, and they found that the dominated hydrogenotrophic populations shifted from *Methanobacteriales* to *Methanomicrobiales* in both reactor configurations [141].

For *mcrA*-targeted probes/primes, the combination of mlas-derived forward primers (i.e., mlas-mod [126] and mlas-mod2 [127]) and mcrA-rev reverse primer [125] boasts the highest coverage for *mcrA* gene sequences of known methanogens (Table 2). Although the usability of these newly developed primer sets has not yet been applied for quantification of methanogens in anaerobic wastewater treatment process, mlas-mod/mcrA-rev and mlas-mod2/mcrA-rev primer sets were used for the quantification of methanogens in soil environments [16, 126] and pilot-scale pig slurry storage facilities [127], respectively. Angel et al. further employed dual-labeled msa and mcel-rcI probes with mlas-mod/ mcrA-rev primer set for qPCR detection of *Methanosarcina* and *Methanocella*, respectively, in desert soil crusts [126]. Successful detection of methanogens in such ecosystems implies that these primer sets can be used for the investigation of methanogen population dynamics in anaerobic bioreactors.

RNase H method is an alternative way to monitor the methanogen populations [33]. Narihiro et al. developed a total of 40 probes, including newly designed and previously reported probes listed in Table 1. The hybridization condition has been optimized for the specific quantification of methanogens at different taxonomic levels (i.e., order, family, genus, and species) for use in the RNase H method and has been applied to quantitative and comprehensive detection of methanogens in various types of anaerobic bioreactors [33].

FISH analysis has been used for investigating the localization of methanogens in biofilms (sludge granules) from anaerobic

bioreactors [100, 221, 226–228, 263–265]. In anaerobic sludge granules, hydrogenotrophic methanogens are often juxtaposed with syntrophic substrate-degrading bacteria, such as syntrophic propionate-oxidizing bacteria (e.g., members of the genera *Syntrophobacter*, *Syntrophorhabdus*, and *Pelotomaculum*); such close proximity between syntrophic bacteria and methanogens has been observed by FISH with confocal laser scanning microscopy [228, 266–268]. Imachi et al. visualized the proximity between *Pelotomaculum*-related propionate-oxidizing syntroph and *Methanobacteriaceae*-type methanogen in a sludge granule taken from a lab-scale thermophilic UASB reactor with MB1174 probe [268]. In addition, Sekiguchi et al. showed spatial distribution of the *Methanomicrobiales* (MG1200 probe), *Methanobacteriaceae* (MB1174 probe), *Methanosarcinaceae* (MS1414 probe), and *Methanosaetaceae* (MX825 probe) along with filamentous cells of the *Anaerolinea* (*Chloroflexi* subphylum I)-related organisms in two lab-scale UASB reactors [228].

Thus, combined use of clone library/high-throughput sequencing (to gain the big picture of community members), qPCR/RNase H method (to quantify groups of interest), and FISH (to visualize/localize the targeted groups) with abovementioned probes/primers is a recommended approach to explore methanogen populations in anaerobic wastewater treatment processes.

9   Conclusions

As described in this chapter, a vast number of oligonucleotide probes/primers have been developed so far for deciphering and quantifying methanogen populations, covering most parts of the methanogens known to date. A variety of molecular methods have also been developed that are used in combination with the probe/primers. Most recently, a 16S rRNA gene database of ruminal and intestinal methanogen (RIM-DB) that consists of 2,379 nearly full-length chimera-checked 16S rRNA gene sequences in total has been made [269]. Such database will be a useful foundation not only for species-level taxonomic assignment of 16S rRNA gene amplicons generated by high-throughput sequencing but also for the development/evaluation of methanogen-specific probes/primers. Lastly, it should be noted that there are still a number of uncultivated methanogens in various environments and that they should be further isolated and characterized in detail. Detection tools for such uncultured methanogens remain to be developed to further increase in the coverage of our knowledge about methanogens present in environments.

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Primers: Functional Genes for Aerobic Chlorinated Hydrocarbon-Degrading Microbes

Nicholas V. Coleman

Abstract

Bioremediation offers a solution to the problem of chlorinated hydrocarbon pollution. Small chlorinated compounds can be mineralised by aerobic bacteria, acting as carbon and energy sources, and the genes that encode these processes can be detected and monitored by PCR. This provides a rapid, specific, culture-independent, and potentially quantitative tool of enormous utility to bioremediation practitioners. This chapter summarises and evaluates available PCR primers for genes encoding metabolism of organo-chlorines, especially chlorinated alkanes, alkenes, and alkanoic acids. The enzyme families involved include hydrolytic dehalogenases, dehydrochlorinases, monooxygenases, glutathione-*S*-transferases, and corrinoid-dependent enzymes. Although aromatic dioxygenases are important enzymes for degradation of chlorinated aromatic hydrocarbons, this enzyme family is not discussed here. This chapter assumes a basic knowledge of PCR and primer design. The focus will be on the design and use of degenerate primers and on the relationships between genes, bacteria, and chlorinated substrates.

Keywords: Biodegradation, Bioremediation, Chloroalkane, Chloroalkene, Dechlorination, Dehalogenase, Halidohydrolase, Monooxygenase, Organochlorine, qPCR

1 Introduction

Chlorinated hydrocarbons have been widely used as pesticides, solvents, and plastics, and many of these chemicals have become problematic pollutants. Bioremediation is increasingly seen as a viable treatment technology for organochlorines, but there is a need for better methods of monitoring and predicting the underlying microbial activities [1, 2]. PCR offers a useful tool here, since it is rapid, specific, culture independent, and potentially quantitative.

The aerobic biodegradation of large, highly chlorinated hydrocarbons is incomplete and not linked to growth [3, 4], but the smaller, less chlorinated compounds (e.g. chloromethane) can be mineralised and act as carbon and energy sources [5–7]. The greater solubility, bioavailability, and biodegradability of the small organochlorines make these compounds more attractive targets for

bioremediation, and thus the focus of this chapter will be PCR primers for genes encoding metabolism of smaller compounds, such as chloromethanes, chloroethanes, and chloroethylenes. The enzyme families involved in these processes include hydrolytic dehalogenases, dehydrochlorinases, monooxygenases, glutathione-*S*-transferases, and corrinoid-dependent enzymes.

PCR is an excellent tool but it needs to be applied carefully. Good basic principles include thorough reading of the literature, careful primer design, and/or independent evaluation of existing primers, the use of positive and negative controls, and, of course, good hands-on training, especially in aseptic technique. The need for caution is perhaps best explained by a casual list (below) of unexpected things that can happen in PCR, compiled from experiences in the author's own laboratory.

Examples of Unexpected PCR Results

- Amplified products are the wrong size and the wrong sequence.
- Amplified products are the correct size but the wrong sequence.
- Amplified products are the wrong size but the correct sequence.
- Reverse primer mis-primes and instead acts as forward primer.
- Forward primer mis-primes, giving two products, both from the correct target gene.
- Inhibitors prevent amplification despite careful purification of DNA.
- Amplification of lab bacteria sequences (e.g. *E. coli*) from unexpected locations.
- Primers look good on the computer, but fail in the lab.
- Primers look bad on the computer, but work in the lab.

This chapter assumes a basic knowledge of PCR and primer design. Issues such as dimers, hairpins, melting, and annealing temperatures will not be discussed at length here, although these are certainly important considerations. The focus will instead be on the design and use of degenerate primers and on broader issues such as the relationships between genes, bacteria, and chlorinated substrates – these impact strongly on the interpretation of PCR data.

The chapter is organised into sections based on different catabolic genes; this corresponds to some extent to organisation based on different organochlorine compounds. Two case studies from the author's laboratory describe in detail some approaches to primer design and evaluation. All of the primers discussed in this chapter are compiled in Table 1.

Table 1
Primers for organochlorine metabolic genes in aerobic bacteria

Gene[a]	Pollutant(s)	Primer name	F/R	Primer sequence (5'-3')	Fold degeneracy	Annealing temp used	Product size (bp)	Type of PCR	Successful templates	References
SDIMO groups 3,4,5,6	Chlorinated aliphatics	NVC57	F	CAGTCNGAYGARKCSCGNCAYAT	512	55	1,100/ 810/ 720/ 420	Regular PCR, nested PCR	Pure cultures, enrichments, soils, sediments	[14, 15]
		NVC65	F	CARATGYTNGAYGARGTNCGNCA	1,024					
		NVC58	R	CGDATRTCRTCDATNGTCCA	144					
		NVC66	R	CCANCCNGGRTAYTTRTTYTCR AACCA	1,024					
etnC	VC	NVC105	F	CAGGAGTCSCTKGACCGTCA	4	55	360	Regular PCR	Pure cultures, enrichments	[15, 29]
		NVC106	R	CARACCGCCGTAKGACTTTGT	4	55	360			
		RTC_F	F	ACCCTGGTCGGTGTKSTYTC	8	55	106	qPCR	Groundwater	[30]
		RTC_R	R	TCATGTAMGAGCCGACGAAGTC	2					
		MRTC_F	F	ACACTCGTCGGCGTTGTTTC (+7 others)	8	55	106	qPCR	Pure cultures, groundwater	[32]
		MRTC_R	R	TCATGTACGAGCCGACGAAGTC (+5 others)	6					
etnE	VC	CoM-F1L	F	AACTACCCSAAYCCSCGCTGGTACGAC	8	60	891	Regular PCR	Pure cultures	[25]
		CoM-R2E	R	GTCGGCAGTTTCGGTGATCGTGC TCTTGAC	1					
		RTE_F	F	CAGAAYGGCTGYGACATYATCCA	8	55	151	qPCR	Groundwater	[30]
		RTE_R	R	CSGGYGTRCCCGAGTAGTTWCC	16					
		MRTE_F	F	CAGAATGGCTGTGACATTATCCA (+5 others)	6	55	151	qPCR	Pure cultures, groundwater	[32]
		MRTE_R	R	CTGGTGTGCCGGAGTAGTTTCC (+8 others)	9					
cmuA	Halomethanes	929f	F	AACTAGCTGCTGAGGTTGGCTAY AAYGGNGG	16	Not specified	741	Regular PCR	Pure cultures, enrichment culture	[37]
		1669r	R	CAACGTATACGGTGGAGGA GTTNGTCATNAC	16					
		cmuA802f	F	TTCAACGGCGAYATGTATCCYGG	2	55	807	Regular PCR	Pure cultures, enrichment cultures, SIP fractions, soil	[38]

Table 1
(continued)

Gene[a]	Pollutant(s)	Primer name	F/R	Primer sequence (5'-3')	Fold degeneracy	Annealing temp used	Product size (bp)	Type of PCR	Successful templates	References
		cmuA1609r	R	TCTCGATGAACTGCTCRGGCT	4					
		cmuA802f	F	TTCAACGGCGAYATGTATCCYGG	2	55	984	Regular PCR	Pure cultures, phyllosphere	[41]
		cmuA1802r	R	TTVGCRTCRAGVCCGTA	36						
		cmuA802f	F	TTCAACGGCGAYATGTATCCYGG	2	60	167	qPCR	Phyllosphere	[41]
		MF2	R	CCRCCRTRTAVCCVACYTC	144						
dcmA	DCM	Cfor	F	ATSATCYKGCRTCMCAGC	32	50	441–450	Regular PCR	Pure cultures, enrichments, sludge	[47]
		Crev	R	TMAGCMAGTAWTYCTA	16						
		Dmfor	F	AAAAAAACATCTAGAGAATGA CAACCGTGCGC	1	55	1,225	Regular PCR	Pure cultures, enrichments, sludge, bioreactors	[43, 47, 48]
		DMrev	R	AAAAAAAAAGGATCCGGT CATCGAAGGAATGC	1						
HLD	Many haloalkanes	Hld95f	F	TGCATTGCCCCGGAYYWNRTNGG	256	60.5	650	Regular PCR	Pure cultures, groundwater	[62]
		Hld320r	R	TTTCTTCAGGCTGATCTTCC TGNRNRWARTG	256						
dhlA	DCA	DHM-F	F	GGCGAGCCCACCTGGAGYTAC	2	60	950	Regular PCR	Groundwater	[64]
		DHM-R	R	GWMKYGTCRGGGAARGGCGC	64						
		DHLA-319F	F	CTTGCACTAATCGAACGGCTTG	1	55	284	Regular PCR	Pure cultures	[53]
		DHLA-603R	R	AGCTTCGGTCAGTGTGGGCG	1						
		DHLA-380F	F	GATTTTTGGGGGCTGACCTTA	1	60	168	qPCR	Membrane bioreactor, groundwater	[53]
		DHLA-548R	R	GATGGCGTAACCAGATCGTA	1						
dhaA	Chlorobutane	Not specified	F	AAAATCGCCATGGCAGAAATCGGTA	1	58	950	Regular PCR	Pure cultures	[66]
		Not specified	R	TGGACAATCGGACCATGGCGTGAACC	1						
linA	HCH	LinA forward	F	CGCCTCGAGATGAGTGATCT AGACAGACTTG	1	55	480	Regular PCR	Soil	[73]
		LinA reverse 2	R	CGCGGATCCTCACGATTTTTG CAACAGAGCC	1						

Gene	Compound	Primer	Dir.	Sequence (5′–3′)		Annealing (°C)	Product	Method	Environment	References
		Not specified	F	GCGGATCCGCATGAGTGATC TAGACAGACTT	1	Not specified	Not specified	TRFLP	Soil	[74]
		Not specified	R	GCCTCGAGTTATGCGCCGGAC GGTGCGAAATG	1	Not specified				
		Not specified	F	AGCTCAACGGATGCATGAACT	1	Not specified	Not specified	qPCR	Soil	[74]
		Not specified	R	GGCGGTGCGAAATGAATG	1	Not specified				
		linA-F11	F	TAGACAGACTTGCAAGCCGG	1	55	400/370	Regular PCR, nested PCR	Soil	[71]
		linA-F33	F	CGCGATTCAGGACCTCTACT	1	55				
		linA-F42	F	GGACCTCTACTTCTGACAAGC	1	55				
		linA-R396	R	TCTCTAAGCGCAACGCATGC	1	55				
		linA-R418	R	CCAGCGGGGTGAAATAGTTC	1	55				
		linAf	F	GCGGATCCGCATGAGTGA TCTAGACAGACTT	1	50	471	Regular PCR	Soil	[75]
		linAr1	R	GCCTCGAGTTATGCGCCGGACG GTGCGAAATG	1	50				
linB		linB-2F	F	CTGATCGCCTGTGACCTGAT	1	Not specified	390	Regular PCR	Pure cultures	[72]
		linB-3R	R	CCATCTCCGCTTCGCTTAAG	1	Not specified				
	HCH	LinB forward	F	CGCCTCGAGATGAGCCTCGGGCGCAAAG	1	55	900	Regular PCR	Soil	[73]
		LinB reverse	R	CGCGGATCCTTATGCTGGGCGCCAATCG	1	Not specified				
		Not specified	F	GCGGATCCGCATGAGCCT CGGCGCAAAGCCA	1	Not specified	Not specified	TRFLP	Soil	[74]
		Not specified	R	GCCTCGAGTTATGCTGGGGCG CAATCGCCGGAC	1	Not specified				
		Not specified	F	ACCACGGGCGAATGC	1	Not specified	Not specified	qPCR	Soil	[74]
		Not specified	R	ACCGTGATTTCGGGTCTGGTTT	1	Not specified				
dehI	Chloropropionate	dehI_for1	F	ACGYTNSGSGTGCCNTGGGT	128	70–51 (touchdown)	230/504	Regular PCR	Pure cultures, enrichments, sludge, water	[80–82, 85, 86]
		dehI_rev1	R	AWCARRTAYTTYGGATTRCCRTA	128					
		dehI_rev2	R	SGCMAKSRCNYKGWARTCACT	2,048					

(continued)

Table 1
(continued)

Gene[a]	Pollutant(s)	Primer name	F/R	Primer sequence (5'-3')	Fold degeneracy	Annealing temp used	Product size (bp)	Type of PCR	Successful templates	References
dehII	Chloroacetate, chloropropionate	dehII$_{for1}$	F	TGGCGCVCARMRDCARCTBGARTA	864	55	422	Regular PCR, nested PCR, qPCR	Pure cultures, enrichments, sludge, sediments, water	[80–82, 85–87]
		dehII$_{rev1}$	R	TCSMADSBRTTBGASGANACRAA	6,912					
		Xantho	F	TTGTCTCGATCAGGTCGC	1	62	Not specified	qPCR, nested PCR	Wastewater, enrichments	[85]
			R	ACGACTACCGCGACTTCTG	1					
		Delfia	F	TGCGAAGCGTACCTAAACCT	1	61	Not specified	qPCR, nested PCR		
			R	GGCTTGAACACTTCGACCTC	1					
		Ultra	F	TGAAATGGTCGCAATCGAA	1	62	Not specified	qPCR, nested PCR		
			R	CGCTCATTTCGAACTCAAACTC	1					
		Pseudo	F	AGCCTGTCCCTGGGGATG	1	67	Not specified	qPCR, nested PCR		
			R	GAGCAGGTCGTCAGCCA	1					
		Afipia	F	CTCAAATACGATCCCGCGGG	1	61	Not specified	qPCR, nested PCR		
			R	TACGCCGAGCTTCTGTTCGAT	1					
dhlB	Chloroacetate	dhlB 314	F	TCTGGCGCGCAGAAGCAGCTGG	1	60	330	Regular PCR	Wastewater	[90]
		dhlB 637	R	CGGGCTTGGCAATCGACGCTGATG	1					
tfdA	2,4-D, MCPA	tfdAr	F	ACGGAGTTCTGYGAYATG	4	59	370	Regular PCR, qPCR	Pure cultures	[99, 101–104]
		tfdAf	R	AACGCAGCGRTTRTCCCA	4					
		tfdA**F	F	GAGCACTACGCRCTGAAYTCCCG	4	64	220	Regular PCR or qPCR	Pure culture, soil	[105]
		tfdA**R	R	CTTCGGCCACCGGAAGGCCT	1					
		tfdAα**F	F	ACSGAGTTCKSCGACATGCG	8	65	350	Regular PCR	Pure culture	[105]
		tfdAα**R	R	GCGGTTGTCCCACATCAC	1					

Gene	Substrate	Primer		Sequence (5′–3′)		T_m		Amplicon	Method	Sample	References
		Not specified	F	GAGCACTACGCRCTGAAYTCCCG	4	64		Not specified	qPCR, RTqCR	Soil	[106, 107]
			R	GTCGCGTGCTCGAGAAG	1						
		TfdAα52F	F	GGCGTCGATCTGCGCAAGCC	1	70		360	Regular PCR, qPCR	Soil	[109]
		TfdAα408R	R	GTTGACGACGCGCGCGACA	1						
		TfdAα421F	F	ACSGAGTTCGSIGAYATSC	64	63.6	64	360	Regular PCR	Soil	[109]
		TfdAα779R	R	CAGCGGTTGTCCCACATCAC	1						
		TfdA421dF	F	ACSGARTTCKSIGACATGC	64	53	64	360	Regular PCR, qPCR	Soil	[109]
		TfdA778vkR	R	AGCGGTTGTCCCACATCAC	1						
atzA	Atrazine	Af	F	ACGGGCGTCAATTCTATGAC	1	60	1	200	qPCR	Pure cultures	[121–123]
		Ar	R	CACCCACCTCACCATAGACC	1						
		Not specified	F	CCATGTGAACCAGATCCT	1	52	1	500	Regular PCR	Pure cultures, enrichments	[111–113, 118]
		Not specified	R	TGAAGCGTCCACATTACC							
		atzA F	F	AATTCTATGACTGGCTGTTC	1	60	1	92	qPCR	Water, sediment	[120]
		atzA R	R	CGCACAATACAACCTCAC	1						
		AtrzA1:1	F	CCATGTGAACCAGATCCT	1	55	1	528/444	Nested PCR	Soil	[119]
		Inner F	F	GCACGGACGTCAATTCTA	1						
		Inner R	R	CGCATTCCTTCAACTGTC	1						
		AtrzA1:2	R	TGAAGCGTCCACATTACC	1						

[a]Encoded functions of the genes are as follows: *SDIMO* soluble di-iron monooxygenase alpha subunit, *etmC* ethene monooxygenase, *etmE* epoxyalkane-coenzyme M transferase, *cmuA* halomethane dehalogenase, *dcmA* dichloromethane dehalogenase, *HLD* hydrolytic dehalogenase, *dhlA* 1,2-dichloroethane dehalogenase, *dhaA* haloalkane dehalogenase, *linA* lindane (hexachlorocyclochexane) dehalogenase, *linB* tetrachloro-cyclohexadiene dehalogenase, *dehI* type 1 haloacid dehalogenase, *dehII* type 2 haloacid dehalogenase, *dhlB* chloroacetate dehalogenase, *dehI* type 1 haloacid dehalogenase, *tfdA* alpha-ketoglutarate-dependent 2,4-dichlorophenoxyacetate dioxygenase, *atzA* atrazine dechlorinase

2   Case Study: Primer Design for Soluble Di-iron Monooxygenase (SDIMO) Genes

Monooxygenase (MO) enzymes catalyse the first step in aerobic attack on many hydrocarbons and chlorinated hydrocarbons. These enzymes are also of interest to biocatalysis [8, 9]. Many distinct MO types exist in bacteria, perhaps the most diverse and most widespread is the soluble di-iron monooxygenase (SDIMO) family [10], which includes enzymes of critical significance to biogeochemistry [11] and enzymes active on many important chlorinated pollutants [5, 12, 13]. The SDIMOs are found in both Proteobacteria and Actinobacteria. Six SDIMO groups can be defined based on gene sequences [14], and these loosely correlate to types of growth substrates (Fig. 1).

The author's laboratory designed degenerate primers for SDIMOs in a project aimed at retrieving novel MO genes for bioremediation and biocatalysis [14, 15]. The experimental strategy was to use PCR to amplify MO genes from soil, enrichments, and isolates. The test substrate for these experiments was ethene (ethylene, C_2H_4), which is oxidised by the SDIMO enzyme EtnABCD. This enzyme and related alkene MOs have very high enantioselectivity [16] and are thus of special interest for biocatalysis. Primers were needed that could retrieve sequences related to *etnC*, without
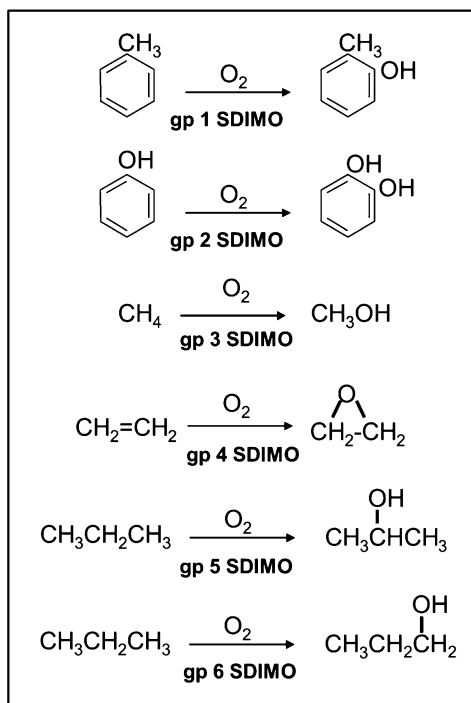
Fig. 1 *Relationships between SDIMO groups and substrates*. Reactions are representative only, not a complete list. Compiled based on [10, 11]

retrieving non-specific sequences, even with very complex templates.

The first step was to choose which SDIMO subunit(s) to target for PCR. These are multicomponent enzymes (4–6 subunits), of which the large (alpha) hydroxylase subunit is the most conserved. There are arguments for PCRs spanning multiple MO genes; these would retrieve more sequence data, and this approach offers more priming sites, but the problem is that this depends on the gene order being conserved, which is not in the SDIMO family as a whole. Thus, only the alpha subunit was targeted.

The next step was to make a sequence alignment of SDIMO alpha subunits. Alignments were made using protein rather than DNA; this is a better approach for identifying homologous regions, especially if sequence homology is low. Using protein sequences for primer design gives broader-spectrum primers which retrieve more sequence diversity, since this approach makes no assumptions about codon usage. The drawback is that primers will be degenerated, which reduces their efficiency.

The SDIMO alignment (Fig. 2) indicates that there are only two amino acid (a.a.) motifs that are absolutely conserved (2 x DEXRH); these are the iron-binding sites, and they are approx. 100 a.a. apart. A very broad range PCR that targets these two sites is possible in theory, but there were two problems with this approach. Firstly, the use of two similar a.a. motifs as both forward and reverse priming sites leads to problems with heterodimer formation, and secondly, this approach only retrieves a small portion of the gene, which was not ideal for the purpose of biocatalyst prospecting.

If the focus is restricted to just groups 3, 4, and 5 SDIMOs, two further conserved motifs become available; these are WFEX(N/H/K)YPGW and WT(I/L)DD(I/L/V)R (Fig. 2). These motifs are attractive for the design of reverse primers, since the 3′ end of each primer is at a tryptophan (W), which is one of the only two nondegenerate a.a.s (W = TGG, M = ATG). This means that the critical 3′ end of the primer is nondegenerate, increasing the specificity of the PCR. With these two further motifs available as reverse priming sites, both DEXRH motifs can be used as forward priming sites, giving five possible PCR strategies, i.e. two regular PCRs, one nested PCR and two semi-nested PCRs.

Different a.a.s are better or worse for degenerate primer design, depending on how degenerate the code is. M and W are the best (unique codons); then C, N, D, E, Q, H, K, F, and Y are good (two codons); then T, P, A, G, and V are average (four codons); and S, R, and L are bad (six codons). The overall degeneracy of a particular a.a. motif can make or break the primer design process, e.g. SSSSSSS has 279936-fold degeneracy (6^6), which is too high to be an effective PCR primer. Inspection of the conserved regions in the SDIMO alignment (Fig. 3) shows some problems here, especially the X residue (any a.a.), which translates to NNN, and a degeneracy of 64.
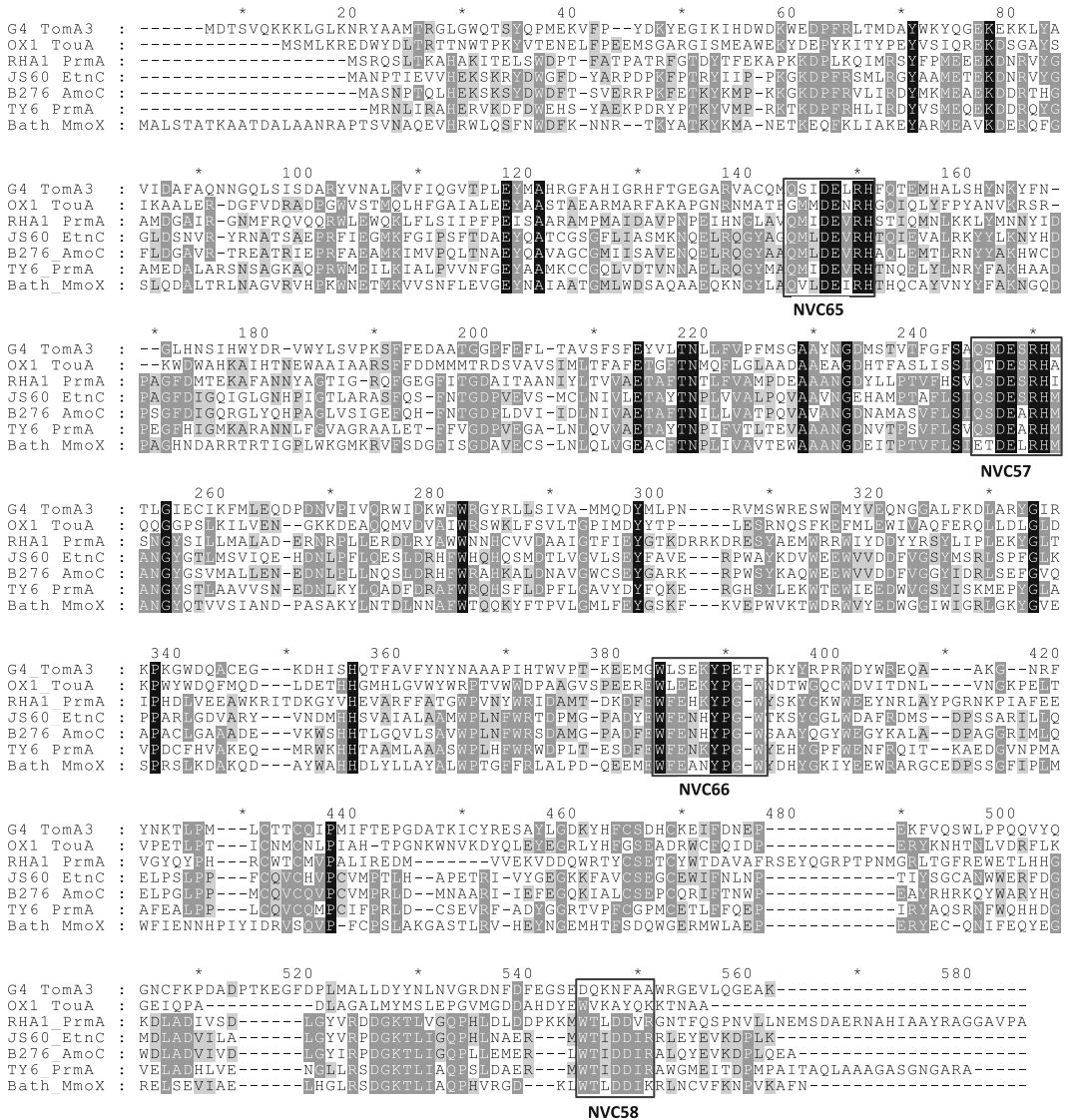
Fig. 2 *Alignment of SDIMO alpha subunits.* Conserved regions are shaded *black* (100% conserved), *dark grey* (60% conserved), or *light grey* (40% conserved). Amino acid motifs targeted by PCR primers are *boxed* and labelled with the primer name. Further information about sequences and strains can be obtained from the NCBI protein database entries, as follows: G4_TomA3, AAK07411 (group 2); OX1_TouA, CAA06654 (group 1); RHA1_PrmA, ABG92277 (group 5); JS60_EtnC, AAO48576 (group 4); B276_AmoC, BAA07114 (group 4); TY6_PrmA, BAF34294 (group 6); Bath_MmoX, P22868 (group 3). Note that in this alignment and later alignments, the sequences are named using the format XXX_AbcD, where XXX is the strain name, and AbcD is the enzyme name

Excessive degeneracy in this case was handled by 'cheating' a little (Fig. 3). For example, the X residue was replaced by the specific a.a. found in EtnC. The justification for this was that for this study, it was of primary importance to ensure that ethene MO genes were retrieved. Although some positions have EtnC-bias, the primers overall are targeted at SDIMO conserved regions and

```
Q(V/M/Q)(L/V/I)DE(I/V/F)RH
           ⇩  Simplify to EtnC sequence
NVC65      QMLDEVRH
           ⇩  Translate to DNA
           CARATGYTNGAYGARGTNCGNCA
```

```
           QSDE(S/A)RH(M/I)
NVC57         ⇩  Translate to DNA,
              high-GC bias  at (S/A)
           CAGTCNGAYGARKCSCGNCAYAT
```

```
           WFEX(N/H/K)YPGW
           ⇩  Simplify to EtnC sequence
NVC66      WFENKYPGW
           ⇩  Translate to DNA
           TGGTTYGARAAYAARTAYCCNGGNTGG
           ⇩  Reverse complement
           CCANCCNGGRTAYTTRTTYTCRAACCA
```

```
           WT(I/L)DD(I/L/V)R
              ⇩  Simplify to EtnC sequence
NVC58      WTIDDIR
              ⇩  Translate to DNA
           TGGACNATHGAYGAYATHCG
              ⇩  Reverse complement
           CGDATRTCRTCDATNGTCCA
```

Fig. 3 Design strategy for degenerate SDIMO alpha subunit primers

were hypothesised to retrieve more than just *etnC* (this turned out to be true).

Another 'cheat' was used to handle the S/A position (WSN + GCN → DSN, 24-fold degenerate). In this case, changes were made based on inspection of a DNA alignment and by assuming a high GC codon usage at the third position (group 4 SDIMOs are nearly exclusive to Actinobacteria). These tweaks give KCS (four-fold deg.) and keep the overall primer degeneracy manageable (max. 1024-fold). For a systematic approach to handling degeneracy in primer design, the reader is referred to the CODEHOP programme [17], which yields oligos with a degenerate 3′ end but conserved 5′ end.

The new SDIMO primers were first tested on ethene enrichment cultures. The 2-primer PCRs (NVC65-NVC66 or NVC57-NVC58) gave products of the expected size from most samples, and 17/23 clones sequenced were *etnC*-like SDIMO genes [15]. The two-primer PCRs failed to amplify anything from unenriched environmental samples, but the 4-primer nested PCR did work with these, giving strong products of the expected size in 12/13 cases. Sequencing of 45 nested PCR clones revealed that all were SDIMOs. Very diverse genes were recovered, including SDIMO groups 3, 4, 5, and 6 [15].

It was unclear why the nested PCR was essential for detecting SDIMO genes in unenriched samples in this study, since other

catabolic genes (many examples below) do not require this level of amplification to be detectable. This finding could be due to the low lysis efficiency of SDIMO-containing bacteria in environmental samples (perhaps mycobacteria?), the low efficiency of the very degenerate primers used, the low abundance of the SDIMO-containing bacteria, or a combination of these factors.

3 Primers for *etnC* and *etnE* in Vinyl Chloride (VC)-Degrading Bacteria

VC is a pollutant found in groundwater and waste gases and is of particular concern since it is a known human carcinogen [18]. VC is used for manufacture of PVC plastic, but the major source of VC contamination is biogenic production from higher-chlorinated ethenes (tetra- and trichloroethene (PCE, TCE)), which are the primary pollutants. The PCE and TCE can be reductively dechlorinated by anaerobes, ideally yielding ethene, but this process may 'stall', yielding *cis*-dichloroethene (cDCE) or VC as persistent end products [19].

Aerobic bacteria can cometabolise all of the chlorinated ethenes [20] and can grow on cDCE and VC [5]. The activity of such bacteria is likely to be significant in the field [21, 22]. Many bacteria that can grow on VC have been isolated, including *Mycobacterium*, *Nocardioides*, *Pseudomonas*, and *Ochrobactrum* [5, 23]. The VC degradation pathway is only partially characterised [24–26]; the SDIMO enzyme EtnABCD attacks VC, making VC epoxide; then the EtnE enzyme (epoxyalkane-coenzyme M transferase) acts on the epoxide.

The *etnC* and *etnE* genes can be used for detection of VC degraders, but a major complicating factor is that these genes are identical or near identical to genes in ethene-oxidising bacteria, which are ubiquitous. This issue also impacts on interpretation of enrichment experiments, since VC-assimilating bacteria can evolve spontaneously from ethene assimilators [27]. Multiple point mutations can give rise to the VC-assimilating phenotype [28]; these changes are subtle (1 bp) and occur at variable locations, making a diagnostic PCR impossible. For the moment, it must be accepted that primers for VC-assimilating bacteria will also amplify ethene-assimilating bacteria. This is not entirely inappropriate, since ethene oxidisers can cometabolise VC, and they represent a pool of potential VC assimilators.

The SDIMO primers discussed above are not appropriate for detection of VC-degrading bacteria, although they will detect *etnC* genes. More specific *etnC* primers were designed (NVC105 – NVC106) to allow tracking of this gene in enrichment cultures and isolates [15] and for screening fosmid libraries [29]. The isolates examined in that study contained a diverse mix of SDIMO genes, so it was important to ensure specificity of the *etnC* primers. These were designed based on alignments such as Fig. 4 to target regions

```
              *          20          *          40          *          60          *
JS60-EtnC  : MANPTIEVVHEKSKRYDWGFDYARPDPKFPTRYIIPPKGKDPFRSMLRGYAAMETEKDNRVYGGLDSNVRYRNAT
NBB4-EtnC  : MANPTIEVVHEKSKRYDWGFDYARPDPKFPTRYIIPPKGKDPFRSMLRGYAAMETEKDNRVYGGLDSNVRYRNAT
JS623-EtnC : MASPKLEVVHEKSKRYDWGFDYAKPDPKFPTRYIIPPKGKDPFRSMLRGYAAMESEKDNRVYGGLDSNVRYRNAT
NBB3-EtnC  : MASPKLEVVHEKSKRYDWGFDYARPDPKFPTRYIIPPKGKDPFRSMLRGYAAMESEKDNRVYGGLDSNVRYRNAT
JS614-EtnC : MVAPKLETVHEKSKRYDWGFDYAKPDPKFPSRYIIPPKGKDPFRVMMRGYAAMENEKDNRVYGALDSNVRYRNAN
B276-AmoC  : MAS-NPTQLHEKSKYDWDFTSVERRPKFETKYKMPKKGKDPFRVLIRDYMKMEAEKDDRTHGFLDGAVRTREAT
M156-PmoC  : MAS-DATQLHEKTKAYDWDFTSVEQKPKFETKYKLPKKGKDPFRLLVRDYMKMEAEKDDRTYGFLDGAIRTREAT

              80          *          100         *          120         *          140         *
JS60-EtnC  : SAEPRFIEGMKFGIPSFTDAEYQATCGSGFLIASMKNQELRQGYAGQMLDEVRHTQIEVALRKYYLKNYHDPAGF
NBB4-EtnC  : SAEPRFIEGMKFGIPSFTDAEYQAVCGSGFLIASMKNQELRQGYAGQMLDEVRHTQIEVALRKYYLKNYHDPAGF
JS623-EtnC : SAEPRFIEGMKFGIPSFTDAEYQAVCGAGFLIASMKNQELRQGYAGQMLDEVRHTQIEVALRKYYLKNYHDPAGF
NBB3-EtnC  : SAEPRFIEGMKFGIPSFTDAEYQAVCGSGFLIASMKNQELRQGYAGQMLDEVRHTQIEVALRKYYLKNYHDPAGF
JS614-EtnC : LAEPRFMEAMKFAVPALTDAEYQAVCGAGFLISSVKNQELRQGYAGQMLDEVRHAQLEINLRKYYLKNYHDPAGF
B276-AmoC  : RIEPRFAEAMKIMVPQLTNAEYQAVAGCCMIISAVENQELRQGYAAQMLDEVRHAQLEMTLRNYYAKHWCDPSGF
M156-PmoC  : KMEPRFVELMKMILPILTNAEYQAVAGCCMIISAINNQEMRQGYAAQMLDEVRHAQLEMALRNFYVKHYHDPAGF

              160         *          180         *          200         *          220
JS60-EtnC  : DIGQIC-LGNHPIGTLARASFQSFNTGDPVEVSMCLNIVLETAYTNPLVVALPQVAAVNGEHAMPTAFLSIQSDE
NBB4-EtnC  : DIGQIC-LGNHPIGTLARASFQSFNTGDPVEVSMCLNIVLETAYTNPLVVALPQVAAVNGEHAMPTAFLSIQSDE
JS623-EtnC : DIGQIC-LGNHPVGTLARASFQPFNTGDPVEVSMCLNIVLETAYTNPLVVALPQVAAVNGEHAMPTAFLSIQSDE
NBB3-EtnC  : DIGQIC-LGNHPIGTLARASFQSFNTGDPVEVSMCLNIVLETAYTNPLVVALPQVAAVNGEHAMPTAFLSIQSDE
JS614-EtnC : DIGQIALG-NHPIGTLARASFQPFNTGDPIEVAMCLGVVLETAYTNPLVVALPQVAMVNGDHAMPTTFLSIQSDE
B276-AmoC  : DIGQRCLY-QHPAGLVSIGEFQHFNTGDPLDVIIDLNIVAETAFTNILLVATPQVAVANGDNAMASVFLSIQSDE
M156-PmoC  : DLGQVCLY-QHPAGLLSIGEFQHFNTGDPLDCIIDLNIVVETAFTNILLVAMPQVAVRNGDNALATTMLSIQSDE

              *          240         *          260         *          280         *          300
JS60-EtnC  : SRHMANGYGTLMSVIQEHDNLPFLQESLDRHFWHQHQSMDTLVGVLSEYFAVERPWAYKDVWEEWVVDDFVGSYM
NBB4-EtnC  : SRHMANGYGTLMSVIQEHDNLPFLQESLDRHFWHQHQSMDTLVGVLSEYFAVERPWAYKDVWEEWVVDDFVGSYM
JS623-EtnC : SRHMANGYGTLMSVIQEHDNLPFLQESLDRHFWHQHQSMDTLVGVLSEYFAVERPWAYKDVWEEWVVDDFVGSYM
NBB3-EtnC  : SRHMANGYGTLMSVIQEHDNLPFLQESLDRHFWHQHQSMDTLVGVLSEYFAVERPWAYKDVWEEWVVDDFVGSYM
JS614-EtnC : SRHMANGYATLMACLESTENVPFLQESLERHFWHQHMSMDTLVGVVSEYYAVNRPWAYKDVWEEWVVDDFVGSYM
B276-AmoC  : ARHMANGYCSVMALLENEDNLPLLNQSLDRHFWRAHKALDNAVGWCSEYGARKRPWSYKAQWEEWVVDDFVGGYI
M156-PmoC  : SRHMANGYCAIMSVLGEPDNVPMINESLERHFWHAHKSLDAAIGWGSEYGAHDRAWSYKDQWDEWVVDDFIGGFV
                                       └───────┘        └─────────┘                  └───────
                                        NVC105            RTC_F                        RTC_R

              *          320         *          340         *          360         *
JS60-EtnC  : SRLSPFGLKPPARLGDVARYVNDMHHSVAIALAAMWPLNFWRTDPMGPADYEWFENHYPGWTKSYGGLWDAFRDM
NBB4-EtnC  : SRLSPFGLKPPARLGDVARYVNDMHHSVAIALAAMWPLNFWRTDPMGPADYEWFENHYPGWTKSYGGLWDAFRDM
JS623-EtnC : SRLSPFGLKPPARLGDVARFVDEMHHSVAIALAAMWPLNFWRTDPMGPADYEWFENHYPGWTKSYGGLWDAFRDM
NBB3-EtnC  : SRLSPFGLKPPARLGDVARFVNDMHHSVAIALAAMWPLNFWRTDPMGPADYEWFENHYPGWTKSYGGLWDAFRDM
JS614-EtnC : NRLAPYGLKPPERLPDVARFVEDMHHSVAIALAAIWPLNFWRIDPMGPADYEWFENHYPGWTARYGGLWDAYREM
B276-AmoC  : DRLSEFGVQAPACLGAAADEVKWSHHTLGQVLSAVWPLNFWRSDAMGPADFEWFENHYPGWSAAYQGYWEGYKAL
M156-PmoC  : DRLSEFGLTPPSRLAAAAEEVTWTHHTYGQVLSAVWPLNFWRSDAMGPADFEWFENNYPGWSAAYQGYWEAYKEL
                                                                                └──────────┘
                                                                                   NVC106

              380         *          400         *          420         *          440         *
JS60-EtnC  : SDPSSARILLQELPSLPPFCQVCHVPCVMPTLHAPETRIVYGEGKKFAVCSEGCEWIFNLNPTIYSGCANWWERF
NBB4-EtnC  : SDPSSARILLQELPSLPPFCQVCHVPCVMPTLHAPETRIVYGEGKKFAVCSEGCEWIFNLNPTIYSGCANWWERF
JS623-EtnC : SDPSSARILLQELPALPAFCQVCHVPCVVPSIHAPETRIVYGEGKKFAVCSEGCEWIFNLNPTIYSGCANWWERF
NBB3-EtnC  : SDPSSARILLQELPALPAFCQVCHVPCVVPSIHAPETRIVYGEGKKFAVCSEGCEWIFNLNPTIYSGCANWWERF
JS614-EtnC : SDPSSGRLLMQELPALPPFCQVCHVPCVMPRIDAPETRIFDHEGKRYAVCSEGCDWIFKLNPTIYTGCANWWERF
B276-AmoC  : ADPAGGRIMLQELPGLPPMCQVCQVPCVMPRLDMNAARIIEFEGQKIALCSEPCQRIFTNWPEAYRHRKQYWARY
M156-PmoC  : ADPAGGHIMLQELPGLPPMCQVCQTPCVIPRLDTNTVRIVELDGQNYALCSEGCEWIFGKWPDAYKDRKQLWERY

              460         *          480         *          500
JS60-EtnC  : DGMDLADVILALGYVRPDGKTLIGQPHLNAERMWTIDDIRRLEYEVKDPLK---
NBB4-EtnC  : DGMDLADVILALGYVRPDGKTLIGQPHLNADRMWTIDDIRRLEYEVKDPLNSTW
JS623-EtnC : DGMDLADVILALGYVRPDGKTLIGQPHLNAERMWTIDDIRRLHYEVKDPLK---
NBB3-EtnC  : DGMDLADVILALGYVRPDGKTLIGQPHLNAERMWTIDDIRRLEQYEVKDPLK---
JS614-EtnC : DGMDLADVILALGYVRPDGKTLIMGQPHLNADRMWTIDDIRALKYEIKDPLRV--
B276-AmoC  : HGWDLADVIVDLGYIRPDGKTLIGQPLIEMERLWTIDDIRALQYEVKDPLQEA-
M156-PmoC  : DGWDLADVVLDLGYVRPDGHTLIGQPLLKMDRFWTIDDIRRLGYEVKNPMLTI-
```

Fig. 4 *Alignment of alpha subunits of ethene and propene monooxygenases.* Conserved regions are shaded *black* (100% conserved), *dark grey* (80% conserved), or *light grey* (60% conserved). Amino acid motifs targeted by PCR primers are *boxed* and labelled with the primer name. The propene MO of *Xanthobacter* Py2 (group 1 SDIMO) is not shown, as it is quite divergent in sequence from the Actinobacteria alkene MOs (group 4 SDIMOs). Further information about sequences and strains can be obtained from the NCBI protein database entries, as follows: JS60-EtnC, AAO48576; NBB4-EtnC, ACZ56346; JS623-EtnC, ACM61846; NBB3-EtnC, AEV73508; JS614-EtnC, AAV52084; B276-AmoC, BAA07114; M156-PmoC. AAS19484

unique to *etnC* that are not conserved with the nearest distinct SDIMO, i.e. the propene MO genes *amoC/amoC.*

Primers have also been designed for the *etnE* gene (CoM-F1L and CoM-R2E) to retrieve this gene in isolates that grow on ethene and VC. These primers have low degeneracy (eight- and onefold), but were effective in retrieving a large fragment of etnE (891 bp) from all ten of the VC/ethene-oxidising bacteria tested [25]. Some non-specific products were seen from some strains. These primers also retrieved the coenzyme M transferase gene from the propene-oxidising *Rhodococcus* (*Gordonia*) B-276. These primers have not yet been tested with metagenomic DNA.

Effective qPCR primers for *etnC* and *etnE* (RTC_F, RTC_R, RTE_F, RTE_R) have now been developed [30]; this study is recommended reading for workers new to qPCR, since the primer design and testing process and the gene copy calculations are clearly described. The qPCR enabled detection of both *etnC* and *etnE* in groundwater at 10^3–10^5 genes/L. The ratio of *etnC:etnE* genes unexpectedly varied between samples (from 1.1 to 17.8) – this may indicate that *etnE* can be found associated with genes other than *etnC* [31], or it could be an artefact arising from different relative priming efficiencies with different templates.

The *etnC* and *etnE* primers were later modified to minimise bias towards certain gene types [32]; these second-generation primers (MRTC_F, MRTC_R, MRTE_F, MRTE_R) were designed using a different strategy, i.e. individual representation of each sequence variant. The modified primers gave increased detection levels of *etnC* and *etnE* in groundwater compared to the first-generation primers and also reduced the *etnC:etnE* gene ratio closer to the theoretically expected 1:1 level.

4   Primers for *cmuA* in Halomethane-Degrading Bacteria

Chloromethane (methyl chloride) and bromomethane (methyl bromide) have both biotic and anthropogenic sources and have a major impact on atmospheric chemistry, since they act as ozone-depleting agents [7]. Methyl bromide is used as an agricultural biocide for pre-planting soil fumigation [33]. Many Alphaproteobacteria can degrade methyl halides, including *Aminobacter*, *Hyphomicrobium*, *Leisingera*, *Methylobacterium*, and *Roseovarius* [7, 34].

The halomethane dehalogenase which initiates metabolism of chloro- and bromomethane has a corrinoid-dependent (CmuA) and a tetrahydrofolate-dependent (CmuB) component [34–36]. CmuA has been more widely used as a molecular marker (Fig. 5). McAnulla et al. [37] were the first to design degenerate primers for *cmuA* (929f -1669r) – these gave sequences from all nine pure cultures tested and from an enrichment. Miller et al. designed new primers
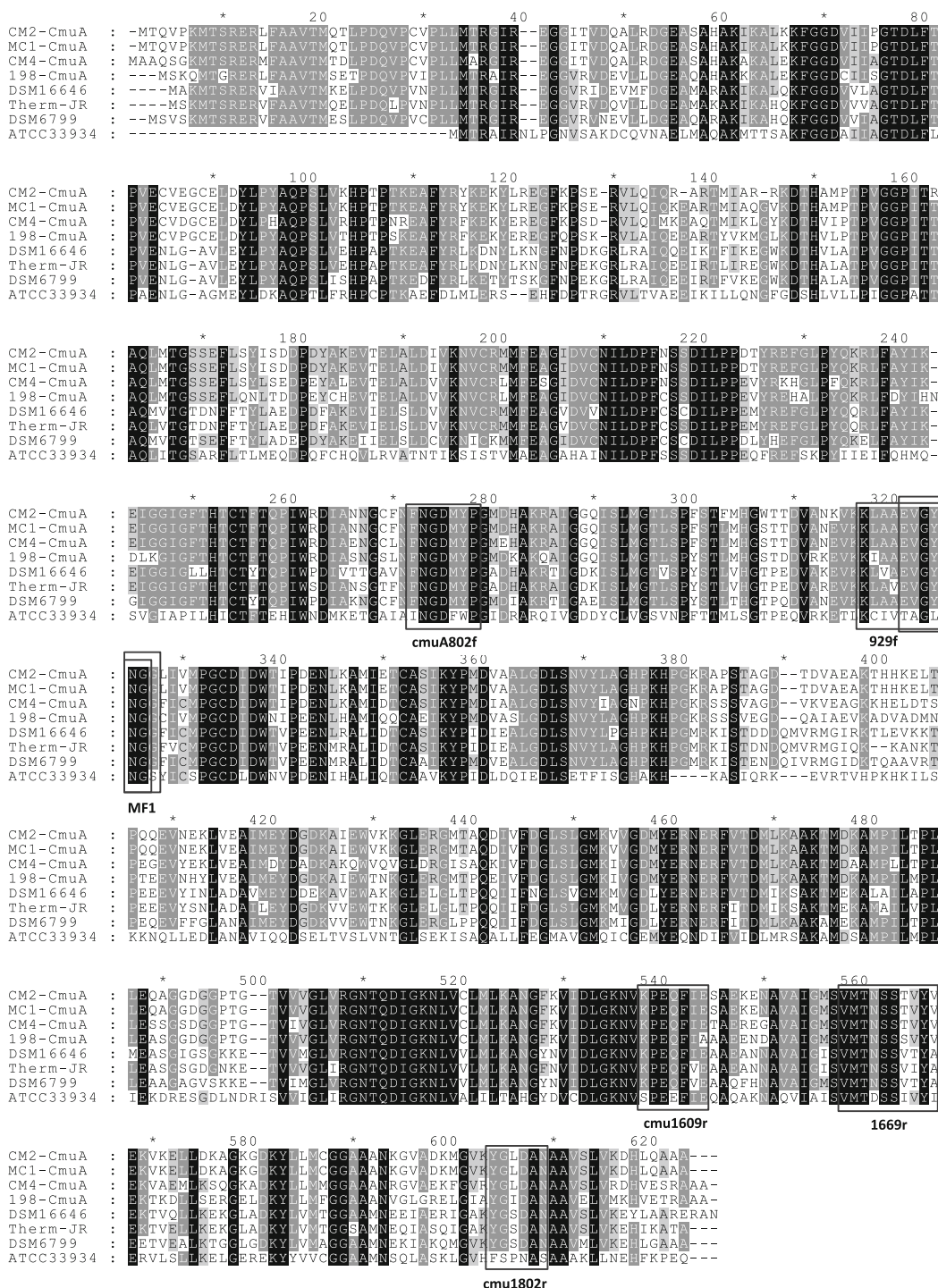
Fig. 5 *Alignment of representative CmuA enzymes and genomic homologues.* Conserved regions are shaded *black* (100%), *dark grey* (80%), or *light grey* (60%). Amino acid motifs targeted by PCR primers are *boxed* and labelled with the primer name. Further information about sequences and strains can be obtained from the NCBI protein database entries, as follows: CM2-CmuA, AAK01347; MC1-CmuA, CCB65493; CM4-CmuA, CAB39403; 198-CmuA, CAH18515; DSM16646, ADL08278; Therm-JR, ADG81034; DSM6799, AFM28033; ATCC33934, EX93664

(cmuA802f – cmuA1609r) [38] – these have subsequently been widely used for detection of halomethane degraders, e.g. with heavy DNA fractions in C13-SIP studies [38, 39], in soil samples and isolates [39], and in marine enrichments and isolates [34, 40].

Two new reverse primers (cmuA1802r and MF2) were developed for a study of chloromethane degraders in the phyllosphere [41]. This study developed a qPCR for *cmuA*, which was found in all *Arabidopsis* leaf samples examined, at eight copies per ng DNA, which was estimated as 0.1% of the total bacteria based on 16S rDNA qPCR. One possible problem with this study was that the qPCR standard curves were done with genomic DNA of *M. extorquens* CM4; is this accurate for plasmid-borne genes such as *cmuA* (see GenBank NC_011758)? Are the plasmids lost from the culture at some rate? Is the plasmid copy number constant under all growth conditions?

While all the experimentally characterised CmuA enzymes are from Alphaproteobacteria, fairly close homologues (63–69% inferred a.a. identity) are predicted from the genomes of several Deltaproteobacteria and Firmicutes, including *Desulfomonile tiedjei*, *Desulfurispora thermophile*, *Thermincola potens*, *and Thermosediminibacter oceani*, and a more distant homologue is found in the Gammaproteobacterium *Vibrio orientalis* (43% identity) (Fig. 5). It is not known whether these *cmuA* homologues encode functional dehalogenating enzymes – this issue needs to be resolved to clarify whether new *cmuA* primers are needed.

5   Primers for *dcmA* in Dichloromethane (DCM)-Degrading Bacteria

DCM is a widely used solvent and a problematic pollutant in groundwater [42] and waste gases [43]. Several types of methylotrophic Alphaproteobacteria (*Methylobacterium*, *Methylophila*, *Methylorhabdus*, *Ancylobacter*) are capable of aerobic growth on DCM as a carbon and energy source [44]. The aerobic DCM biodegradation pathway is unusual in that only a single enzyme is required to convert DCM into a central metabolite (formaldehyde) [45]. This key enzyme DcmA is a glutathione-*S*-transferase. Although there are three possible initial aerobic attack mechanisms for DCM (glutathione-*S*-transferase (GST), monooxygenase, and hydrolase [46]), the GST-mediated pathway is the only one seen in degraders isolated on DCM as carbon source.

There are 11 DcmA proteins or predicted proteins currently in GenBank, which show >98% a.a. identity to each other, with one exception, which is the DcmA of *Methylophilus leisingeri* DM11, at 58% a.a. identity to the others. The very high sequence conservation of *dcmA* genes means that nondegenerate primers can be used to detect *dcmA*-containing bacteria in different contexts, which makes PCR easier. To date, there are no reports of a qPCR assay

for dcmA, although this should be straightforward to develop given the high sequence conservation.

Two different primer sets have been described for amplification of *dcmA* genes; these are Cfor/Crev [47] and DMfor/DMrev [43, 47, 48] – these primer sets have been successfully used to retrieve *dcmA* from pure cultures, enrichments, activated sludge, and bioreactors. Note that while the degenerate Cfor/Crev pair allows amplification of both common (DM4-like) and divergent (DM11-like) DCM dehalogenases, the nondegenerate DMfor/DMrev primers amplify only DM4-like sequences.

6   Primers for *dhlA* in 1,2-Dichloroethane (DCA)-Degrading Bacteria

1,2-Dichloroethane (DCA) is used industrially for synthesis of VC and thus PVC plastic, and DCA is a common groundwater pollutant [49]. Many aerobic bacteria can grow on DCA as a carbon and energy source; the majority of these are Alphaproteobacteria (e.g. *Xanthobacter*, *Ancylobacter*, *Starkeya*) [50–54], although other types of DCA degraders have also been reported (e.g. *Pseudomonas*, *Polaromonas*, *Azoarcus*, *Klebsiella*, *Bacillus*) [55–59]. The majority of aerobic DCA degraders use a hydrolytic pathway initiated by DhlA dehalogenase [50–53], but in two cases, a monooxygenase is used [55, 56], and in two cases, the mechanism is unknown [57, 59]. Here, the focus will be only on the hydrolytic DhlA-mediated pathway.

The DhlA enzyme is a hydrolytic dehalogenase (HLD), also known as a haloalkane halidohydrolase [60]. These enzymes are part of an exceedingly diverse superfamily of alpha/beta-hydrolase fold proteins [61]; these include also haloacid dehalogenases (see below), epoxide hydrolases, lipases, proteases, peroxidases, and esterases. Due to genomic and metagenomic sequencing, the haloalkane dehalogenase family is constantly growing; a conservative estimate based on BLAST indicates that there are currently at least 150 HLD homologues in cultivated bacteria, most of which are experimentally uncharacterised. It must be noted that while some other HLDs can attack DCA, only the *dhlA* gene to date has been linked to growth on DCA.

There is very high sequence diversity within the HLD family (Fig. 6), but it is possible to design primers to capture at least some of this diversity in metagenomic DNA. Kotik and Famerova [62] used the CODEHOP approach [17] to make primers that could retrieve a broad range of HLDs. While in silico work suggested several feasible primer sets, only one was proven to work in practice (Hld95f – Hld320r). The PCR had good specificity, as 35/36 sequenced products from groundwater DNA template were HLDs; these were diverse and different to homologues in cultured bacteria.

DhlA sequences from ten different bacterial isolates are available [51–54]. Despite the fact that these belong to different genera, and
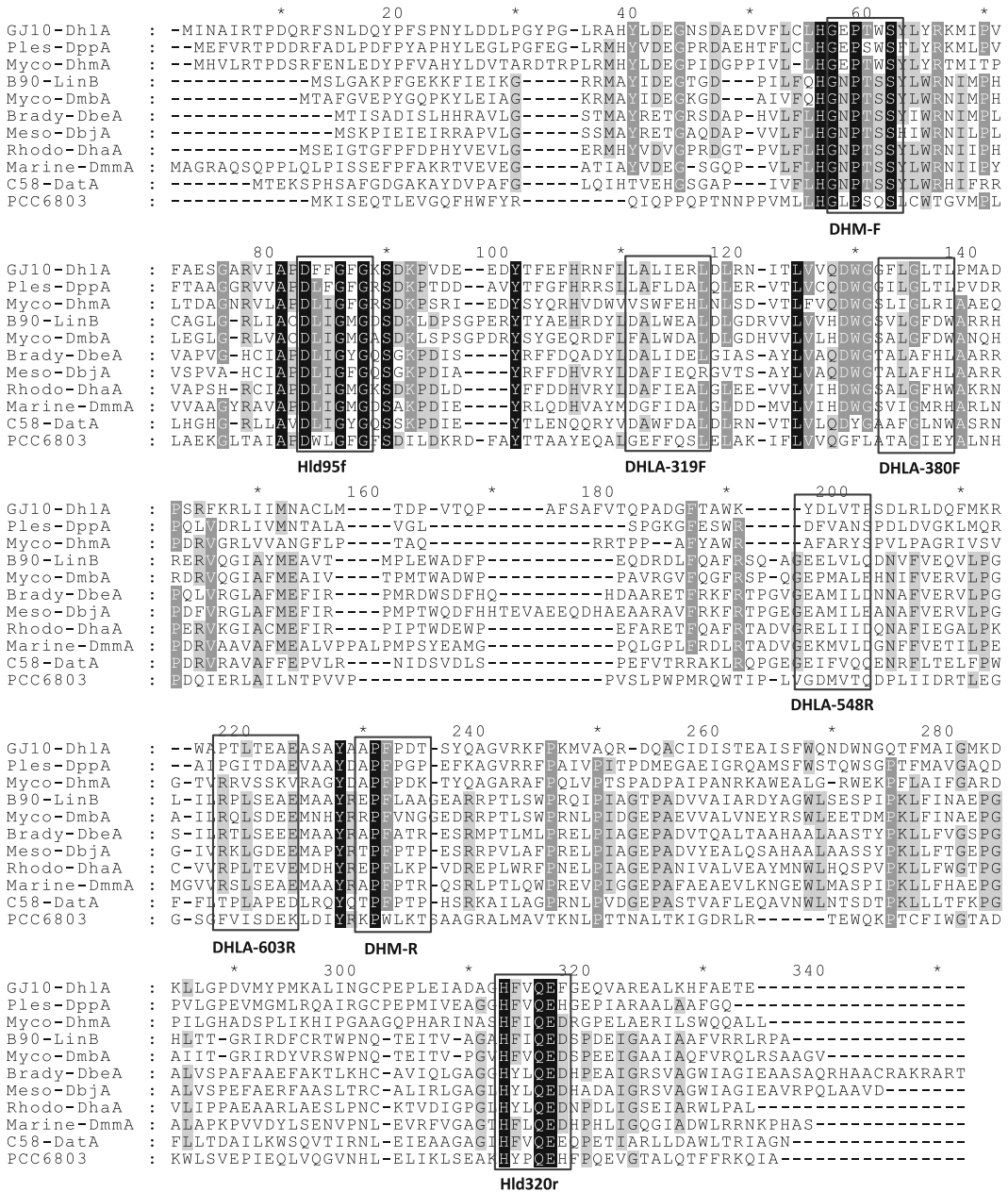
Fig. 6 *Alignment of representative hydrolytic haloalkane dehalogenase (HLD) proteins.* Conserved regions are shaded *black* (100%), *dark grey* (80%), or *light grey* (60%). Amino acid motifs targeted by PCR primers are *boxed* and labelled with the primer name. Further information about sequences and strains can be obtained from the NCBI protein database entries, as follows: GJ10-DhlA, AAA88691; Ples-DppA, 2XT0_A; Myco-DhmA, CAC41377; B90-LinB, AAN64241; Myco-DmbA, CAH04659; Brady-DbeA, BAJ23986; Meso-DbjA, 3A2M_A; Rhodo-DhaA, AAC15838; Marine-DmmA, 3U1T_A; C58-DatA, BAJ23993; PCC6803, BAA17121

were isolated in distant locations over a 30-year period (1983–2013), the *dhlA* gene is 100% identical in all cases. This pattern is consistent with horizontal gene transfer [63]. The practical significance of the 100% identity of *dhlA* genes is that there is no need for degenerate primers, which simplifies the task of primer design. The same logic applies also to the *linA*, *linB*, and *atzA* genes (see below). However, it is possible to incorporate degeneracies to capture related HLDs; this was done in a study of aquifer sediments [64], which used degenerate primers based on *dhlA* and *dhmA*.

7 Case Study: Development of *dhlA* qPCR for Pilot-Scale Membrane Bioreactor

The Coleman lab has been working on DCA bioremediation at the Botany Industrial Park site in Sydney, Australia. Currently, a groundwater treatment plant (GTP) at the site removes DCA by air stripping and thermal oxidation. This method is effective but expensive, and ideally, a lower-cost, lower-energy method such as bioremediation could be used [65]. A membrane bioreactor (MBR) (Fig. 7) was installed to test the feasibility of DCA bioremediation, and the site managers wanted to be able to detect and enumerate DCA-degrading bacteria in the MBR.

This challenge was approached using a mix of culture-based methods (enrichments, isolations) and DNA-based methods (PCR, qPCR, pyrosequencing) [53]. Enrichments confirmed that aerobic DCA degraders were present in the GTP, and five DCA-degrading bacteria (*Xanthobacter*, *Starkeya*, *Leifsonia* spp.) were isolated. Four of these contained *dhlA*, based on PCR screening with the
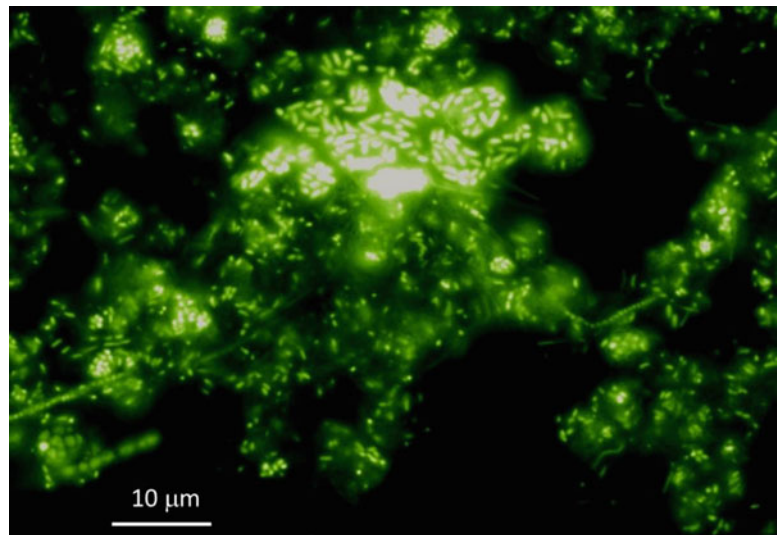

Fig. 7 *Bacterial community in membrane bioreactor (MBR) used for dhlA qPCR study.* Fluorescence microscopy image after acridine orange staining

DHLA-319F/DHLA-603R primers. In light of these results, a *dhlA*-targeted qPCR was devised to enable monitoring of the DCA-degrading bacteria in the MBR.

The job of making *dhlA* primers is simplified by the 100% sequence identity of all *dhlA* genes detected to date. The only constraints were the usual issues of minimising hairpin and dimer formation, optimising and matching Tms, and obtaining a product of the appropriate size for qPCR (100–300 bp). Standard curves were generated for both 16S rDNA and *dhlA*. The linear range of the qPCR assays was 10^4–10^9 and 10^2–10^9 gene copies for 16S and *dhlA*, respectively, and the quantitation limits were 4.4×10^6 and 7.44×10^4 copies of 16S and *dhlA* genes, respectively, per mL of sample. Melt curves indicated a single amplicon of the expected size was made in both cases. After validation of primers with purified genomic DNA from a DCA-degrading *Xanthobacter* isolate, meta-genomic DNA samples were tested.

The 16S and *dhlA* gene abundance in the MBR was studied over a 137-day time course, including a 67-day initial period where the feed water for the MBR was gradually switched from air-stripped water to raw groundwater. The *dhlA* gene was initially undetectable, but rose to a maximum of 5.1×10^7 copies/mL on day 124 – this provides evidence that the bacterial community in the MBR was adapting to the presence of increasing DCA concentrations in the feed water.

The abundance of *dhlA* in the MBR at each time point was correlated to the abundance of each bacterial genus (from 16S pyrosequencing). The only genus that gave a positive correlation was *Azoarcus* ($R^2 = 0.68$). Interestingly, an *Azoarcus* isolate that can grow on DCA as carbon source under anaerobic denitrifying conditions has been previously described [59], but the genetics were not known. Correlating the catabolic gene qPCR data with 16S pyrosequencing data, as described here, is a useful general approach and can give clues to the phylogeny of microbes that contain catabolic genes of interest.

The qPCR method is well suited to a geographical survey, and the Coleman lab have subsequently used the *dhlA* primers to perform a qPCR analysis of groundwater from monitoring wells at the Botany Industrial Park site (Munro and Coleman, unpublished data). In this analysis, *dhlA* genes were detected in several ground-water samples (up to 10^3 genes/mL), but they were certainly not ubiquitous, despite the extensive DCA contamination at the site.

8   Primers for *dhaA* in Haloalkane- and Haloalkene-Degrading Bacteria

DhaA is a HLD enzyme that initiates bacterial growth on C3-C8 haloalkanes, 1,3-dichloropropene, and 1,2-dibromoethane. DhaA has only 32% a.a. identity to DhlA (the 1,2-dichloroethane

hydrolytic dehalogenase), but is clearly part of the same hydrolase family. The *dhaA* gene is 100% identical across multiple haloalkane- and haloalkene-degrading bacterial isolates from geographically distant locations [66] and, in some cases, phylogenetically distant clades (e.g. *Rhodococcus*, *Mycobacterium*, and *Pseudomonas*) [67, 68].

Despite the importance of *dhaA* for biodegradation of halogenated pollutants, there appear to be no reports to date of a *dhaA*-specific PCR or qPCR applied to environmental samples, microcosms, or enrichment cultures. PCR has been used to confirm the presence of *dhaA* genes in pure cultures [66], but note that the primers used (shown in Table 1) were not specifically designed for this purpose. Further primers for *dhaA* would be straightforward to design, given the 100% sequence conservation of this gene seen to date.

9   Primers for *linA* and *linB* in Hexachlorocyclohexane (HCH)-Degrading Bacteria

The gamma isomer of HCH was widely used as an insecticide (Lindane), and this compound is a highly persistent pollutant [69]. Several strains of *Sphingomonas* and *Sphingobium* can grow on γ-HCH as a carbon and energy source. The *LinA* dehydrochlorinase and the *LinB* hydrolytic dehalogenase together mediate the first four steps of this pathway [70]. The *linA* and *linB* genes are highly conserved and uniquely associated with the HCH biodegradation pathway. This situation is very well suited to a PCR or qPCR approach.

Kuramochi et al. [71] developed a PCR and nested PCR method using nondegenerate primers linA-F11/linA-R418 to retrieve *linA* genes. The *linA* gene was amplified from the soil that the archetypal HCH degrader UT26 was derived from, and from 66 clones sequenced, all were *linA*, with >99% identity to the UT26 sequence. Yamamoto et al. [72] used the primers of Kuramochi et al. to retrieve *linA* genes and also designed new primers (linB-2F/linB-3R) to retrieve *linB* from five isolates from HCH-contaminated soil.

Because of the very high conservation of *linA* and *linB* sequences, full-length genes can be amplified from metagenomic DNA and then directly used for cloning and expression [73]. Cloned *linA* and *linB* genes (300 and 400 clones, respectively) were screened for activity on gamma-HCH and delta-HCH in a high-throughput colorimetric chloride assay. Eight *linA* variants and eight *linB* variants with higher dehalogenase activity than the control (B90A) were sequenced; these varied from each other by 1–17 a.a. (*linA*) or 1–6 a.a. (*linB*).

Gupta et al. [74] developed qPCRs for *linA* and *linB* genes and used these to monitor bacterial communities during pilot-scale bioremediation of HCH-contaminated soils. The qPCR revealed that both *linA* and *linB* were undetectable at the start of the

experiment, but after biostimulation, these genes increased to 2,000–5,000 copies/ng of soil DNA, and in the case of *linB*, this increase was sustained for 360 days over four sampling points. Manickam et al. [75] used the linAf and linAr1 primers to amplify and sequence *linA* genes from 8 of 11 HCH-contaminated soil samples tested, giving products with 92–98% identity with *linA* from sphingomonads.

An additional intriguing alternative to enable culture-independent access to *linA* and *linB* genes is a PCR targeting IS6100 [76] which is very commonly associated with HCH bio-degradation genes [77] and believed to be one of the prime movers in assembling and mobilising the genes of this pathway. This approach enabled recovery of three separate PCR amplicons from soil samples, which among them contained the *linB*, *linD*, *linE*, *linR*, and *linF* genes.

Despite the success of all the above studies with *linA* and *linB*, there is still scope for improvement. It is time to revisit primer design in light of expanded sequence databases, and there is potential for primer design with other genes in the HCH degradation pathway.

10   Primers for *dehI* and *dehII* in Haloacid-Degrading Bacteria

Halogenated alkanoic acids occur in nature [78] and also as metabolites of xenobiotics such as haloalkanes and haloaromatics [6]. Some haloacids are used as herbicides, such as 2,2-dichloropropionic acid (2,2-DCPA, Dalapon). Detection of genes for haloacid metabolism is of interest to many areas of bioremedia-tion, and the genes involved provide some interesting challenges for primer design. The chlorinated substrates considered here will include 2,2-DCPA and also monochloroacetic acid (MCA), dichloroacetic acid (DCA), and chloropropionic acid (MCPA). These are all metabolised by hydrolytic haloacid dehalogenases (HADs) *dehI* and *dehII*; these genes are found in diverse bacteria including Proteobacteria, Actinobacteria, and Firmicutes [79].

A landmark study by Hill et al. yielded the first degenerate PCR primers for HADs [80]. These authors realised that nearly all the HAD genes could be divided into two evolutionarily distinct groups (*dehI* or *dehII*) (Figs. 8 and 9) and that these two groups could be targeted separately by degenerate PCR, even though primers targeting all HADs were not possible. The study was successful in amplifying six new *dehI* genes and seven new *dehII* genes from various haloacid-degrading cultures [80]. The PCRs revealed multiple *dehI* and *dehII* genes in some strains; this trend was confirmed in a later study by Marchesi et al. [81], using the same primers on new isolates from pristine soils.
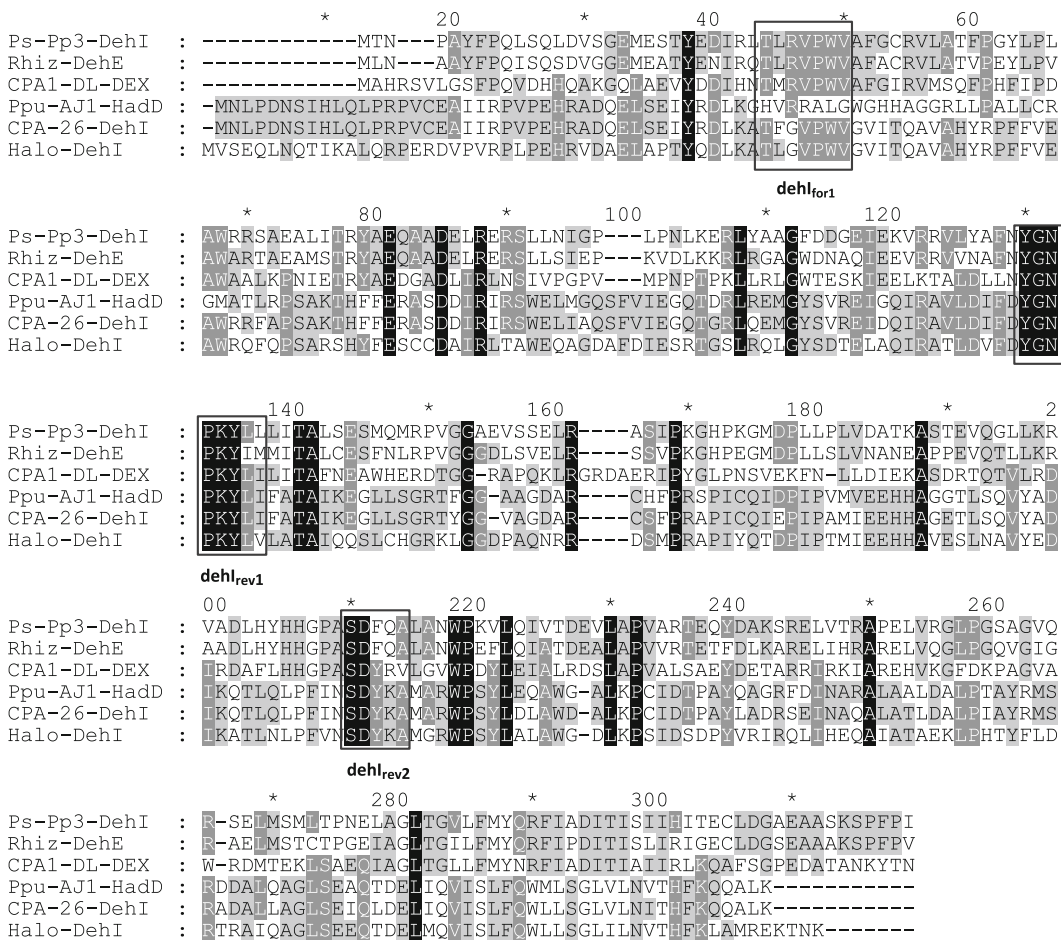
Fig. 8 *Alignment of representative haloacid dehalogenase family I proteins (Dehl)*. Conserved regions are shaded *black* (100%), *dark grey* (60%), or *light grey* (40%). Amino acid motifs targeted by PCR primers are *boxed* and labelled with the primer name. Further information about sequences and strains can be obtained from the NCBI protein database entries, as follows: Ps-Pp3-Dehl, AAN60470; Rhiz-dehE, CAA75670; CPA1-DL-DEX, BAF64754; Ppu-AJ1-HadD, AAA25831; CPA-26-dehl, ADL27927; Halodehl, WP_019017884. The DehD of *Rhizobium* and DehHI of *Moraxella* were omitted from the alignment due to their very low sequence identity to the other enzymes

The usefulness of the degenerate *dehI* and *dehII* primers designed by Hill et al. was confirmed in a PCR survey of *dehI* and *dehII* genes in activated sludge, 2,2-DCPA enrichments, and 2,2-DCPA-degrading isolates [82]. Some of the *dehI* and *dehII* genes recovered were similar to those seen in previous pure cultures such as *P. putida* Pp3, but others were divergent. Notably, isolates from this study had different *deh* gene types to those detected by PCR in the sludge or enrichment cultures from which they were derived. The loss of genetic diversity upon isolation is a common theme of this and related PCR surveys and points to the continuing need for innovative isolation approaches, e.g. [83, 84].

```
                                          *        20        *        40        *        60        *
GJ10-DhlB        : --MIKAVVFDAYGTLFDVQSVADATERAYPGRGEYITQVWRQKQLEYSWLRAIMGRYADFWGVTREALAY
Py2-DhlB         : --MIKAFVFDAYGTLFDVQSVADVTEQAFPGHGEIITQIWRMKQLEYSWLRSLMGDYRDFWTVTREALTY
Bjap-DhlB        : -MSIKAVVFDAYGTLYDIQSVADVTEDAFPCYGEIITQVWRIKQLEYTWLRSLMQRYQDFDAVTREALAY
USDA110-DhlB     : -MSLKAVVFDAYGTLYDIQSVADITEDAFPCYGEIITQVWRIKQLEYTWLRSLMQRYQDFDAVTRDSLAY
Oligo-OM4        : -MTIKAFVFDAYGTLYDIQSVATVTEEAFPCYGDIITQIWRMKQLEYTWQRSMMQRYADFSVVTRESLVY
ATCC-53690       : -MSIKGIVFDAYGTLYDVQTVSAVTDKAFPCFGDYITQVWRMKQLEYTWLRTMMGDYQDFWACTRDALSY
Pput-AJ1-HadL    : MKNIQGIVFDLYGTLYDVHSVQACEEVYPCQGDAISRLWRQKQLEYTWLRSTMGRYVNFEKATEDALRF
Bpro-0530-JS666  : MHAIKAVVFDLYGTLYDVYSVRTSCERIFPCQGEMVSKMWRQKQLEYTWMRTLMGQYQDFESATLDALRY
Ps-YL-L-DEX      : MDYIKGIAFDLYGTLFDVHSVVGRCDEAFPCRGREISALWRQKQLEYTWLRSLMNRYVNFQQATEDALRF
Ps-WS-L-DEX      : MNHIKGIAFDLYGTLYDVHSVAQACDAFPEARGREISALWRQKQLEYTWLRSMMNRYVNFAGERCDEALRF
Ps-CBS3-DehC2    : MQEIRGVVFDLYGTLCDVHSVAQLCGQYFPERCTEISIMWRQKQLEYSWLRSTMGQYVSFPQATEDALVF
Morax-DehH2      : MKKIEAIAFDMYGTLYDVHSVVDACEKQYPCKGKDISVLWRQKQLEYAWLRCTMGQYIKFEEATANALTY
Pse-CBS3-DehC1   : MDPIRACVFDAYGTLLDVNTAVMKHAHDIGCCAEELSSLWRQRQLEYSWTRTLMGRYADFWQLTEALDF
Burk-DehIVa      : MDSLRACVFDAYGTLLDVHSAVMRNADEVGASAEALSMWRQRQLEYSWTRTLMHQYADFWQLTDEALTF
                                                       dhlB314     dehIIfor

                                          *        80        *       100        *       120        *       140
GJ10-DhlB        : TLGTLGLEPDESFLADMAQAYNRLTPYPDAAQCLAELA--PLKRAILSNGAPDMLQALVANAGLTDSFDA
Py2-DhlB         : TLGVLGLSPTATLFDDIAEAYNRLAPYPDAVAALGGLS--GVHRAILSNGSPQMLDPLVGGSALRDLIET
Bjap-DhlB        : TLRVLGLAYDSEAFERVIEKYLHLDLYPDATSALAALR--PRQLAILSNGSPDMLNALVRNSGLDRLLDA
USDA110-DhlB     : TLRILGLAYEREEFERVIEKYLHLDLYPDATSALAALK--PRQLAILSNGSPDMLNGLVRNTKLDRLLDA
Oligo-OM4        : SLKLLGLTYSDDAFERIMDKYVHLDLYPDAKAALAALS--GYKLAILSNGSSGMLNDLVKNTGLDQVLDA
ATCC-53690       : TLKTIGLTADQKHFDEIAEAYNNLKPYPDAKEALMALQ--GYRLAILSNGSPGMLDALTRNSGLDKHLET
Pput-AJ1-HadL    : TCTHLGLSLDDETHQRLSDAYLHLTPYADTADAVRRLKAAGLPLGIISNGHSCIEQVVTNSEMNWAFDQ
Bpro-0530-JS666  : TCGSLGLALDADGEAHLCSEYLSLTPFADVPQALQQLRAAGLKTAILSNGSRHSIRQVVGNSGLTNSFDH
Ps-YL-L-DEX      : TCRHLGLDLDARTRSTLCDAYLRLAPFSEVPDSLRELKRRGLKLAILSNGSPQSIDAVVSHAGLRDGFDH
Ps-WS-L-DEX      : ACRHLRLELDGQTSNALCDAYLQLAPFDEVPSVLRELRGRGLKLAILSNGSPHSINAVVSNAGLRGEFDH
Ps-CBS3-DehC2    : VCNALNLKLREDTRIALCNEYLNIKPYREVRSALESLRSGAVPLAILSNGSAHSIQSVVGNAGIEHFFSH
Morax-DehH2      : TCNQMKLDCDEGSAMRLTEEYLRLKPFPEVRGALRALRQRGMRLAILSNGSTETIHDVVHNSGVEGEFEH
Pse-CBS3-DehC1   : ALESFGLLERTDLKNRLLDAYHELSAYPDAVGTLGALKAAGFTTAILSNGNNEMLRGALRAGNLTEALDQ
Burk-DehIVa      : ALRTYHLLEDRKGLKDRLMSAYKELSAYPDAAETLEKLKSAGYIVAILSNGNDEMLQAALKASKLDRVLDS

                                          *       160        *       180        *       200        *
GJ10-DhlB        : VISVDAKRVFKPHPDSYALVEEVLGVTPAEVLFVSSNGFDVGCAKNEGFSVARVARLSQEALAREL---V
Py2-DhlB         : TLSVDEKRAFKPDPRAYELVEERLGVKPHEVIFVSSNGFDIACAKRFGFRVARIARLPQAALAQEVAEEM
Bjap-DhlB        : TLSVDAKKIFKPSPAAYQLIGEVLGCTAPDEVLFVSSNPWDVACAKSFGLNVAWIERVTPEAMALACVE--
USDA110-DhlB     : TLSVDAKKVFKPSPAAYELIGEVLGCTAPDEVLFVSSNPWDVACAKSYGLNVAWIERVTPEAMALACVE--
Oligo-OM4        : TLSIDSERIFKPAPQAYTLIEKKLCMKPSDVMFVSSNPFDVACAKSFGLNVAWIERVTPQAMAAEFKP--
ATCC-53690       : WLSVDPVKAFKPDMRAYEPVHAQMCLARDEVLFVSSNGFDVCCAKRFGFRVARIERVTPKALNKEIRD--
Pput-AJ1-HadL    : LLSVEDVQYFKPDSRVYSLAEKRMCFPKENILFVSSNAWDASAASNFGFPVCWINRQ-------------
Bpro-0530-JS666  : LLSVDEVRLFKPHQKVYELAMDTLHLGKSEILFVSCNSWDATCAKYFGYPVCWINRS------------
Ps-YL-L-DEX      : LLSVDPVQYYKPDNRVYELAEQALGLDRSAILFVSSNAWDATGARYFGFPTCWINRT-----------
Ps-WS-L-DEX      : LLSGDVHYWSFKPDNRVYEIAESAFGLHRSAILFVSSNAWDATGARYFGFPTCWVNRT----------
Ps-CBS3-DehC2    : LLSADEVSVSKPSPAAYELAEKRLKVVRSKLLFVSSNAWDASGARHFGFQVCWVNRS----------
Morax-DehH2      : LLSVDSARAYKPHPLAYELGEEAFGISRESILFVSSNPWDVSCAKAFGYQVCWTNRY---------
Pse-CBS3-DehC1   : CLSVDEIKIYKPDPRVYQFACDRLDVRPSEVCCFVSSNAWDIGCAGAFGFNTVRINRI--------
Burk-DehIVa      : CLSADDLKIYKPDPRIYQFACDRLGCVNPNEVCCFVSSNAWDLGCAGKEGFNTVRINRI--------
                      dhlB637                            dehIIrev1

                                          220        *       240        *       260
GJ10-DhlB        : SGTIAPLTMFKALRMREETYAEAPD-FVVPALGDLPRLVRGMAGAHLAPAV
Py2-DhlB         : TGALRPVTFYKALRTQEEALGYAPD-FTVSSLADLSRLAVAA---------
Bjap-DhlB        : NELVAPLTMFKALRTQMDELGFAPD-HRVRSLSELPTIVARP---------
USDA110-DhlB     : NELVAPLTMFKAIRTQMDELGFAPD-HRVRALSELPSII-----------
Oligo-OM4        : GAPVAPVTMFKAVRTQMDELGFLPD-HRIRALAELPKVVGK----------
ATCC-53690       : ASVISPSTMFKATRMRIEDIGHGPD-HVVASLKELPTVLETMENAA-----
Pput-AJ1-HadL    : -------------NGAFDELDAKET-HVVRNLAEMSNWLVNSLD-------
Bpro-0530-JS666  : -------------NGVFDQLGVVPD-IVVSDVGVLASRFSPVDEAA-----
Ps-YL-L-DEX      : -------------GNVFEEMGQTPD-WEVTSLRAVVELFETAAGKAEKG--
Ps-WS-L-DEX      : -------------GNSFEEMGQVRN-HEVTGLHGLAKLFESPVGKLGNV--
Ps-CBS3-DehC2    : -------------RNTFEQLGRERPD-HVISGLDELPNLLNFASADR----
Morax-DehH2      : -------------GFAFDELGQTPD-FTVPVMDAIVHLIAV---------
Pse-CBS3-DehC1   : --------------NKPQEYSFAPQRHQLSSLSELPQLLLR-LTQ------
Burk-DehIVa      : --------------GNPPEYEFALKHQVNSLSELWPLLAKNVTKAA----
```
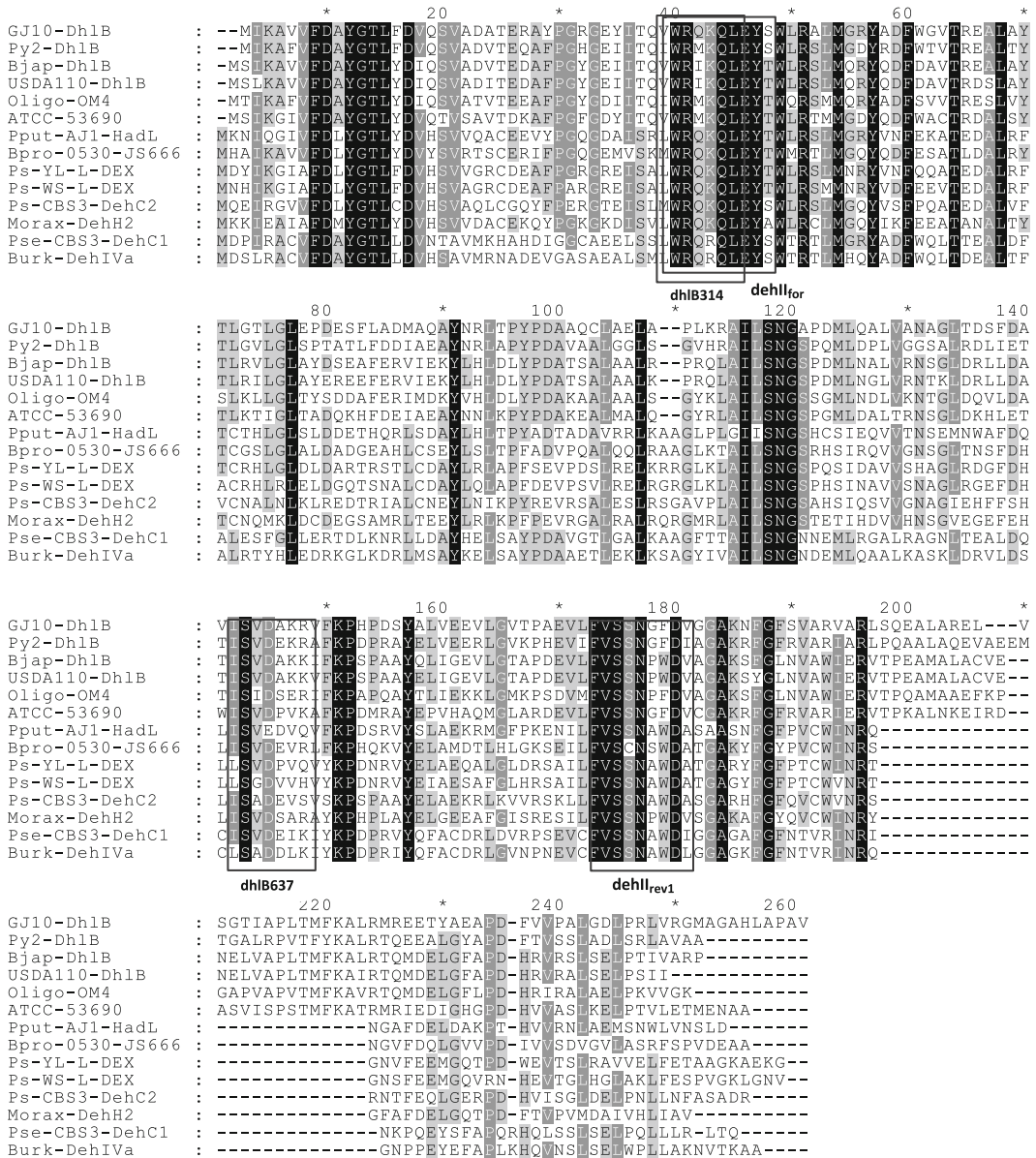
Fig. 9 *Alignment of representative haloacid dehalogenase family II (DehII) proteins.* Conserved regions are shaded *black* (100% conserved), *dark grey* (80% conserved), or *light grey* (60% conserved). Amino acid motifs targeted by PCR primers are *boxed* and labelled with the primer name. Further information about sequences and strains can be obtained from the NCBI protein database entries, as follows: GJ10-DhlB, AAA27590; Py2-DhlB, YP_001415886; BJap-DhlB, WP_018648446; USDA110-DhlB, BAC52825; Oligo-OM4, WP_012562239; ATCC-53690, EKS30519; Pput-AJ1-HadL, HADL_PSEPU; Bpro-0530-JS666, ABE42492; Ps-YL-L-DEX, HAD_PSEUY; Ps-WS-L-DEX, BAD91552; Pse-CBS3-DehC2, HAD2_PSEUC; Morax-DehH2, DEH2_MORSB; Pse-CBS3-DehC1, HAD1_PSEUC; Burk-DehIVa, 2NO5_A

The study of Marchesi and Weightman [82] highlights a useful general approach to metagenomic PCR that our laboratory has also adopted at times [15]; i.e. applying a single primer pair across a related set of environmental samples, enrichment cultures, and isolates. This gives information about the level of representation of different genes in cultured vs. uncultured bacteria, it facilitates PCR troubleshooting by enabling study of a gradient of template complexity, and it can be used to guide enrichment and isolation strategies.

The degenerate *dehI* and *dehII* primers of Hill et al. have been used to survey drinking water and water isolates [85, 86] – there is interest in this area due to the production of haloacetates as by-products of disinfection. Grigorescu et al. [86] isolated 35 new haloacetate degraders (MCA, DCA, TCA) and screened them with *dehI* and *dehII* primers; this revealed that *dehII* genes were more common than *dehI*. Leach et al. [85] had similar good results with the same primers and again showed that both *dehI* and *dehII* genes could be amplified in many cases from water, enrichments, and isolates and that *dehII* genes were more prevalent.

Leach et al. [85] found that the dehII$_{for1}$ – dehII$_{rev1}$ primers were unsuitable for qPCR due to secondary products and designed a set of nondegenerate *dehII* qPCR primers, which were used to quantify *dehII* in enrichments. The qPCR was not sensitive enough to detect *dehII* genes in wastewater directly, but this was enabled qualitatively by a nested PCR using the degenerate primers, then the specific primers. Despite the adverse findings of Leach et al., one study did use the dehII$_{for1}$ – dehII$_{rev1}$ primers for qPCR [87] and was apparently successful at detecting 10^4–10^5 *dehII* genes per mL, but in light of the findings of Leach et al. above, a sizeable fraction of these must be expected to be artefacts.

The use of highly degenerate primers for qPCR is not recommended, and in cases such as this, the burden of proof is on the investigator to show that the primers work as expected. A large representative fraction of the amplicons must be sequenced, to get a sense of the specificity of the primers with the particular template type (e.g. enrichments, groundwater, soil) before the PCR or qPCR can be applied as meaningful analytical tools.

11 Primers for *dhlB* in DCA-Degrading Bacteria

DhlB is a type of dehII enzyme (see above section) that removes chloride from chloroacetate as part of the hydrolytic DCA biodegradation pathway [88, 89]. The dehII$_{for1}$ – dehII$_{rev1}$ primers discussed above can retrieve *dhlB*-like genes from metagenomic DNA [82], and there are two further examples in the literature of PCR amplification of *dhlB* from metagenomes, but both these studies

(below) are of a preliminary nature. To date, *dhlB* has only been found in Alphaproteobacteria and Betaproteobacteria.

A PCR approach was used to detect *dhlB* in paper mill effluent [90], using nondegenerate primers dhlB-314 and dhlB-637, which were designed based on the *Xanthobacter* GJ10 sequence. This PCR gave the expected product from one of seven chloroacetate-utilising pure cultures tested and gave products from metagenomic DNA, although these were not sequenced. Another PCR survey studied *dhlB* in aquifer sediments from a chlorinated ethene-contaminated site [64]. An 850 bp product was obtained from all samples studied, and these were 100% identical to *dhlB* of *Xanthobacter* GJ10. Unfortunately, the primers used for *dhlB* amplification in this study were not described.

To date, there is still only one experimentally characterised *dhlB* gene, from *Xanthobacter* strain GJ10, but close homologues (>90% a.a. identity) exist in the genomes of *Xanthobacter* Py2 and *Xanthobacter* 126, which are not known to be haloacid degraders. The Coleman lab has sequenced a *dhlB* gene from a DCA-degrading *Xanthobacter* isolate (EL4), which is 91% identical to the GJ10 *dhlB* (Munro and Coleman, unpublished data). Fortin et al. amplified a *dhlB* gene from an *Ancylobacter* strain [90], which reportedly had 88% DNA identity to GJ10 *dhlB*, but this sequence is not available. An alignment of all four available *Xanthobacter* DhlB enzymes is shown in Fig. 10.

Another candidate *dhlB* gene is Bpro_0530 in *Polaromonas* JS666, which is believed to encode chloroacetate metabolism in this cDCE-degrading organism [56, 91, 92]. Strain JS666 was recently shown to grow on DCA [56], so Bpro_0530 is a *dhlB* gene by the fairly strict working definition above, which requires it to act in a DCA pathway. However, the inclusion of Bpro_0530 as a *dhlB* causes problems for primer design, since the predicted enzyme has only 51% a.a. identity to GJ10 DhlB, and is phylogenetically closer to other types of HADs such as HadL and L-DEX (*see* Fig. 10).

If our interest is in identifying bacteria capable of growth on chlorinated pollutants such as DCA and cDCE, our instinct may be to capture as much HAD and HLD gene diversity as possible in our primer design. But the need to cast a wide net needs to be tempered by the knowledge that in many cases, bacterial genomes will contain multiple HAD and HLD homologues, and inevitably, some of these are not going to be involved in the process of interest. Caution is needed in interpreting HAD and HLD PCRs; one organism may contain multiple hydrolases, and one hydrolase may metabolise multiple substrates.

After consideration of all the above, the dehII$_{for1}$ – dehII$_{rev1}$ primers [80] are still a valid choice for detection of *dhlB*-like genes, especially if detection of 'divergent' *dhlB*s (such as that of JS666) is required. These primers must be used with caution due to their high degeneracy. If the target is constrained to just *Xanthobacter-*

```
                    *           20          *           40          *           60
EL4  : MIKAVVFDAYGTLFDVQSVADATEQAYPGRGDYITQVWRQKQLEYSWLRALMGRYADFWSVTREA
126  : MIKAVVFDAYGTLFDVQSVADATEQAYPGRGEYITQVWRQKQLEYSWLRALMGRYADFWSVTREA
GJ10 : MIKAVVFDAYGTLFDVQSVADATERAYPGRGEYITQVWRQKQLEYSWLRALMGRYADFWGVTREA
Py2  : MIKAFVFDAYGTLFDVQSVADVTEQAFPGHGEIITQIWRMKQLEYSWLRSLMGDYRDFWTVTREA
                                            dehII_for
```

```
        *           80          *          100          *          120          *
EL4  : LAYTLGTLGLEPDKAFLAGMAEAYNRLTPYPDAARCLAELAPLKRAILSNGAPDMLRALVGNAAL
126  : LAYTLGTLGLEPDEAFLAGMAQAYNHLTLYPDAARCLAEMAPLKRAILSNGAPDMLRALVANAGL
GJ10 : LAYTLGTLGLEPDESFLADMAQAYNRLTPYPDAAQCLAELAPLKRAILSNGAPDMLQALVANAGL
Py2  : LTYTLGVLGLSPTATLFDDIAEAYNRLAPYPDAVAALGGLSGVHRAILSNGSPQMLDPLVGGSAL
```

```
           140          *          160          *          180          *
EL4  : TDSFDAVISVDVKRVFKPHPEAYALVEEVLGVKPAEVIFVSSNGFDVGGAKSFGFRVARVARLPR
126  : ADSFDAVISVDAKRVFKPHPEAYALVEEVLGVTPAEVIFVSSNGFDVGGAKNFGFRVARVARLPR
GJ10 : TDSFDAVISVDAKRVFKPHPDSYALVEEVLGVTPAEVIFVSSNGFDVGGAKNFGFSVARVARLSQ
Py2  : RDLIETTISVDEKRAFKPDPRAYEVEERLGVKPHEVIFVSSNGFDIAGAKRFGFRVARIARLPQ
                                            dehII_rev1
```

```
     200          *          220          *          240          *
EL4  : EALARELL---ASGSIAPLTMFKALRMQEEAYAEAPDFVVPLGDLPRLVREMAGARLAPAV
126  : EALARELL---ASGAIAPLTVFKALRMQEETYAEAPDFVVPALGDLPRLIAETAGGTR----
GJ10 : EALARELL---VSGTIAPLTMFKALRMREETYAEAPDFVVPALGDLPRLVRGMAGAHLAPAV
Py2  : AALAQEVAEEMTGALRPVTFYKALRTQEEALGYAPDFTVSSLADLSRLAVAA---------
```

Fig. 10 *Alignment of DhlB proteins from Xanthobacter strains.* Conserved regions are shaded *black* (100% conserved), *dark grey* (80% conserved), or *light grey* (60% conserved). Amino acid motifs targeted by PCR primers are *boxed* and labelled with the primer name. Further information about sequences and strains can be obtained from the NCBI protein database entries, as follows: GJ10-DhlB, AAA27590; Py2, ABS66229; 126, WP_024277583; EL4 (pending)

like *dhlB* genes (Fig. 10), then more specific primers could be made; e.g. qPCR primers could target MIKA(V/F)VF (forward) and KQLEYSW (reverse) (141 bp product). Alternatively, FVSSNGF could be used as a reverse priming site with the same forwards primer to give a 525 bp product in a regular PCR.

12 Primers for *tfdA* in 2,4-ᴅ-Degrading Bacteria

The phenoxy herbicides include 2,4-dichlorophenoxyacetic acid (2,4-ᴅ), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 2-methyl-4-chlorophenoxyacetic acid (MCPA), and 2-(2-methyl-4-chlorophenoxy)propionic acid (mecoprop, MCPP), among others. These herbicides all contain a chlorinated aromatic hydrocarbon core, and they can be persistent pollutants under certain conditions [93]. Many Proteobacteria and a few Firmicutes are known which can grow on 2,4-ᴅ, and this herbicide is a model compound for studies of bacterial catabolic diversity [94].

The TfdA enzyme encodes an α-ketoglutarate-dependent oxygenase [95], which cleaves the ether linkage in 2,4-ᴅ; this is the first step in the herbicide biodegradation pathway and neutralises the herbicidal activity. Homologues of TfdA are found in many, but not all, bacteria isolated on 2,4-ᴅ and other phenoxy herbicides [96, 97], and genes similar to *tfdA* are also seen in genomes of bacteria with no

known exposure to herbicides [98]. Other key genes in the 2,4-D pathway have been used as PCR targets, such as *tfdB* [99] and *tfdC* [100], but the below discussion focusses only on primers for *tfdA*.

There is substantial sequence diversity in *tfdA* genes, and primer design is not trivial. The first serious effort to make degenerate primers for *tfdA* [99] used a collection of 25 different 2,4-D degraders as the test set for primer validation. The primers gave products of the expected size from 17 strains – these were classified into four different RFLP groups, although none were sequenced. Later work confirmed these primers amplified genuine *tfdA* genes and demonstrated their usefulness for diverse templates [101–103]. The primers of Vallaeys et al. have been used directly for qPCR [104], although a later study [105] indicated that they were unsuitable for this.

Baelum et al. [105–108] made several generations of *tfdA* primers for PCR, qPCR, and RT-qPCR of soil DNA. They showed that the response of bacteria to 2,4-D can be detected transiently as mRNA, that exposure to herbicide increases the count of *tfdA* genes in soil, and that that enrichment changes the *tfdA* gene types present. Zaprasis et al. [109] revisited primer design for *tfdA* and tested three new *tfdA* primer sets for PCR and qPCR. They found that *tfdAα* genes similar to those in Alphaproteobacteria dominate in unenriched soil samples, rather than classical *tfdA* genes like that in *Cupriavidus* JMP134.

Note that some phenoxy herbicide-degrading bacteria use alternative enzymes to initiate the degradation pathway; these include TftAB, CadAB, RdpA, and SdpA [103, 110] – alternative primers are needed for these, which are not further discussed here.

13 Primers for *atzA* in Atrazine-Degrading Bacteria

Atrazine is a widely used triazine herbicide; other chemicals in this family include propazine, simazine, ametryn, and prometryn. All of these herbicides can be biodegraded by bacteria as sources of carbon and/or nitrogen. Isolates that biodegrade triazines include Proteobacteria and Actinobacteria. A chlorohydrolase (AtzA or TrzN) initiates the pathway, and then two other hydrolases (AtzB, AtzC) act to yield cyanuric acid [111–115], which is broken down by AtzD, AtzE, and AtzF to give urea [116].

PCR primers have been designed for *atzA*, *atzB*, *atzC*, and *trzN*. Here, only *atzA* primers will be discussed, since this has been most widely used as a biomarker, but note there are good arguments for including the other genes in any study aimed at detecting atrazine-degrading bacteria. Many of the studies discussed below also included *atzB*, *atzC*, and *trzN* primers, and the reader is directed to these studies for further information. Note also that an alternative atrazine degradation pathway is initiated by a p-450 monooxygenase [117]; this is not discussed further here.

de Souza et al. [111] were the first to develop PCRs for *atzA*, *atzB*, and *atzC*, and they showed that these genes were >99% conserved in five independent atrazine-utilising isolates [111]. The same primers were later used to investigate atrazine-degrading enrichments [113, 118] and to survey culture collections [112] – the latter study showed that of 83 atrazine-degrading isolates surveyed, 82 had *trzN*, 75 strains had atzB and atzC, and only one had *atzA*, but note that this sample set was almost all Gram-positive bacteria (*Arthrobacter*, *Nocardioides*).

A semi-quantitative nested PCR method for detection of *atzA* was developed by Shapir et al. [119]. This study was from before the era of real-time PCR and uses an MPN-type approach based on dilutions to estimate gene copy numbers; these were seen to increase from 10^2 to 10^5 per g soil in response to atrazine amendment. A similar trend was seen by Sherchan and Bachoon [120], using a more modern qPCR method; in that case, *atzA* numbers were increased in soil from a site with higher atrazine levels (golf course) compared to soil from a more pristine site (forest).

An RT-qPCR assay was developed and used to investigate the regulation of expression of atz genes in *Pseudomonas* and *Chelatobacter* strains [121] – this showed that in both bacteria, the expression of the *atzA* gene was upregulated to some extent by addition of atrazine. The same primers were used in a qPCR study of soil [122] and in an RT-qPCR study of soil and earthworms [123] – interestingly, the latter study could not detect *atzA* expression in situ, but could detect *atzD* expression, emphasising one of the advantages of doing several parallel qPCRs, each for a different gene in the degradation pathway.

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Primers That Target Functional Genes of Organohalide-Respiring Bacteria

Yue Lu*, Siavash Atashgahi*, Laura A. Hug, and Hauke Smidt

Abstract

Halogenated organic hydrocarbons are problematic environmental pollutants that can be reductively dehalogenated by organohalide-respiring bacteria (OHRB) in anoxic environments. This energy-conserving process is mediated by reductive dehalogenases (RDases). To amplify the diversity of reductive dehalogenase-encoding genes, degenerate primers have been designed, most of which target the conserved regions of the encoded protein sequences of the catalytic subunit, RdhA. In addition, specific primer sets have been developed and widely used to quantify and characterise OHRB and the reductive dehalogenase homologous (*rdh*) genes in the environment. The specific primers have been applied to multiple molecular techniques including regular and quantitative PCR (qPCR), Southern blot hybridisation, terminal restriction fragment length polymorphism (T-RFLP) and reverse transcriptase PCR (RT-PCR). The hunt for novel *rdhA* genes has benefited greatly from next-generation sequencing techniques, including primer-dependent amplicon sequencing and primer-independent metagenomic analyses. This chapter provides an overview of most primers targeting RDase-encoding genes described to date and their applications, and it discusses the developing trend of leveraging primer-(in)dependent techniques for better understanding of OHRB and their RDase gene pools.

Keywords: Degenerate primers, Halogenated hydrocarbons, Organohalide-respiring bacteria, Reductive dehalogenase

1 Introduction

Halogenated organic compounds (organohalides) are problematic environmental chemicals that over the last century have been widely produced and used for industrial applications and chemical manufacturing. As a result of accidental or indiscriminate disposal, organohalides are among the most abundant soil and groundwater contaminants. Microorganisms have evolved different strategies to take advantage of organohalides, greatly impacting the compounds' environmental fates. Under anaerobic conditions,

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*Author contributed equally with all other contributors.

organohalides can be reductively dehalogenated, which, depending on the degree of dehalogenation, can lead to their detoxification or render them susceptible for further (bio)transformation [1]. During this process, known as organohalide respiration (OHR), the organohalides are used as terminal electron acceptors in an energy-conserving respiratory metabolism. The chemically stable halogen–carbon bond is unlocked by replacing the halogen atom with hydrogen and liberating it as a halide [2]. This process is mediated by organohalide-respiring bacteria (OHRB), some of which are extreme niche specialists with OHR as their only metabolism. This group of obligate OHRB comprises the organohalide-respiring members of *Chloroflexi* (including strains of *Dehalococcoides mccartyi*, *Dehalogenimonas* and *Dehalobium*) and *Firmicutes* (strains of *Dehalobacter*) [2, 3]. It should however be noted that recent studies showed fermentative growth of a few *Dehalobacter* spp. on chloromethane [4, 5]. Moreover, single cell genomic studies of marine *Dehalococcoidia* did not reveal any evidence for catabolic reductive dehalogenation, indicating that microorganisms closely related to known obligate OHRB do not rely on OHR for energy conservation, but rather utilise organic matter degradation pathways [6, 7]. Compared to obligate OHRB, facultative OHRB have versatile metabolisms encoded by relatively large genomes and are capable of using a broad variety of electron acceptors for respiratory growth that include but are not limited to organohalides. This group comprises phylogenetically diverse proteobacterial OHRB such as members of the genera *Geobacter*, *Desulfuromonas*, *Anaeromyxobacter*, *Desulfomonile*, *Desulfovibrio* and *Sulfurospirillum* and also the *Desulfitobacterium* spp. belonging to the *Firmicutes* [2, 3].

The key enzymes of OHR are reductive dehalogenases (RDases). For several of these enzymes, the catalytic subunits have been purified and biochemically characterised, which are referred to as RDase catalytic subunit (as opposed to RdhA if only predicted based on sequence homology) [8–15]. These studies, combined with PCR-based [14–18] and genomic analyses [19–21], have revealed a conserved operon structure for RDase-encoding genes that consist of *rdhA*, coding for the catalytic subunit RdhA; *rdhB*, coding for a small putative membrane anchor (RdhB) that (presumably) locates the A subunit to the outside of the cytoplasmic membrane; and a variable set of accessory genes, the majority of which has been predicted to code for proteins involved in regulation and maturation of Rdh synthesis [2]. In general, the catalytic subunits (RdhAs) are characterised by two iron–sulphur clusters and a cobamide as cofactors in the active holoenzyme. As an exception to this rule, the chlorobenzoate RDase of *Desulfomonile tiedjei* contains a heme cofactor [8], and *Sulfurospirillum multivorans* was shown to specifically require 'norpseudo-B$_{12}$' as the corrinoid cofactor [22].

Another conserved feature among the catalytic subunits is the presence of an N-terminal Tat (twin-arginine translocation) signal peptide. After cofactor incorporation and folding of the cytoplasmic precursors of an RdhA enzyme, the Tat signal peptide is necessary for secretion of the mature protein through the cell membrane where it is located at the exocytoplasmic face of the cytoplasmic membrane [23].

2   Phylogenetic Context of *rdhA*

The genomes of OHRB encode either few (1–3) or many (10–40) distinct RDase gene operons. In general, larger numbers of *rdhA* genes are encoded on genomes of obligate OHRB, with fewer present on genomes of OHRB with versatile metabolism [3]. The expansion of the gene family through gene duplication has led to multiple non-identical *rdhA* genes per genome and across diverse, unrelated genera resulting in a complicated evolutionary history for *rdhA*. A tree of RdhA protein sequences from known microorganisms (not including sequences retrieved from environmental samples) shows limited correlation between microorganism taxonomy and RDase phylogeny (Fig. 1). Most phyla with multiple OHRB do not form monophyletic clades on the RdhA tree, and, even within clades comprised of a single phylum or group, the branching order of the RdhA does not usually follow the corresponding 16S rRNA-based taxonomy tree. In addition, microorganisms within the same genus often do not have the same RdhA homologues in their genomes. Taken together, the evolutionary relationships between the *rdhA* genes can only be explained using a combination of vertical inheritance, gene duplication and lateral gene transfer [2].

Most interesting from a functional perspective is the placement of the biochemically characterised RDases, which are distributed across the tree with little apparent relationship between proteins with shared substrate specificities when they are present in different phyla. The five characterised tetrachloroethene-reducing enzymes (PceA) are located in four distinct clades (Fig. 1), indicating their low sequence similarity despite shared specificity. In contrast, dichloroethane RDase DcrA and the chloroform RDase CfrA from *Dehalobacter* strains DCA and CF, respectively, share extremely high sequence identity (98%) but do not have overlapping substrate specificities (Fig. 1) [24]. The lack of correlation between sequence similarity and substrate specificity hinders the ability to predict substrates for novel genes, as well as to design primers targeting specific functions of interest.
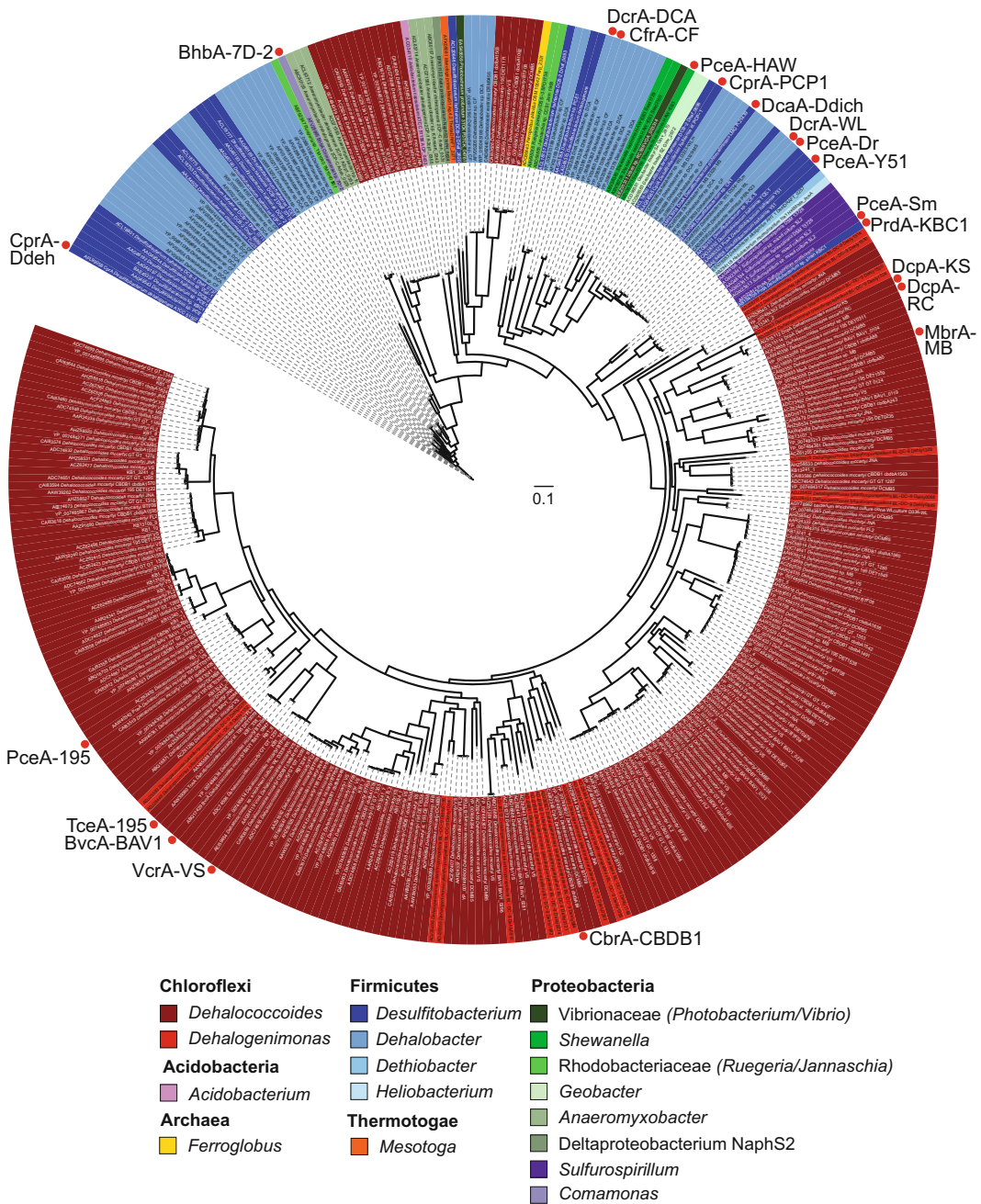
Fig. 1 Maximum likelihood tree of RdhA amino acid sequences from known microorganisms. RdhAs are coloured by genus affiliation of the source microorganism or, in two cases, order affiliation. Functionally characterised RdhAs are highlighted with red circles and named according to Table 1. The tree was constructed from 354 full-length RdhA sequences. Sequences were aligned using MUSCLE [41], and the alignment was iteratively refined using HMMER 3.0 [42] for ten iterations. Alignment columns were removed if they contained >90% gaps, and the ends of the alignment trimmed to remove trailing ends. The tree was generated using RAxML [43, 44] under the PROTGAMMAWAG model of evolution and visualised using iTOL [45, 46]. Microorganism information and protein NCBI accession numbers are included in each RdhA gene name on the tree

3   Molecular Characterisation of RDase-Encoding Genes

Initial identification of *rdh* gene sequences was performed by classical reverse genetic approaches based on N-terminal amino acid sequences obtained from purified catalytic subunits [11, 16]. Subsequent availability of RDase-encoding gene sequences has made it possible to design degenerate primers for PCR-based *rdh* sequence retrieval. This has been particularly helpful for strains of *D. mccartyi* whose low biomass yields have hindered protein-based identification in the past. In recent years genomic- and metagenomic-based approaches have also been applied for sequence-based retrieval and characterisation of *rdh* genes (Fig. 2).

4   Degenerate Primers for Characterisation of RDase Genes

Degenerate primers are primer mixes with degenerate positions instead of a single sequence with specified bases. The primer degeneracy allows amplification of multiple loci simultaneously from
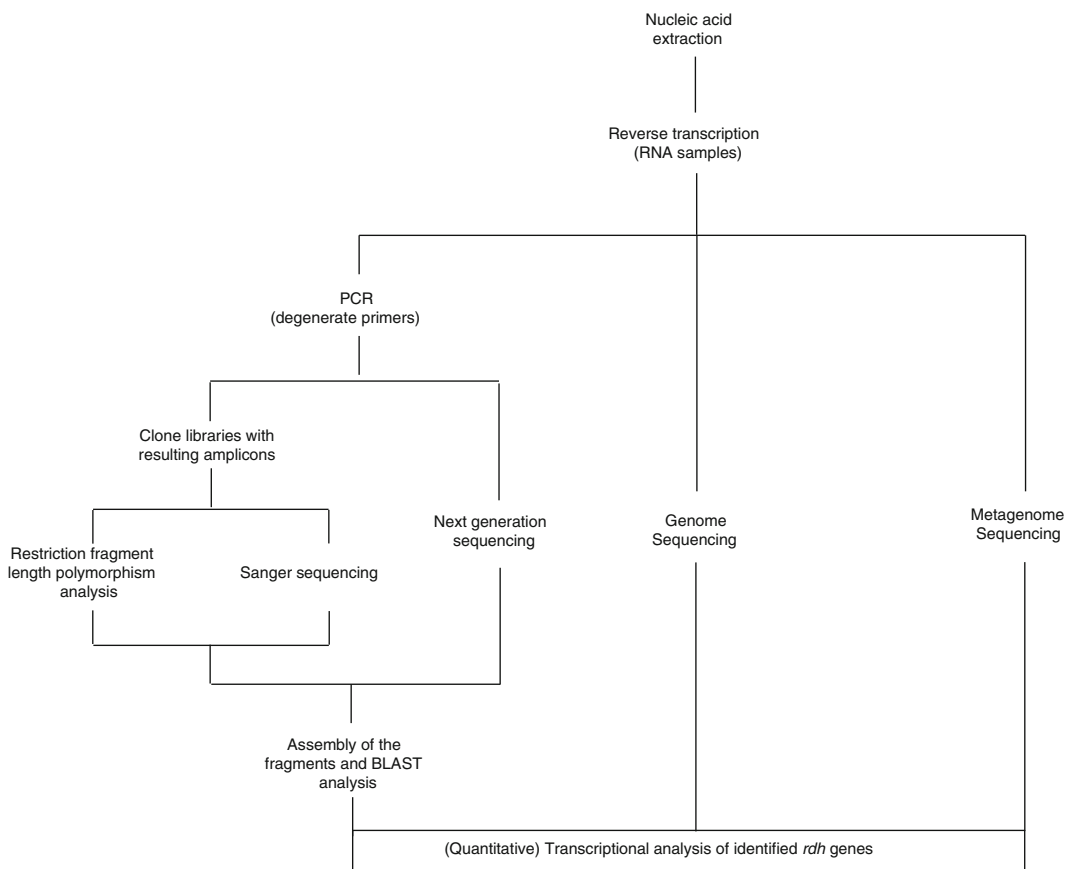
Fig. 2 Overview of the methods used for molecular characterisation of RDase-encoding genes

different microorganisms, targeting sequences with similar but not identical motifs. Degenerate primers are widely used to amplify *rdhA* genes, especially when the aim is to amplify genes from uncultivated microorganisms for which genomic information is not available. Degenerate primers are usually designed based on conserved regions of RDase catalytic subunit-encoding genes by aligning amino acid sequences found in sequence databases like GenBank [25] and using specific approaches including consensus-degenerate hybrid oligonucleotide primer (CDHOP) [26]. The first set of degenerate primers (primer pair 1, Table 2) for an RDase gene was designed based on internal peptides in 1998, targeting a 1,200 bp long region of the PCE dehalogenase from *Dehalospirillum multivorans* [16] (later reclassified as *Sulfurospirillum multivorans* [27]) (Table 2). In the following years, with increased availability of homologous sequences of RDase-encoding genes, multiple degenerate primer sets were designed and applied for *rdh* detection, mostly targeting conserved sequences of *rdhA* [12, 14, 15, 28, 29] (Fig. 3, Table 2). von Wintzingerode et al. [30] developed primers (primer pairs 13–16, Table 2) binding to sequence motifs encoding the conserved regions of PceA (*Sulfurospirillum multivorans*) and CprA (*Desulfitobacterium dehalogenans*) (Fig. 3), which enabled amplification of *cprA*-like gene fragments from *Desulfitobacterium hafniense*, *Desulfitobacterium* sp. strain PCE1 and *Dehalobacter restrictus*. Expanding this technique further, Smidt [31] designed multiple sets of highly degenerate oligonucleotide primers (primer pairs 17–20, Table 2) based on twin-arginine signal peptides, iron–sulphur clusters and five additional highly conserved sequence motifs (Fig. 3), which were used to target *rdhA* genes from different *ortho*-chlorophenol- and chloroethene-dechlorinating OHRB. Of all primers, the RRF2 and B1R primers (primer pair 4, Table 2) became one of the most popular sets [17] [32]. Originally used to identify the *bvcAB* gene from *D. mccartyi* BAV1, this primer set targets the RRXFXK motif of the Tat signal peptide (RRF2) (Fig. 3) and the WYEW motif internal to the downstream associated *rdhB* gene (B1R), yielding amplicons of 1.5–1.7 bp containing almost complete *rdhA* genes (Table 2). Using this primer set, Krajmalnik-Brown et al. [17] found seven *rdhA* genes in *D. mccartyi* BAV1. Later, the RR2F/B1R pair was widely used to detect *rdhAs* from *D. mccartyi* isolates and enrichments, including 13 *rdh* loci in strain CBDB1 [32], 14 in strain FL2 [32], 14 in the mixed culture KB1 [33], 8 in the enrichment culture TUT2264 [34] and 4 from TCE-contaminated groundwater samples [35]. Although the RR2F/B1R primer set was designed based on 17 *rdhAB* genes in the genome of *D. mccartyi* 195 [17], the primer set fails to amplify *tceA*-like genes [18]. Furthermore, it should be noted that the *rdh-A* and *rdh-B* genes are not always oriented in an A–B direction in the *rdh* operon [36]. To circumvent this shortcoming, Chow et al. [18] designed a complimentary primer set RDH F1C/RDH R1C (primer pair 7, Table 2)
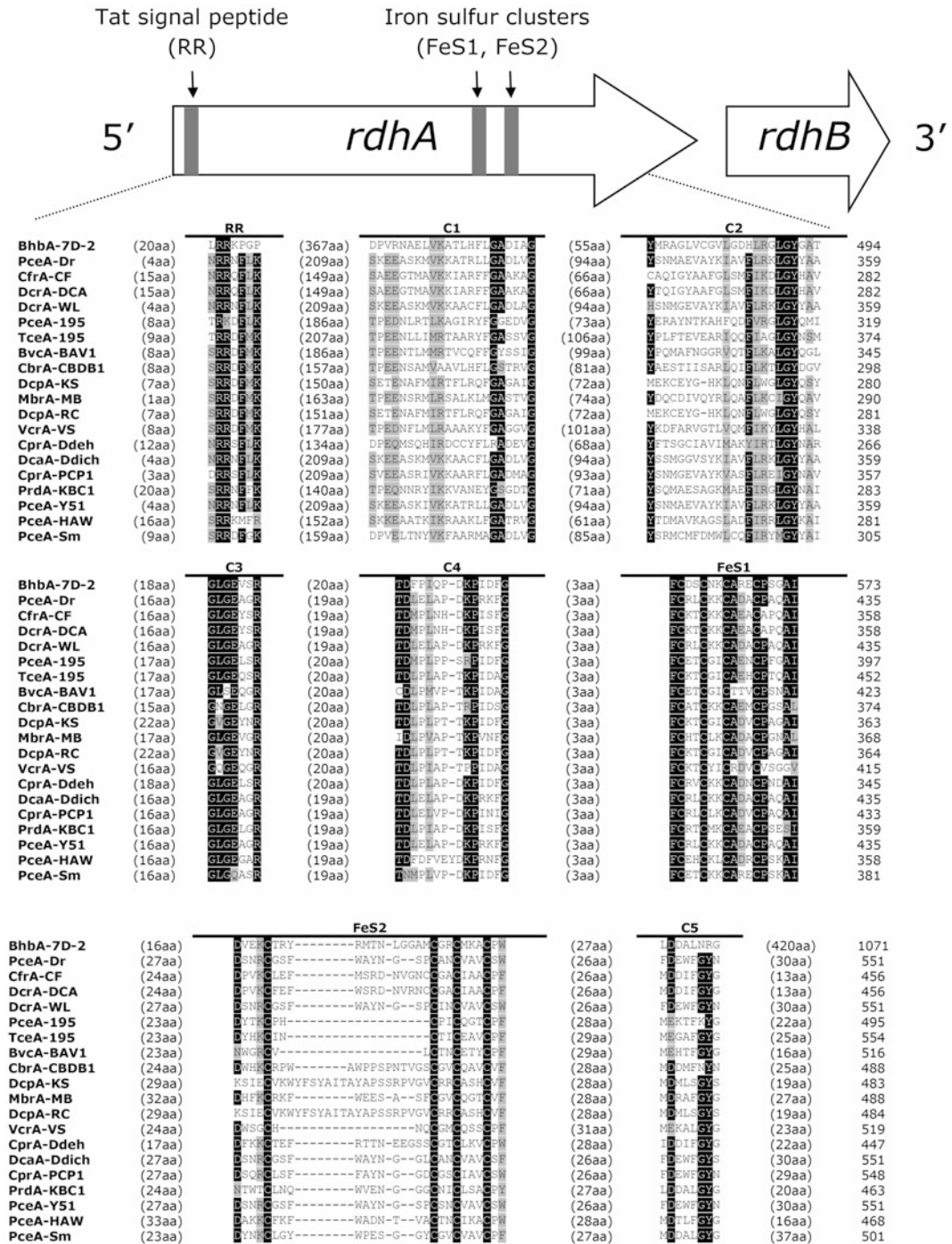
Fig. 3 Physical map of *rdhAB* operon (*top*) and primary sequence alignment of the functionally characterised RdhAs (*bottom*). Note that in some cases, the order of genes in the operon is reversed (i.e. *rdhBA*, rather than *rdhAB*). ClustalW [52] multiple sequence alignment was conducted by using BioEdit version 7.2.5 (http://bioedit.software.informer.com/). The conserved sequence motifs among experimentally characterised reductive dehalogenases (RR, two Fe–S and C1–C5) are indicated with black backgrounds. The functionally characterised RdhAs are named according to Table 1

Table 1
Functionally characterised RDase catalytic subunits

Code	Protein	Microorganism	Protein NCBI accession numbers	References
BhbA-7D-2	BhbA	*Comamonas* sp. 7D-2	AFV28965.1	[68]
PceA-Dr	PceA	*Dehalobacter restrictus* PER-K23	CAD28790.2	[14]
CfrA-CF	CfrA	*Dehalobacter* sp. strain CF	AFQ20272.1	[24]
DcrA-DCA	DcrA	*Dehalobacter* sp. strain DCA	AFQ20273.1	[24]
DcrA-WL	DcrA	*Dehalobacter* sp. strain WL	ACH87594.1	[39]
PceA-195	PceA	*Dehalococcoides mccartyi* 195	AAW40342.1	[69]
TceA-195	TceA	*Dehalococcoides mccartyi* 195	AAF73916.1	[28]
CbrA-CBDB1	CbrA	*Dehalococcoides mccartyi* CBDB1	CAI82345.1	[70]
DcpA-KS	DcpA	*Dehalococcoides mccartyi* KS	AGS15112.1	[57]
MbrA-MB	MbrA	*Dehalococcoides mccartyi* MB	ADF96893.1	[18]
DcpA-RC	CbrA	*Dehalococcoides mccartyi* RC	AGS15114.1	[57]
VcrA-VS	VcrA	*Dehalococcoides mccartyi* VS	AAQ94119.1	[15]
CprA-Ddeh	CprA	*Desulfitobacterium dehalogenans* JW/IU-DC1	AAD44542.1	[11, 58]
DcaA-Ddich	DcaA	*Desulfitobacterium dichloroeliminans* DCA1	CAJ75430.1	[38]
CprA-PCP1	CprA	*Desulfitobacterium hafniense* sp. PCP-1	AAQ54585.2	[55]
PrdA-KBC1	PrdA	*Desulfitobacterium* sp. strain KBC1	BAE45338.1	[71]
PceA-Y51	PceA	*Desulfitobacterium* sp. strain Y51	AAW80323.1	[29]
PceA-HAW	PceA	*Shewanella sediminis* HAW-EB3	ABV38373.1	[63]
PceA-Sm	PceA	*Sulfurospirillum multivorans* DSM 12446	AAC60788.1	[16]

targeting the coding region of the twin-arginine motif and conserved PIDDG motif, respectively (Fig. 3, Table 2). With a product size of 1,200 bp, this primer pair targeted only the catabolic subunit gene (*rdhA*) and (at the time of design) could cover ~90% of the known *rdhAs* in *D. mccartyi* populations. Using this primer set, the authors detected seven putative *rdhA* genes from *D. mccartyi* strain MB [18]. Another potential problem with the RR2F/B1R primer pair is that, although the N-terminal Tat signal peptide motif is highly conserved among RdhAs, it is also found in numerous other exported proteins and as such is not unique to this class of proteins [23]. Meanwhile Regeard et al. [26] had designed seven degenerate primers (primer

Table 2
Degenerate primer sets targeting RDase genes

Primer pair	Targeted RDase genes	Name	Primer sequence (5'-3')	Targeted region	Annealing T (°C)	Size (bp)	Targeted OHRB
1	pceA	Unnamed [16]	GGIGAGGTIAAGCCITGGTT	Internal peptides: 'GEVKPWF'[a]	45	1,200	Sulfurospirillum multivorans [16]
		Unnamed [16]	GTCCCAIACYTCIGTDATRTT	Internal peptides: 'NITEVWD'[a]			
2	tceA	TFRO [28]	GCIAAYAARGTIAAYAA YCAYCCNTGGTGGG	N-terminal: 'ANKVNNHPWW'[a]	60	491	Dehalococcoides mccartyi 195 [28]
		TREV [28]	CCYTCCCAYTTIGGRTARTT NGTNGT	Internal peptide: 'TTNYPKWEG'[a]			
3	vcrA	Nterm13F [15]	ACVAARGAYCARCCDTGGTA	N-terminal: 'TKDQPWY'[a]	47	292	Dehalococcoides mccartyi VS [15]
		Intern4R [15]	GGYTGBGTIACKGCRTARAA[b]	Internal peptide: 'FYAVTQP'[a]			
4	bvcA	RRF2 [17]	SHMGBMGWGATTTYAT GAARR	Twin-arginine motif: 'RRXFXK'	48	1,500	Dehalococcoides mccartyi [17]
	bvcB	B1R [17]	CHADHAGCCAYTCRTACCA	Associated anchoring protein motif: 'WYEY'	48	1,700	Dehalococcoides mccartyi CBDB1 and FL2 [32]
					48	~1,500	Dehalococcoides-containing mixed culture KB1 [33]
					48	~1,700	Environmental sample containing Dehalococcoides spp. [35]
					–	>1,520	Enrichment culture TUT2264 [34]
					48	1,500–1,700	Dehalococcoides spp. [18]

(continued)

Table 2
(continued)

Primer pair	Targeted RDase genes	Name	Primer sequence (5′-3′)	Targeted region	Annealing T (°C)	Size (bp)	Targeted OHRB
5	rdhA	fdehal [32]	CARGGXACXCCXGARGA	Internal peptides: 'QGTPE(D/E)'^c	–	500	Dehalococcoides mccartyi CBDB1 [32]
		rdehal [32]	RSXCCRAARTCXATXGG	Internal peptide of C4 region: 'P(I/M)DFG (P/L/A/V)'^c			
6	rdhA	mern2 [32]	NNNTTYCAYGAY YTNGAYGAM	Upstream of RR motif: 'XFHD(F/L)D (D/E)'^c	–	~1,000	Dehalococcoides mccartyi CBDB1 [32]
		mern5 [32]	NCCNGCRTCDATN GGNNNNNN	Internal peptide of C4 region: 'XXPIDAG'^c			
7	rdhA	RDH F1C [18]	TTYMVIGAYITIGAYGA	Conserved twin-arginine motif	47	1,200	Dehalococcoides spp. [18]
		RDH R1C [18]	CCIRMRTYIRYIGG	Conserved motif: 'PIDD'			
8	pceA	NTERM [29]	CIGAYATIGTIGCICCIAT	N-terminal: 'ADIVAPI'^a	46	1,000	Desulfitobacterium sp. Y51 [29]
		INT14[29]	YTTRTCIGGIGCIARYTC	Internal peptide of C4 region: 'VYTDLELAPDK'			
9	pceC	Unnamed [12]	GCIGARGTTTAYAAYAARGA	N-terminal: 'AEVYNKD'^a	43	81	Coculture DPH-1 [12] (containing Desulfitobacterium hafniense strain JH1 [47])
		unnamed [12]	GTRTCRTTISIRAARTARTG	N-terminal: 'HYFSDDA'^c			

	Gene	Primer [ref]	Sequence	Peptide/region	Temp	Size (bp)	Source
10	pceA	DR3f [14]	GAYATIGTIGCICCIATIAC	N-terminal: 'DIVAPIT'	50	1,126	*Dehalobacter restrictus* PER-K23 [14]
		DR4r [14]	CCRAARTCIATIG GYTTRTCIGG	Internal peptide of C4 region: 'PDKPIDFG'			
11	rdh	DebfrdhF [48]	ATGGGNGARATHAAYMG	Peptide 'MGEIN(S/R)'[c]	–	~1,100	*Dehalobacter* sp. TCP-1 [48]
		DebfrdhR [48]	TCNGCRCAYTTYTTRCA	Iron–sulphur cluster binding motif: 'CKKCA(D/E)'[c]			
12	rdh	RRF2 [17]	SHMGBMGWGATTTYAT GAARR	Twin-arginine motif: 'RRXFXK'[c]	48	1,000	WL cultures (containing *Dehalobacter* sp. WL) [39]
		RD7r [26]	AANGGRCAIACIGCIWCRCA	Iron–sulphur cluster peptide: 'C(V/E)AVCP'			
13	cprA/pceA-like gene	dehaloF3 [30]	ATCGWTSMRGGTAT	Internal peptide 'I(D/V)(A/Q)GI'[c]	50	~600	*Desulfitobacterium dehalogenans, Sulfurospirillum multivorans, Desulfitobacterium* sp. PCE1 and *Dehalobacter restrictus* [30]
		dehaloR2 [30]	TYTGTACCATAGCC	C-terminal: 'GYGT(K/E)'[c]			
14	cprA/pceA-like gene	dehaloF4 [30]	YMTKGGTTAYWATGC	Internal peptide of C2 region: 'LGYNA'[c]	50	~450	DCP-dechlorinating batch culture [30]
		dehaloR3 [30]	AGCCMAANRYATCATC	Internal peptide of C5 region: 'DD(A/I)(L/F)G'[c]			

Table 2
(continued)

Primer pair	Targeted RDase genes	Name	Primer sequence (5'-3')	Targeted region	Annealing T (°C)	Size (bp)	Targeted OHRB
15	cprA/pceA-like gene	dehaloF5 [30]	GGTTGCATTGCYGTCAT	Internal peptide of C2 region: 'GCIAVI'^{c}	50	425	*Desulfitobacterium dehalogenans*, *Desulfitobacterium hafniense*, *Desulfitobacterium* sp. PCE1 and *Dehalobacter restrictus* [30]
		dehaloR4 [30]	TGCTTYATGGAACCAGG	C-terminal: 'SWFHQA'^{c}			
16	cprA/pceA-like gene	dehaloF4 [30]	YMTKGGTTAYWATGC	Internal peptide of C2 region: 'LGYNA'^{c}	50	~500	*Desulfitobacterium dehalogenans*, *Sulfurospirillum multivorans*, *Desulfitobacterium* sp. PCE1 and *Dehalobacter restrictus* [30]
		dehaloR2 [30]	TYTGTACCATAGCC	C-terminal: 'GYGTX'^{c}			
17	cprA	D1 [31]	AAYMGVMGVAA YTTYCTBAA	Twin-arginine motif: 'NRRNFLK'	35–40	–	*Desulfitobacterium dehalogenans* and *Desulfitobacterium* sp. PCE-1 [31]
		D8 [31]	GCRCAYTTYTTRCA	Iron–sulphur cluster binding motif: 'CKKCA'			

No.	Gene	Primer	Sequence	Target region/peptide	Size	bp	Organism
18	*rdhA*	D2 [31]	GCIGAYATHGTIGCICC	N-terminal: 'ADIVAP'	35–40	–	*Desulfitobacterium hafniense, Desulfitobacterium frappieri* strains PCP-1 and TCE1 [31]
		D7 [31]	ATIGGYTTRTCIGG	Internal peptide of C4 region: 'PDKPI'			
19	*rdhA*	D4 [31]	GGIGCIGAYYTIGTIGG	Internal peptide of C1 region: 'GADLVG'	35–40	–	*Desulfitobacterium dehalogenans, Desulfitobacterium* sp. PCE-1 and *Sulfurospirillum multivorans* [31]
		D7 [31]	ATIGGYTTRTCIGG	Internal peptide of C4 region: 'PDKPI'			
20	*rdhA*	D4 [31]	GGIGCIGAYYTIGTIGG	Internal peptide of C1 region: 'GADLVG'	35–40	–	*Desulfitobacterium dehalogenans, Desulfitobacterium* sp. PCE-1 and *Sulfurospirillum multivorans* [31]
		D8 [31]	GCRCAYTTYTTRCA	Iron–sulphur cluster binding motif: 'CKKCA'			
21	*cprA*	DHU1080F [49]	TWGYYCCYRMYAARCCYA TYGA	Internal peptide of C4 region: 'LXPX(N/S)P(S/L)'c	55	450	2-Bromophenol-degrading consortium [49]
		DHU1350R [49]	CCRTAGCCVAAKATWTCA TCMAT	Internal peptide of C5 region: ' (M/I)D (D/E)I(F/L)GYG'c			

Table 2
(continued)

Primer pair	Targeted RDase genes	Name	Primer sequence (5′-3′)	Targeted region	Annealing T (°C)	Size (bp)	Targeted OHRB
22	*rdhA*	DHAR1000F [49]	GWAGCAGGTYTRGGASAA	Internal peptide of C2 region: '(V/E)AGLG(Q/E)'c	55	350	2-Bromophenol-degrading consortium [49]
		DHU1350R [49]	CCRTAGCCVAAKATWTCATCMAT	Internal peptide of C5 region: '(M/I)D(D/E)I(F/L)GYG'c	46	No amplicon	Tidal Flat *Chloroflexi* Cluster [50]
23	*pceA*	ceRD2Lf/ceRD2Sf [26]	GCAGCACGCCTTTTGGI GCIKMIYTNGTIGG/GCA GCACGCCTTTTGGIGC IKMIWSIGTIGG	Internal peptide of C1 region: 'AARLFGA(D/S)(L/S)VG'	47	750–800	*Desulfitobacterium* sp. PCE-1, *Sulfurospirillum multivorans* and *Dehalobacter restrictus* [26]
		RD7r [26]	AANGGRCAIACIGCIWCRCA	Iron–sulphur cluster peptide: 'C(V/E)AVCP'	47	800–1,150	1,2-DCA-enriched culture 6VS (*Desulfitobacterium dichloroeliminans* DCA1) [37, 38]
					48	–	WL cultures (containing *Dehalobacter* sp. WL) [39]
24	*pceA*	RD5f [26]	CCIRMIAARCCIATIRAIK YNGG	Internal peptide of C4 region: 'P(D/T)KPI(D/K)(A/F)G'	47	200 (expected), no amplicon	*Desulfitobacterium* sp. PCE-1, *Sulfurospirillum multivorans* and *Dehalobacter restrictus* [26]
		RD7r [26]	AANGGRCAIACIGCIWCRCA	Iron–sulphur cluster peptide: 'C(V/E)AVCP'			

No.	Gene	Primer	Sequence	Region	Annealing temp. (°C)	Product size (bp)	Organisms
25	*pccA*	RD4f [26]	GGNYTIGGISARGCITCIMG	Internal peptide of C3 region: 'GLG(E/Q)ASR'	50	350 (expected), no amplicon	*Desulfitobacterium* sp. PCE-1, *Sulfurospirillum multivorans* and *Dehalobacter restrictus* [26]
		RD7r [26]	AANGGRCAIACIGCIWCRCA	Iron–sulphur cluster peptide: 'C(V/E)AVCP'			
26	*pccA*	ceRD2Lf [26] /ceRD2Sf [26]	GCAGCACGCC TTTTGGIGCIKMIYTN GTIGG/GCAGCACG CCTTTTTGGIGCIKMI WSIGTIGG	Internal peptide of C1 region: 'AARLFGA (D/S)(L/S)VG'	50	450 (expected), no amplicon	*Desulfitobacterium* sp. PCE-1, *Sulfurospirillum multivorans* and *Dehalobacter restrictus* [26]
		RD4r [26]	CKIGAIGCYTSICCIARNCC	Internal peptide of C3 region: 'GLG(E/Q) ASR'			
27	*pccA*	RD4f [26]	GGNYTIGGISARGCITCIMG	Internal peptide of C3 region: 'GLG(E/Q) ASR'	50	150 (expected), no amplicon	*Desulfitobacterium* sp. PCE-1, *Sulfurospirillum multivorans* and *Dehalobacter restrictus* [26]
		RD5r [26]	CCIRMITYIATIGGYTT IKYNGG	Internal peptide of C4 region: 'P(D/T)KPI (D/K)(A/F)G'			
28	*pccA*	ceRD2Lf [26]/ ceRD2Sf [26]	GCAGCACGCCTTTTT GGIGCIKMIYTNGTIGG/ GCAGCACGCCTTTTT GGIGCIKMIWSIGTIGG	Internal peptide of C1 region: 'AARLFGA (D/S)(L/S)VG'	51	550	*Sulfurospirillum multivorans* and *Dehalobacter restrictus* [26]
		RD5r [26]	CCIRMITYIATIGGYTTIK YNGG	Internal peptide of C4 region: 'P(D/T)KPI (D/K)(A/F)G'			

Table 2
(continued)

Primer pair	Targeted RDase genes	Name	Primer sequence (5′-3′)	Targeted region	Annealing T (°C)	Size (bp)	Targeted OHRB
29	*pceA*	Unnamed [51]	GAYAARCCNATHGAYTTYGG	Internal peptide of C4 region: 'PDKPIDFG'[c]	–	330	Environmental DNA [51]
		Unnamed [51]	CCRTANCCNARNGCRTCRTC	Internal peptide of C5 region: 'DDALGYG'[c]			

Abbreviations of degenerate nucleotides: I = inosine; R = A or G; K = G or T; M = A or C; S = C or G; W = A or T; Y = C or T; B = C, G or T; D = A, G or T; V = A, C or G; H = A, C or T; N or X = A/C/G/T

[a]The internal peptides published as the binding motif for primer design in the original publication are adjusted to the actual length of the primer

[b]The sequence of the Intern4R primer in the original publication by Müller et al. [15] represents the coding strand, rather than the reverse complement, and is therefore not correct as confirmed by the author. The primer sequence given in the table is therefore the reverse complement of the originally published sequence

[c]The binding motif of the targeted internal peptide was not shown in the original publication. The amino acid sequences shown here are the (possible) binding motifs according to genetic code translation of primer sequence and amino acid sequences alignment of reductive dehalogenases in Fig. 3

pairs 23–28, Table 2) based on four conserved regions of chloroethene RDase catalytic subunit-encoding amino acid sequences from *Desulfitobacterium* sp. PCE-1, *S. multivorans* and *D. restrictus* and chlorophenol RDase catalytic subunit-encoding amino acid sequences from *Desulfitobacterium dehalogenans*, *D. hafniense* strain DCB-2 and *Desulfitobacterium chlororespirans* (Table 2). This suite of degenerate primers did not utilise the Tat signal sequence, reducing non-specific amplification, but also resulted in shorter amplicons than the primers developed by Chow et al. [18]. The primers were originally used to estimate the diversity of *rdhA* from tetra- and trichloroethene-dechlorinating enrichments [26] and later to detect *rdhA* from *Desulfitobacterium dichloroeliminans* DCA1 [37, 38], *Dehalobacter* sp. WL [39] and sub-seafloor sediments [40].

It is evident from the alignment of *rdhA* genes that one primer pair cannot cover all possible sequences. Accordingly, Futagami et al. [40] used an array of degenerate primer sets designed to target different *rdhAs* [17, 26, 30, 32]. Using this approach, 32 putative *rdhA* phylotypes were detected from marine subsurface sediments [40]. In a further step, Wagner et al. designed 13 degenerate primer pairs to monitor the expression of all 32 *rdhA* genes present in the genome of *D. mccartyi* CBDB1 [53]. Although ideal in order to cover a range of different *rdhAs*, application of such an array of degenerate primers would dramatically increase the workload in subsequent clone library construction. Besides, most of the degenerate primers were designed based on the limited number of RDase sequences available at the time, which could potentially overlook distantly related novel *rdhAs*. To adequately cover the full diversity of *rdhA* homologous sequences displaying lower similarity, a suite of degenerate PCR primers (comprising 44 phylogeny-derived groups) targeting 255 RDase catalytic subunit genes was recently designed and applied to environmental and enrichment culture samples [54]. Subsequent application of Illumina HiSeq next-generation sequencing identified a much broader diversity of *rdhA* gene sequence than was previously accounted for [54].

5   Specific Primers and Application Potential

In addition to degenerate primers, a broad range of specific primers targeting selected RDase genes have been designed (Table 3). Usually, specific primers are developed and used for different purposes than the more exploratory degenerate primer sets, including analysis of gene expression in *S. multivorans* [16] and *Desulfitobacterium* sp. Y51 [29], preparation of probes for Southern blot hybridisation [38], detection of *Sulfurospirillum rdhA* genes by T-RFLP [56] and detection of known chloroethene RDase genes [26]. Additionally, specific primers were used for putative *rdhA* detection from

Table 3
Nondegenerate primer sets designed for specific RDase genes

Target gene	Target OHRB	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)	Application potential	Comments	References
pceA	*Sulfurospirillum multivorans*	Unnamed (ATAGACCATGGAA AGAAAAAAAGC CTGAACTCTC)	Unnamed (GCAAGGATCCT CATGATTTTTTA ACCCTATCC TTTCTAAAGC)	–	Expression of RDase		[16]
pceAB	*Sulfurospirillum multivorans*	Unnamed (ATAGACCATGG AAAAGAAAAA AAGCC TGAACTCTC)	Unnamed (AGCTGGATCCTTA ACGCTTAAGCTT TTCCCAT AAAATATATG)	–	Expression of RDase		[16]
tceA	*Dehalococcoides mccartyi* 195	797 F (ACGCCAAAGT GCGAAAAGC)	2490R (TAATCTATT CCATCCTTTCTC)	1,693	Entire *tceA* sequencing		[28]
cprAB	*Desulfitobacterium dehalogenans*	PS1 (CATTOCTTGGA TCCTTTTGACTA CGG)	PS2 (CCGGAATTC GATTATCTG CCTTAGG)	1,884	*cprAB* identification		[31]
pceAB	*Sulfurospirillum multivorans*	PS3 (TGGAAAAGAAAA AAAAGCCTGAACTC)	PS4 (CTTCATCA GCGCTTTGAA TTCCACTCC)	1,874	*pceAB* identification		[31]
pceA	*Desulfitobacterium* sp. Y51	PCEAF (GGCGGGGGAT CCAATGGGAGAA ATCAAC)	PCEAR (GGC GGGTCGACTT GTTTTATAGA CTCAG)	1,700	Expression of RDase		[29]
pceAB	*Dehalobacter restrictus*	PCE1f (ATGCAATTA TTATTAAGGAGG AAG)	PCE2r (AGCAG AAATAGTATC CGAACT)	–	Entire *pceAB* sequencing	1,656 bp for *pceA* and 318 bp for *pceB*	[14]

Gene	Organism	Forward primer	Reverse primer	Size (bp)	Target	References
pceA	Dehalobacter restrictus PER-K23 and Desulfitobacterium spp. strains TCE1 and PCE-1	SpDr11f (TTGGATGA GGCCTTGAAGC)	SpDr9r (GCGCTG CATAATAG CCAAGC)	618	Known CE-RDases[a] detection	[26]
pceA	Dehalobacter restrictus PER-K23 and Desulfitobacterium spp. strains TCE1 and PCE-1	SpDr1f (CGTTGGAC CTATTCCACCTG)	SpDr1r (CAAGAACGAA GGCAATCACA)	199	Known CE-RDases[a] detection	[26]
pceA	Sulfurospirillum multivorans	SpSm1f (TCGTTGCAG GTATCGCTATG)	SpSm1r (TTCAACAGCA AAGGCAACTG)	194	Known CE-RDases[a] detection	[26]
tceA	Dehalococcoides ethenogenes 195	SpDe1f (GCTTTTGGCG GTGATGATAAG)	SpDe1r (GTTATAGCCAA GGCCTGCAA)	194	Known CE-RDases[a] detection	[26]
rdhA	Sulfurospirillum multivorans	SpSmAL3f (CCCCT GAGTATGGACCA ATG)	SpSmAL3r (GTAGCGGCCA TTTGCATTAT)	199	Known CE-RDases[a] detection	[26]
rdhA	Desulfitobacterium sp. strains PCE1 (forward) and Dehalobacter restrictus PER-K23 (reverse)	SpDPAS9f (TGAAAAA GGGCCTTATGCTA)	SpDPAS9r (CAATTCCGAA ACCATTCAGG)	199	known CE-RDases[a] detection	[26]
vcrAB	Dehalococcoides mccartyi VS	Unnamed (CTATGAAGGCC CTCCAGATGC)	Unnamed (GTAACAG CCCCAATATG CCAAGTA)[b]	–	vcrAB identification	[15]
bvcA	Dehalococcoides mccartyi BAV1	bvcA F (TGCCTCAA GTACAGGTGGT)	bvcA R (ATTGTGTGA GGACCTACCT)	839	putative bvcA gene identification	Another 7 primer pairs targeting $rdhA1_{BAV1}$-$rdhA7_{BAV1}$ [17]

(continued)

Table 3
(continued)

Target gene	Target OHRB	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)	Application potential	Comments	References
cprAB	*Desulfitobacterium hafniense* strain PCP-1	HafRdA1-G (TGC GGC ACT TTT CTT GAT CGC)	HafRdA1 (GTC TTA AGC AAA TGA CGC AGC)	2300	*cprAB* sequencing		[55]
rdhA1	*Dehalococcoides*-containing mixed culture KB1.	rdhA1_246f (ATCGGAGC TGCACAAGTAGG)	rdhA1_336r (TCTTGTGAG CGGTGTCTTTG)	–	Putative *rdhA* identification	Another 13 primer pairs targeting *rdhA2-rdhA14*	[33]
dcaA	*Desulfitobacterium dichloroeliminans* DCA1	DcaF (CAGGCAAG AAAGATACGG)	DHL-REV (CAGGCTCAT TAGCTATTTCA)	282	Southern blot hybridisation		[38]
rdhA	*Sulfurospirillum* spp.	Sul-rdhA-f (TTRGTRGG TRTTGCAAGATT)	Sul-rdhA-r (CTTGTCCTA AACCTGCTTC)	–	T-RFLP		[56]
rdhA	ACT-3 enrichmnet culture (containing *Dehalobacter* sp.)	rdhA_23f (AAGAGATT GTAGAAGCAGCGG)	rdhA_1383r (CTTAGTAAAT GGGCAAGCAGC)	1,371	Sanger sequencing		[24]
cfrA	ACT-3 enrichmnet culture (containing *Dehalobacter* sp.)	cfrA-413f (CCCGAACCTCT AGCACTTGTAG)	cfrA-531r (ACGGGCAAA GCTTGCACGA)	–	Putative *rdhA* identification		[24]
dcrA	ACT-3 enrichmnet culture (containing *Dehalobacter* sp.)	dcrA-424f (AGCACTCA GAGAGCGTTT TGC)	dcrA-533r (CAACG GCCCAGCT TGCAT)	–	Putative *rdhA* identification		[24]

| dcpA | Dehalococcoides mccartyi RC and KS, Dehalogenimonas lykanthroporepellens BL-DC-9 | dcpA-360 F (TTGCGTGA TCAAATTG GAGCCTGG) | dcpA-1449R (TTTAAAC AGCGGGCAGGT ACTGGT) | 1,089 | Putative rdhA identification | [57] |

^aCE-RDases = chloroethene reductive dehalogenases
^bThe sequence of this primer in the original publication by Müller et al. [15] was missing a 'C' as confirmed by the author. The primer sequence given in the table is therefore corrected

various OHRB encoding *tceA* [28], *bvcA* [17], *vcrA* [15], *cfrA* [24], *dcrA* [24] and *dcpA* [57] (Table 3). Some primer sets have been designed to obtain complete *rdhAB* sequences of specific reductive dehalogenases, including *pceAB* [14, 16, 31], *vcrAB* [15] and *cprAB* [31, 55].

To connect the presence of specific *rdhA*s with OHR activity, multiple primer sets were developed for *rdhA* quantification (Table 4). For instance, by using *D. mccartyi*-specific primer sets targeting *bvcA* (BVC925F/1017R), *vcrA* (Vcr1022F/1093R) and *tceA* (TceA1270F/1336R), Ritalahti et al. [62] were able to link abundance and identity of *D. mccartyi* populations to different organohalide electron acceptors. From this, the authors were able to predict the composition of a *D. mccartyi* community using the quantitative results of *rdhA* and 16S rRNA gene-targeted quantitative PCR (qPCR). qPCR assays with *rdhA* targeted primers have also been used for monitoring of bioremediation in polluted sites [38, 57, 62]. Building upon this knowledge, microfluidics-based, moderately to massively parallel qPCR approaches were recently developed for covering much of the known *rdhA* sequence space. The method was helpful in quantitative analysis of *rdhA* repertoires and identification of closely related populations of OHRB [64, 65]. It should be noted that to date, all known RDase and 16S rRNA genes identified in the genomes of known *D. mccartyi* strains occur as single copies (unlike *Desulfitobacterium* spp. and *Dehalobacter* spp. strains with multiple copies of 16S rRNA genes), suggesting that qPCR data can be converted from gene copy numbers to cell numbers, albeit with the restriction that actively growing cultures might contain multiple genomes per cell.

Primers have additionally been designed for reverse transcription, which, in conjunction with qPCR, was applied to quantify specific *rdh* transcripts from microbial samples. From this, genes encoding the A and B subunits of reductive dehalogenases were found to be co-transcribed [38, 39, 66], confirming previous Northern blot hybridisation and gene-spanning RT-PCR examinations of *Desulfitobacterium dehalogenans* transcripts [58]. Nevertheless, the accuracy of RT-PCR is limited by inefficiencies in reverse transcription and loss during sample processing. To account for this, Johnson et al. [60] introduced an exogenous internal reference mRNA for normalisation of RT-PCR, thereby improving the accuracy of quantification and allowing quantification of mRNA loss during specific steps, including RNA extraction, RT-PCR and qPCR.

Table 4
Specific primer sets designed for quantitative PCR (qPCR) and RT-PCR (reverse transcriptase PCR)

Target gene	Target OHRB	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)	Application potential	Probe	References
cprA	Desulfitobacterium dehalogenans	BG475 (GGCAGGTC TGGGAGAATTG)	BG476 (GTCTCTGTAC CATAGCCAAAG)	–	RT-PCR	–	[58]
cprA	Desulfitobacterium dehalogenans and Desulfitobacterium sp. PCE-1	D5(CTBGCNCC BGAYAARCC)	D9 (TCCAVGGR CANAC)	–	RT-PCR	–	[31]
rddA	Desulfitobacterium sp. PCE-1	D4 (GGIGCIGA YYTIGTIGG)	D7 (ATIGGYT TRTCIGG)	–	RT-PCR	–	[31]
vcrA	Dehalococcoides mccartyi VS	vcrAf (TGCTGGTGGC GTTGGTGCTCT)	vcrAr (TGCCCGTCAAA AGTGGTAAAG)	441	RT-PCR	–	[15]
tceA	Dehalococcoides mccartyi 195, FL2; mixed culture YK-TCE1, PM-VC1, RC-VC2 [59]; TCE enriched ANAS culture containing Dehalococcoides [60]	TceA1270F (ATCCAGATT ATGACCC TGGTGAA)	TceA1336R (GCGGGCATATAT TAGGGCATCTT)	66	qPCR and RT-PCR	TGGGCTATG GCGACCGC AGG	[60]
vcrA	TCE-enriched ANAS culture containing Dehalococcoides	unnamed (CTCGGCT ACCGAACGGATT)	Unnamed (GGGCAG GAGGATT GACACAT)	–	qPCR	CGCACTGGTTATG GCAACCACTC	[61]
bvcA	TCE-enriched ANAS culture containing Dehalococcoides	unnamed (GGTGCC GCGACTT CAGTT)	Unnamed (TCGGCACT AGCAGCA GAAATT)	–	qPCR	TGCCGAATTTT CACGACTTG GATGAAG	[61]

(continued)

Table 4
(continued)

Target gene	Target OHRB	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product size (bp)	Application potential	Probe	References
bvcA	*Dehalococcoides mccartyi* BAV1	Bvc925F (AAAAGCACTTGGCTATCAAGGAC)	Bvc1017R (CCAAAAGCACCACCAGGTC)	92	qPCR	TGGTGGCGACGTGGCTATGTGG	[62]
vcrA	*Dehalococcoides mccartyi* GT and VS	Vcr1022F (CGGGGCGGATGCACTATTTT)	Vcr1093R (GAATAGTCCGTGCCCTTCCTC)	71	qPCR	CGCAGTAACTCAACCATTTCCTGGTAGTGG	[62]
dcaA	1,2-DCA-enriched culture 6VS	DHL F1 (GGACCTCGTTGGACTCC)	DHL R1 (GGCAAATCCCATGGCATTA)	383	RT-PCR	–	[38]
dcaA	1,2-DCA-enriched culture 6VS	DHL F2 (GTTAAAAAGGCAGCCTGTT)	DHL R1 (GGCAAATCCCATGGCATTA)	130	RT-PCR	–	[38]
dcaB	1,2-DCA-enriched culture 6VS	DHL F1 (GGACCTCGTTGGACTCC)	DcaB rev (TGGTATTCACGCTCCGA)	1462	RT-PCR	–	[38]
dcaA	*Desulfitobacterium dichloroeliminans* strain DCA1	DH3F (ATTGGGAGAAGCATGCAGGT)	DH3R (GACCACCGTTATAGGCCCAGA)	526	qPCR	–	[38]
rdhA1	WL cultures (containing *Dehalobacter* sp.)	WLrdhA1f (GCAGGAAGATTCTAAAACCTTG)	WLrdhA1r (CACCGAGGTACTGGAAATGA)	484	qPCR and RT-PCR	–	[39]
rdhA2	WL cultures (containing *Dehalobacter* sp.)	WLrdhA2f (AGTTTTTGGAGAGGATGTTGAA)	WLrdhA2r (GGCCAAAGGAATACTCATTGA)	236	qPCR	–	[39]

Gene	Target	Forward primer	Reverse primer	Size	Method	Probe	Ref.
rdhA3	WL cultures (containing *Dehalobacter* sp.)	WLrdhA3f (ATTAAACT GCGTAATGGGTGG)	WLrdhA3r (CAAGGTTT TAGAATCCTCCTGC)	352	qPCR	–	[39]
rdhAB1	WL cultures (containing *Dehalobacter* sp.)	WLrdhA1f (GCAGGAA GATTCTAAAAACC TTG)	WLrdhB1r (CTTTTCAG ATACCGCCAGATTC)	–	RT-PCR	–	[39]
cprA	*Dehalobacter* sp. TCP-1	debcprAF (CATCAG CTGTGCCAATG GAA)	debcprAR (CGGATAC AGCTCGCGTCT TT)	60	qPCR	–	[48]
rdh1	*Shewanella sediminis*	rdh1_F (GCAAGCC ATCTTACCCAT GT)	rdh1_R (CCGTGT CGGTATCGCT AAAT)	200	qPCR	–	[63]
rdh2	*Shewanella sediminis*	rdh2_F (GTTCCAGC GTGGATCGTTAT)	rdh2_R (CCCGGTTA CCAGCAGATAGA)	200	qPCR	–	[63]
rdh3	*Shewanella sediminis*	rdh3_F (GTCCCAGC GTTTATCACGTT)	rdh3_R (TTGCTGG CATGGAATGAATA)	200	qPCR	–	[63]
rdh4	*Shewanella sediminis*	rdh4_F (CGCGGTT GGTTATTCAACTT)	rdh4_R (CACTTCT GGCGTCATCAAGA)	200	qPCR	–	[63]
rdh5	*Shewanella sediminis*	rdh5_F (GGGTCCGA TTAAGCCGGGGCG)	rdh5_R (CATCGGCG CCATCCGGTTCA)	200	qPCR	–	[63]
dcpA	*Dehalococcoides mccartyi* RC and KS, *Dehalogenimonas lykanthroporepellens* BL-DC-9	1257 F (CGATGTGCC AGCCATTGTGTC TTT)	1449R (TTTTAAA CAGCGGGCAG GTACTGGT)	192	qPCR and RT-PCR	ACGTCATCTC AGATGAAGGC AGAGCT	[57]

Abbreviations: R = A or G; Y = C or T; B = C, G or T; V = A, C or G

6   Conclusions

Degenerate primers have been valuable tools for discovery of new *rdhA* sequences. Their application is not costly and does not need complicated bioinformatics data analysis. Specific primers, on the other hand, have been successfully applied for quantitative analysis of OHRB and their biomarkers and functionality proofs of the identified genes. The hunt for new *rdhAs* was moved to a high-throughput approach by combined application of primer-dependent analysis and next-generation amplicon sequencing [54]. Additionally, primer-independent metagenomic surveys are expected to further broaden the diversity of currently known *rdh* genes, as has already been demonstrated in marine subsurface sediments [67]. Combined application of amplicon-based or meta-genomic surveys with high-throughput quantitative analysis methods [65] should be instrumental in obtaining a comprehensive understanding of OHRB and their catabolic reductive dehalogenation gene pools.

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Primers: Functional Genes for Nitrogen-Cycling Microbes in Oil Reservoirs

C.J. Smith, B.A. McKew, A. Coggan, and C. Whitby

Abstract

Microbial communities found in the subsurface are important in the biogeochemical cycling of nitrogen (N) both in the oxidative and reductive processes, and changes in their functional structure might affect the stability of a petroleum reservoir. In petroleum reservoirs, where in situ conditions are predominantly anoxic, denitrification involving the stepwise reduction of nitrate (NO_3^-) via nitrite (NO_2^-) and nitric oxide (NO) to nitrous oxide (N_2O) or dinitrogen gas (N_2) is a major process. Microorganisms may also decompose organic N to ammonium (NH_4^+) by ammonification, which can subsequently be oxidised to NO_3^- via NO_2^- by the process of nitrification. Autotrophic ammonia oxidation is known in three groups of microorganisms: aerobic autotrophic ammonia-oxidising bacteria (AOB) and Archaea (AOA) and anaerobic ammonia-oxidising bacteria (anammox). Since the microorganisms involved in many of these N transformations are taxonomically diverse, 16S rRNA-based methods are generally not suitable. Instead, a common approach has been to target the protein-encoding genes involved in the transformation of N as biomarkers. This chapter describes the common PCR primers that have been used to target the major functional genes involved in the cycling of N, with the key N transformations likely to occur in petroleum reservoirs highlighted throughout.

Keywords: Anammox, Denitrification, Nitrate reductase, Nitrification, Nitrite reductase

1 Introduction

In general, petroleum contains ~0.1–2% nitrogen (N) (in the form of diverse organic compounds), and so it may act as a N pool in petroleum reservoir systems [1]. Nitrogen may also be injected into reservoirs as a method for enhanced oil recovery. In oil reservoirs, microorganisms have a major influence in the biochemical transformations of N, both in the oxidative and reductive processes (Fig. 1). Microbes decompose organic N to ammonium (NH_4^+) (by ammonification), which can be oxidised to nitrite (NO_2^-) and then nitrate (NO_3^-) by nitrification. NO_3^- can be assimilated into cellular biomass, or it can be dissimilated in denitrification, anammox and dissimilatory NO_3^- reduction to NH_4^+ (DNRA),

depending on the environmental conditions. Additionally, denitrifying anaerobic methane oxidation (DAMO) couples anaerobic oxidation of methane (CH_4) with the reduction of NO_2^- to dinitrogen gas (N_2) [2]. In reservoirs where N availability may be limited, N may become available through the decomposition of organic material, whilst denitrification, anammox and DAMO remove fixed N from the system. In this chapter, the common PCR primers used to target the major functional genes involved in the cycling of N are described, with the key N transformations likely to occur in petroleum reservoirs highlighted throughout. The chapter also provides information on common applications of the primers and the environments from which the primers were first used.

2   Microbial Denitrification

Denitrification is a respiratory process that involves the stepwise reduction of NO_3^- via NO_2^- and nitric oxide (NO) to nitrous oxide (N_2O) or N_2 and is a major process in petroleum reservoirs (Fig. 1) [3]. Denitrification is the main biological process for the return of fixed nitrogen into the atmosphere and results in a net loss of nitrogen from the environment. Denitrification involves the metalloenzymes: nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase [4].
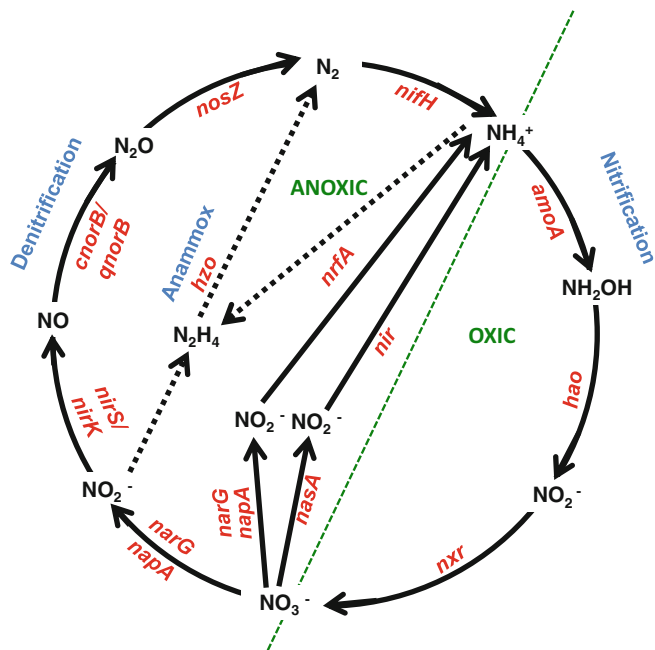
Fig. 1 Nitrogen cycle and key functional genes involved

3 Nitrate Reductase (*narG*, *napA* Genes)

Microorganisms capable of reducing NO_3^- are widespread in the environment [5]. In petroleum reservoirs, hydrogen sulphide (H_2S) produced by sulphate-reducing prokaryotes (SRPs) is a main cause of reservoir corrosion and souring. NO_3^- injection is commonly used by the petroleum industry to mitigate reservoir souring, since NO_3^- and NO_2^- are energetically more favourable terminal electron acceptors than sulphate, and nitrogen cycle-related microorganisms activated by injected NO_3^- may outcompete SRPs for available substrates. Anaerobic oxidation of sulphide to elemental sulphur coupled to the reduction of nitrate to ammonia may also occur.

The reduction of NO_3^- to NO_2^- is catalysed by different nitrate reductases that differ in their cellular location and biochemical properties: the membrane-bound respiratory (NAR), the periplasmic dissimilatory (NAP) [6] and the cytoplasmic assimilatory (NAS) [7]. In general, NAR is more widespread among microorganisms, whilst NAP is restricted to Gram-negative bacteria [8].

Dissimilatory NO_3^- reduction to NO_2^- can be performed by taxonomically diverse microorganisms including members of the Alpha-, Beta-, Gamma- and Epsilonproteobacteria and Archaea [9]. Since microbes capable of NO_3^- reduction are taxonomically diverse, 16S rRNA-based methods are generally not suitable. Thus, many studies have generally focussed on using the *narG* and *napA* genes as biomarkers for the whole NO_3^--reducing community (i.e. denitrifiers and microbes reducing NO_3^- to NH_4^+). However, designing primers specific to all *narG* sequences is problematic due to the high taxonomic diversity of nitrate-reducing prokaryotes [6].

One set of *narG* primers (narG1960f/narG2650r, Table 1) designed to amplify a 650 bp product was used in a direct PCR approach [8]. These primers target a wide range of microorganisms belonging to Proteobacteria and Archaea [8]. Although these primers provided a much higher phylogenetic diversity of the NO_3^--reducing community than previous studies, the authors report non-specific amplification, with 9% of sequences recovered showing no significant identity with *narG* [8]. Later, Gregory et al. [16] designed a more specific nested PCR approach to target a 366 bp fragment of the *narG* gene using the primers T37/T39 (first round of amplification) and W9/T38 (second round of amplification) (Table 1). However, phylogenetic analysis of NO_3^--reducing bacteria and denitrifying bacteria showed that amplification of the *narG* genes results in poor taxonomic resolution when applied to environmental samples and greater resolution may be obtained with the *napA* gene [9, 15].

Table 1
PCR primers for the detection of the nitrate reductase genes (*narG* and *napA*) involved in the reduction of NO_3^- to NO_2^-

Primers	Gene	Specificity	Sequences (5'-3') forward/reverse	~Product length (bp)	Applications/environment primers first used/ relevant to oil reservoirs[a]	Reference
V16/V17	*napA*	Nitrate-reducing bacteria from at least four genera: *Pseudomonas, Paracoccus, Rhodobacter* and *Moraxella*	GCN CCN TGY MGN TTY TGY GG/RTG YTG RTT RAA NCC CAT NGT CCA	296	Nested PCR with V67F/V67R/freshwater sediments	[10]
V67F/V67R	*napA*	Nitrate-reducing bacteria	TAY TTY YTN HSN AAR ATH ATG TAY GG/DAT NGG RTG CAT YTC NGC CAT RTT	414	V67F/V67R used in a non-nested approach in [11]/estuary sediments	[10]
napA-1F/*napA*-1R	*napA*	Nitrate-reducing bacteria, phylotype *napA*-1	GTY ATG GAR GAA AAA TTC AA/GAR CCG AAC ATG CCR AC	111	Q-PCR used with *Taq*Man probe *napA-1* (TM-MGB) AACATGACCTGGAAG/ estuary sediments	[11]
napA-2F/*napA*-2R	*napA*	Nitrate-reducing bacteria, phylotype *napA*-2	GAA CCK AYG GGY TGT TAT G/TGC ATY TCS GCC ATR TT	76	Q-PCR used with *Taq*Man probe *napA-2* (TM-MGB) CTTTGGGGTTCAA/ estuary sediments	[11]
napA-3F/*napA*-3R	*napA*	Nitrate-reducing bacteria, phylotype *napA*-3	CCC AAT GCT CGC CAC TG/CAT GTT KGA GCC CCA CAG	130	Q-PCR used with *Taq*Man probe *napA-3* (TM-MGB) TGGGTTGTTACGA/ estuary sediments	[11]
V17m/napA4r	*napA*	Nitrate-reducing Alpha-, Beta- and Gammaproteobacteria	TGG ACV ATG GGY TTY AAY C/ACY TCR CGH GCV GTR/CCR CA	152	SYBR Green Q-PCR/soils, river sediments, waters, biofilms	[12]
LF716/ SFI173b/ OF604 (forward)/ SR2294 (reverse)	*napA*	Nitrate-reducing Alpha-, Beta-, Gamma-, Delta- and Epsilonproteobacteria	GCN GAR ATG CAC CC/ GTA TGG GNN TNA ACC/ GGN KCH AAY ATG GC/ GWR TGG CCA RTG NTC	1,500–1,800 ~1,000–1,800	Combination of three forward primers with a single reverse primer to amplify *napA* from all five groups of Proteobacteria/human faeces	[13]
napAfl/napArl	*napA*	Nitrate-reducing bacteria	CTG GAC IAT GGG YTT IAA CCA/CCT TCY TTY TCI ACC CAC AT	492	PCR/production water/relevant to oil reservoir	[14] (based on [15])

T37/T39	*narG*	Nitrate-reducing bacteria	CAY GGN GTN AAY TGY CAN GG/TAR TGN GGC CCA NCC NCC NCC	366	Nested PCR/freshwater sediments	[16]
W9/T38	*narG*	Nitrate-reducing bacteria	MGN GGN TGY CCN MGN GGN GC/ACR TCN GTY TGY TCN CCC CA			
narG1960f/narG2650r	*narG*	Proteobacteria, Gram-positive bacteria and Archaea	TAY GTS GGS CAR GAR AA/TTY TCR TAC CAB GTB GC	650	PCR/soils	[8]
1960m2f/2050m2r	*narG*	Nitrate-reducing Proteobacteria	AYG TSG GGC AGG ARA AAC TG/CGT AGA AGA AGC TGG TGC TGT T	110	SYBR Green Q-PCR/soils	[17]
narG-f/narG-r	*narG*	Nitrate-reducing Proteobacteria	TCG CCS ATY CCG GCS ATG TC/GAG TTG TAC CAG TCR GCS GAY	173	SYBR Green Q-PCR/soils, river sediments, waters, biofilms	[12]
narG-1F/*narG*-1R	*narG*	Nitrate-reducing bacteria, phylotype 1 *narG*-1	GAC TTC CGC ATG TCR AC/TTY TCG TAC CAG GTG GC	69	Q-PCR used with *TaqMan* probe *narG*-1 (TM-MGB) TAY TCC GAC ATC GT/estuary sediments	[11]
narG-2F/*narG*-2R	*narG*	Nitrate-reducing bacteria, phylotype 2 *narG*-2	CTC GAY CTG GTG GTY GA/TTY TCG TAC CAG GTS GC	89	Q-PCR used with *TaqMan* probe *narG*-2 (TM-MGB) AAC TTC CGC ATG GA/estuary sediments	[11]
narG328F/narG497R	*narG*	Nitrate-reducing Proteobacteria	GAC AAA CTT CGC AGC GG/TCA CCC AGG ACG CTG TTC	170	Q-PCR/freshwater sediments	[18]
arc-Nred-f/arc-Nred-r	*napA*, *narG*	Nitrate-reducing Archaea	CGA CTG GTA YKC VGA YCT HC/GTC RGY GTK RWA CCA GTS GK	760–1,030	PCR, several bands produced/lakewater	[19]

TM-MGB TaqMan minor groove binding

Ambiguity codes: N = G, A, T or C; W = A or T; Y = C or T; M = A or C; R = G or A; K = G or T; S = G or C; H = A, C or T; D = G, A or T; B = C or G or T; V = A or C or G, I = inosine

a If using primers for Q-PCR, it is advisable to adhere to MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines [20] (for further details, see chapter by [21]

In contrast to the *narG* gene, the *napA* gene corresponding to the Alpha-, Beta-, Gamma- and Epsilonproteobacteria has good congruence with 16S rRNA gene-based taxonomy [9]. Flanagan et al. [10] designed primers for a nested PCR reaction and amplified the *napA* sequences from at least four genera (*Pseudomonas*, *Paracoccus*, *Rhodobacter* and *Moraxella*) (Table 1). Feng et al. [14] used the primers (napAf1/napAr1, Table 1) to detect the *napA* genes in production water samples from oil reservoirs. It is apparent that many bacteria capable of aerobic NO_3^- respiration contain *napA* genes, which is consistent with the physiological evidence for the presence of periplasmic nitrate reductases in these organisms [22]. However, some strains (e.g. *Pseudomonas* sp. strain S3.29) have periplasmic nitrate reductase genes, and although they express the activity, they are incapable of aerobic NO_3^- respiration [10]. Despite this, NO_3^- reduction in the presence of oxygen catalysed by NAP is widespread and makes a significant contribution to the reduction of NO_3^- in oxic/microoxic environments. Thus, primers that target the *napA* genes have been the preferential choice for assessing taxonomic composition of NO_3^--reducing communities in oilfields [14].

Primers (arc-Nred-f,-r) targeting dissimilatory nitrate reductase in Archaea have also been developed (Table 1) and evaluated in silico. These primers were found to perform better when used under low-stringency PCR protocols [19]. However, when these primers were used with the positive control *Halogeometricum borinquense*, additional products were obtained [19].

Nitrite reductases: cytochrome cd_1 nitrite reductase, copper-containing nitrite reductase (*nirS*, *nirK* genes), ferredoxin nitrite reductase (*nirA* gene) and ammonifying nitrite reductase (*nirB* gene).

A key step in the denitrification pathway is the reduction of NO_2^-. Two structurally different but functionally equivalent nitrite reductase enzymes catalyse nitrite reduction. One enzyme is a homotrimeric copper nitrite reductase (NirK), and the other is a homodimeric cytochrome cd_1 nitrite reductase (NirS) [3]. Generally, denitrifiers contain only one of the two types of nitrite reductases. The *nirS* gene is more widely distributed in denitrifiers than the *nirK* gene [23]. Rarefaction analysis has shown that *nirS* total diversity is more constrained than *nirK* [24]. Many of the commonly used *nirS* and *nirK* primers have some limitations, largely due to a lack of known cultivated denitrifiers limiting the number of functional gene sequences available. High sequence divergence at current primer sites and the availability of only a few deep sequencing studies that allows primers to be evaluated in different environmental settings also contribute to these limitations [24].

Ward [25] designed the first PCR primers targeting the *nirS* gene and was based on three sequences. Braker et al. [23] improved on this and designed several primer sets that target both the *nirS* and *nirK* genes (Table 1). Since then, the number of available *nir* sequences has increased considerably, facilitating the construction of additional modified primers [26–30]. The primer sets cd3aF/nirS4R, cd3bF/nirS6R, F3nirS/R4bcd and F3nirS/NirS6R all amplify the *nirS* gene from several species including *Paracoccus denitrificans*, *Pseudomonas aeruginosa*, *Pseudomonas stutzeri* and *Ralstonia eutropha* [31]. F3nirS/nirS6R primer pair has been shown to have good coverage of the Alphaproteobacteria [24]. Hallin and Lindgren [27] designed the PCR primers (F1acd/R4cd) to amplify 800 bp fragments of the *nirS* gene. Although this primer pair did not amplify genes of non-denitrifying strains, the primers had several shortcomings: the *nirS* gene was amplified from one isolate, which could not reduce nitrite [27]; the *nirS* primers were unable to amplify the *nirS* gene from two strains of *P. denitrificans* even though further sequence analysis of this strain showed that the primer sites are included [27, 32]. According to Throbäck et al. [31], the best primer pair for broad-range *nirS* gene amplification for denitrifier community analysis is cd3aF/R3cd, and the fragment size obtained with this primer set makes it suitable for Denaturing Gradient Gel Electrophoresis (DGGE) and Q-PCR. Both cd3af/R3cd and nirS1F/nirS6R have been shown to perform well in targeting *Chloroflexi*, *Deinococcus-Thermus*, *Aquificales* or *Bacteroidetes* [24].

The majority of current *nirK* primers are based on class I CuNIR genes from Alphaproteobacteria [23, 33] and do amplify the class II and III *nirK* sequences that include archaeal *nirK* [34–36]. The primers F1aCu/R3Cu and F1aCu/nirK3R amplify the *nirK* gene from several strains including *Alcaligenes faecalis*, *Blastobacter denitrificans*, *Pseudomonas denitrificans* and *Hyphomicrobium denitrificans* [27, 31]. Throbäck et al. [31]) suggest that F1aCu/R3Cu is the best primer pair for *nirK* gene amplification for denitrifier community analysis due to the broad coverage and the fragment size generated is applicable for DGGE. However, both F1aCu/R3Cu and F1aCu/nirK3R primer sets also amplify the *nirK* genes from ammonia-oxidising bacteria (AOB) such as *Nitrosospira multiformis* [31]. The primers designed by [26] also amplify the *nirK* genes in some betaproteobacterial AOB [37].

The production of NO and N_2O by the reduction of NO_2^- in AOB is intriguing since these organisms require oxygen to oxidise ammonia and generate energy. It has been suggested that in low-oxygen environments, nitrous oxide is substituted for oxygen as a terminal electron acceptor in denitrification-like respiration [38].

Nitrite reduction by AOB was shown initially by the discovery of a copper-containing nitrite reductase from *Nitrosomonas europaea* with similar biochemical characteristics to the copper-containing nitrite reductases found in heterotrophic denitrifiers [26, 39]. Furthermore, *N. europaea* nitrite reductase expression was found to increase in low-oxygen environments, which is similar to some denitrifying nitrite reductases [26]. Although it is uncertain what the physiological role that nitrite reduction plays in AOB, they appear to share a similar mechanism to classical denitrifiers [26].

In contrast, the primer sets nirK1F/R3Cu, nirK1F/nirK3R and nirK1F/nirK5R also target a broad range of microorganisms including *Alcaligenes* sp., *Achromobacter cycloclastes*, *Blastobacter denitrificans*, *Hyphomicrobium denitrificans* and *Rhizobium meliloti* but not the *nirK* genes from *Nitrosospira multiformis* or *Nitrosomonas europaea* [31]. Other *nirK* primers that have been developed include nirK517F/nirK1055R [40]. Indeed, both F1aCu/R3Cu and nirK517F/nirK1055R have been shown to preferentially amplify Alphaproteobacteria [24]. It has been suggested that there is an urgent need for further investigation into *nirK* primer with focus on alternate primer-binding sites [35].

To this end, Wei et al. [41] reassessed *nirS* and *nirK* phylogeny with the view to improving current PCR primer sets. Full-length *nirK* sequences from the phyla *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Nitrospira*, Proteobacteria and *Spirochetes* and the archaeal phyla Euryarchaeota were aligned forming five distinct phylogenetic clusters of *nirK*. Similarly, for *nirS*, full-length sequences from *Bacteroidetes*, NC10, *Planctomycetes* and *Proteobacteria* revealed three distinct clusters of *nirS*. From these alignments, a suite of seven degenerate primers were designed targeting each cluster (*nirK* I–V 430 to 468 bp, *nirS* I–III 430–468 bp). These primers have yet to be used in oil reservoirs, but their use in a range of soils and sediments revealed a greater diversity and abundance (via SYBR Green Q-PCR) of *nirS/nirK* nitrite reducers than has previously been shown.

Primers (arc-NirA-f,-r) targeting the ferredoxin nitrite reductase in Archaea have also been developed (Table 2). These primers were found to have a high affinity to archaeal *nirA* sequences but little complementarity to bacterial ones [19]. However, when these primers were used with the positive control *Halogeometricum borinquense*, additional products were obtained [19]. Other primers targeting ammonifying nitrite reductase gene (*nirB*) for Archaea (arc-NirB-f,-r, Table 2) have also been developed and found to perform better when used under low-stringency PCR protocols [19].

Table 2

PCR primers for the detection of the cytochrome cd_1 nitrite reductase (*nirS*) and copper-containing nitrite reductase (*nirK*) genes involved in the reduction of NO_2^- to nitric oxide (NO)

Primers	Gene	Specificity	Sequences (5′–3′) forward/reverse	~Product length (bp)	Applications/environment primers first used/relevant to oil reservoirs[a]	Reference
KA3-F/KA25R	*nirS*	Nitrite-reducing bacteria	CAC GGY GTB CTG CGC AAG GGC GC/CGC CAC GCG CGG YTC SGG GTG GTA	225	PCR/position[b] 76–83/301–308, *Paracoccus denitrificans*	[32]
nirS1F/nirS6R	*nirS*	Nitrate-reducing bacteria	CCT AYT GGC CGC CRC ART/CGT TGA ACT TRC CGG T	890	PCR, RFLP, DGGE, T-RFLP, probes, position[c] 763–780/1638–1653/activated sludge, freshwater, freshwater sediments	[23]
nirS2F/nirS3R	*nirS*	Nitrite-reducing bacteria	TAC CAC CCS GAR CCG CGC GT/GCC GCC GTC RTG VAG GAA	164	PCR, position[c] 855–874/1002–1019/activated sludge, freshwater, freshwater sediments	[23]
nirS3F/nirS5R	*nirS*	Nitrite-reducing bacteria	TTC CTB CAY GAC GGC GGC/CTT GTT GWA CTC GSS CTG CAC	512	PCR, SYBR Green Q-PCR position[c] 1002–1019/1494–1514/activated sludge, freshwater, freshwater sediments	[23]
nirS4F/nirS6R	*nirS*	Nitrite-reducing bacteria	TTC RTC AAG ACS CAC CGA A/CGT TGA ACT TRC CGG T	336	PCR, position[c] 1317–1336/1638–1653/activated sludge, freshwater, freshwater sediments	[23]
F1acd	*nirS*	Nitrite-reducing bacteria	TAY CAC CCS GAR CCG C		PCR, position[c] 856–871/activated sludge	[27]
cd3F	*nirS*	Nitrite-reducing bacteria	GTN AAY GTN AAR		PCR, position[c] 916–935/seawater, marine sediments	[28]
cd3aF	*nirS*	Nitrite-reducing bacteria	GTS AAC GTS AAG GAR ACS GG		PCR, SYBR Green Q-PCR, position[c] 916–935/seawater, marine sediments	[28]
cd3bF	*nirS*	Nitrite-reducing bacteria	GTG AAC GTS AAG GAR ACS GG		PCR, position[c] 916–935/seawater, marine sediments	[28]
832F/1606R	*nirS*	Nitrite-reducing bacteria	TCA CAC CCC GAG CCG CGC GT/AGK CGT TGA ACT TKC CGG TCG G	774	PCR, RFLP/groundwater	[30]

(continued)

Table 2
(continued)

Primers	Gene	Specificity	Sequences (5′-3′) forward/reverse	~Product length (bp)	Applications/environment primers first used/relevant to oil reservoirs[a]	Reference
Heme832/ heme1606	nirS	Nitrite-reducing bacteria	TCA CAC CCC GAG CCG CGC GT/ AGK CGT TGA ACT TKC CGG TCG G	774	PCR/groundwater	[30]
F1bcd	nirS	Nitrite-reducing bacteria	TAY CAC CCS GAR CCG CG		PCR, position[c] 856–872/soils	[31]
F1dcd	nirS	Nitrite-reducing bacteria	CAC CCS GAR CCG CGC GT		PCR, position[c] 859–875/soils	[31]
nirS3Fa	nirS	Nitrite-reducing bacteria	TTC CTB CAY GAC GGC GGC		PCR, position[c] 1002–1018/soils	[31]
F3nirS	nirS	Nitrite-reducing bacteria	CCG CAC CCG GGB CGY GG		PCR, position[c] 1132–1148/soils	[31]
R3cd	nirS	Nitrite-reducing bacteria	GAS TTC GGR TGS GTC TTG A		PCR, DGGE, position[c] 1322–1341/soils	[31]
R4bcd	nirS	Nitrite-reducing bacteria	CGT TGA AYT TRC CGG TSG G		PCR, position[c] 1636–1654/soils	[31]
F1acd/R4cd	nirS	Nitrite-reducing bacteria including the genera *Pseudomonas* and *Alcaligenes*	TAY CAC CCS GAR CCG C/CGT TGA ACT TRC CGG TSG G	800	PCR, position[c] 856–871/1636–1654/ activated sludge	[27, 31]
nirSCd3aF (modified)/ nirSR3cd (modified)	nirS	Nitrite-reducing bacteria	AAC GYS AAG GAR ACS GG/GAS TTC GGR TGS GTC TTS AYG AA	406	SYBR Green Q-PCR/glacial soils	[42] (modified from [31])
nirS-efF/nirS-efR	nirS	Nitrite-reducing bacteria, phylotype *nirS-e*	CAC CCG GAG TTC ATC GTC/ACC TTG TTG GAC TGG TGG G	172	Q-PCR used with *Taq*Man probe *nirS*-ef (TM-MGB) TGC TGG TCA ACT A/estuary sediments	[11]
nirS-mF/nirS-mR	nirS	Nitrite-reducing bacteria, phylotype *nirS-m*	GGA AAC CTG TTC GTC AAG AC/ CSG ART CCT TGG CGA CGT	162	Q-PCR used with *Taq*Man probe *nirS*-m (TM) TCT GGG CCG ACG CGC CGA TGA AC/estuary sediments	[11]

Primer	Gene	Target	Sequence	Size (bp)	Application/source	Reference
nirS-nF/nirS-nR (also known as nirS6r)	*nirS*	Nitrite-reducing bacteria, phylotype *nirS-n*	AAG GAA GTC TGG ATY TC/CGT TGA ACT TRC CG GT	140	Q-PCR used with *Taq*Man probe *nirS*-n (TM-MGB) ATC CGA AGA TSA/estuary sediments, activated sludge, freshwater, freshwater sediments	[11, 23]
Scnir372F/Scnir845R	*nirS*	Anammox	TGT AGC CAG CAT TGT AGC GT/TCA AGC CAG ACC ATT TGC T	473	PCR/seawater	[43]
nirS263F/nirS950R	*nirS*	Nitrite-reducing bacteria	TGCGYAARGGGGCNACBGGCAA/GCBACRCGSGGYTCSGGATG	687	PCR/soils	[40]
nirS3F/nirS5R	*nirS*	Nitrite-reducing bacteria	CCT AYT GGC CGC CRC ART/GCC GCC GTC RTG VAG GAA	512	Q-PCR/freshwater sediments	[44] (modified from [23])
nirSC1F/nirSC1R	*nirS*	Nitrite-reducing bacteria cluster I	ATC GTC AAC GTC AAR GAR ACV GG/TTC GGG TGC GTC TTS ABG AAS AG	410–420	PCR, SYBR Green Q-PCR soil, sediment	[41]
nirSC2F/nirSC2R	*nirS*	Nitrite-reducing bacteria cluster II	TGG AGA ACG CCG GNC ARG TNT GG/GAT GAT GTC CAC GGC NAC RTA NGG	410–420	PCR, SYBR Green Q-PCR soil, sediment	[41]
nirSC3F/nirSC3R	*nirS*	Nitrite-reducing bacteria cluster III	TTC GCC CTG AAR GAY GGN GG/AGG TGC CCA CGA ANA RNA CNC C	410–420	PCR, SYBR Green Q-PCR soil, sediment	[41]
nirK1F/nirK5R	*nirK*	Nitrite-reducing bacteria	GGM ATG GTK CCS TGG CA/GCC TCG ATC AGR TTR TGG	514	SYBR Green Q-PCR, DGGE, RFLP, probes, position[d] 526–542,1023–1040/activated sludge, freshwater, freshwater sediments	[23]
nirK2F/nirK3R	*nirK*	Nitrite-reducing bacteria	GCS MTS ATG GTS CTG CC/GAA CTT GCC GGT VGY CCA GAC	353	Position[d] 565–581/898–918/activated sludge, freshwater, freshwater sediments	[23]
nirK3F	*nirK*	Nitrite-reducing bacteria	GAA CTT GCC GGT VGY CCA GAC		Position[d] 898–918/activated sludge, freshwater, freshwater sediments	[23]
nirK4R	*nirK*	Nitrite-reducing bacteria	GGR ATR ARC CAG GTT TCC		Position[d] 942–949/activated sludge, freshwater, freshwater sediments	[23]
R3Cu	*nirK*	Nitrite-reducing bacteria	GCC TCG ATC AGR TTG TGG TT		DGGE, position[d] 1021–1040/activated sludge	[27]

(continued)

Table 2
(continued)

Primers	Gene	Specificity	Sequences (5′-3′) forward/reverse	~Product length (bp)	Applications/environment primers first used/relevant to oil reservoirs[a]	Reference
F1aCu/R3Cu	*nirK*	Nitrite-reducing bacteria including the genera *Pseudomonas* and *Alcaligenes*	ATC ATG GTS CTG CCG CG/GCC TCG ATC AGR TTG TGG TT	473	Position[d] 568–584/1021–1040 PCR, DGGE/activated sludge	[27]
Cunir3/Cunir4	*nirK*	Nitrite-reducing bacteria	CGT CTA YCA YTG CGC VCC/GCC TCG ATC AGR TTR TGG	537–540	PCR/various pure cultures	[26]
nirKCu-583F/nirKCu909R	*nirK*	Nitrite-reducing bacteria	TCA TGG TGC TGC CGC GKG ACG G/GAA CTT GCC GGT KGC CCA GAC	348	PCR, RFLP/groundwater	[30]
583F/909R	*nirK*	Nitrite-reducing bacteria	TCA TGG TGC TGC CGC GKG ACG G/GAAC TTG CCG GTK GCC CAG AC	326	PCR/groundwater	[30]
nirK876/nirK1040	*nirK*	Nitrite-reducing bacteria	ATY GGC GGV CAY GGC GA/GCC TCG ATC AGR TTR TGG TT	265	SYBR Green Q-PCR/soils	[45]
nirK876/nirK1040	*nirK*	Nitrite-reducing bacteria	ATY GGC GGV CAY GGC GA/GCC TCG ATC AGR TTR TGG TT	164	SYBR Green Q-PCR (extra C base in some references)/soils	[45, 46]
nirK517f/nirK1055R	*nirK*	Nitrite-reducing bacteria	TTY GTS TAY CAC TGC GCV CC/GCY TCG ATC AGR TTR TGG TT	538	PCR/soils	[40]
nirKC1F/nirKC1R	*nirK*	Nitrite-reducing bacteria cluster I	ATG GCG CCA TCA TGG TNY TNC C/TCG AAG GCC TCG ATN ARR TTR TG	430–468	PCR, SYBR Green Q-PCR/soil, sediment	[41]
nirKC2F/nirKC2R	*nirK*	Nitrite-reducing bacteria cluster II	TGC ACA TCG CCA ACG GNA TGT WYG G/GGC GCG GAA GAT GSH RTG RTC NAC	430–468	PCR, SYBR Green Q-PCR/soil, sediment	[41]
nirKC3F/nirKC3R	*nirK*	Nitrite-reducing bacteria cluster III	CAT CGG CAA CGG CAT GYA YGG NGC/CGA CCA TGG CCG TGG SWN ACR AAN GG	430–468	PCR, SYBR Green Q-PCR/soil, sediment	[41]

Primer	Gene	Target	Sequence	Size (bp)	Application/sample	Ref
nirKC4F/nirKC4R	nirK	Nitrite-reducing bacteria cluster IV	TAC GGT GTG ATC ATC RTS GAT CC/GCA TCA CGC ATG GAA TGA TYS AC	430–468	PCR, SYBR Green Q-PCR/soil, sediment	[41]
arc-NirA-f/arc-NirA-r	nirA	Nitrite-reducing Archaea	AAY MTS CCN CGG AAG TKS AA/AGA ACT CCB TRC CSG TRC AS	660–750	PCR/lakewater	[19]
arc-NirB-f/arc-Nirb-r	nirB	Nitrite-reducing Archaea	ATG CTG AGC CAT TAY ATA GC/CCG TTG TAC TCG GCR CAG TC	680	PCR/lakewater	[19]

TM-MGB TaqMan minor groove binding, *TM TaqMan*

Ambiguity codes: N = G, A, T or C; W = A or T; Y = C or T; M = A or C; R = G or A; K = G or T; S = G or C; H = A, C or T; D = G, A or T; B = C or G or T; V = A or C or G

[a]If using primers for Q-PCR, it is advisable to adhere to MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines [20] (for further details, see chapter by 21.

[b]Positions in *nirS* gene of *Pseudomonas aeruginosa*

[c]Positions in *nirS* gene of *Pseudomonas stutzeri* ZoBell EMBL X56813

[d]Positions in *nirK* gene of *Alcaligenes faecalis* S-6 EMBL D13155

4   Nitric Oxide Reductase (*norB* Gene)

Nitric oxide is produced as an intermediate during respiratory denitrification, when oxidised nitrogen compounds are used as alternative electron acceptors under oxygen-limited conditions [3]. Two types of nitric oxide reductase enzymes catalyse this reaction: one receives the electrons from cytochrome c (cNor) and the other from the quinone pool (qNor). Nitric oxide reductase catalyses the reduction of NO to N_2O and is encoded by the *norB* gene. The *norB* gene is widespread in denitrifying bacteria but has also been found in some AOB [47]. Braker and Tiedje [48] developed *norB* primer sets to detect both types of nitric oxide reductase genes in environmental samples (Table 3). In general, these primer sets allowed a broad detection of the *norB* gene in all the denitrifier strains tested [48]. However, the primer pair targeting qNor (qnorB2F/qnorB5R) yielded *qnorB* amplification products of the correct size for the non-denitrifying strain *Synechocystis* sp. strain PCC6803 [48]. In addition, the primer combination cnorB2F-cnorB7R, which should have generated products of 454 bp, yielded amplification products of unexpected sizes [48].

5   Nitrous Oxide Reductase (*nosZ* Gene)

The reduction of N_2O to N_2 (i.e. the last step in the complete denitrification pathway which represents loss of biologically available N) is catalysed by nitrous oxide reductase, which is encoded by the *nosZ* gene. Although the *nosZ* gene is largely unique to denitrifying bacteria, a few non-denitrifier species capable of reducing nitrous oxide have been identified [3]. In addition, some denitrifiers lack the nitrous oxide reductase enzyme [31]. The *nosZ* gene has been used as a target for the different populations of denitrifying bacteria capable of nitrous oxide reduction [31].

PCR primer sets for the detection of the *nosZ* gene were published by Scala and Kerkhof [50]. These primers were based on three sequences from the cultures *Paracoccus denitrificans* and *Pseudomonas denitrificans*. Different combinations of these primers give different-sized products and can be used in other applications, e.g. DGGE and Restriction Fragment Length Polymorphism (RFLP) (Table 3). More degenerate *nosZ* gene primers were later developed [52]. The primer sets nosZ-F/nosZ-R, No661F/Nos1527R, Nos661F/Nos1773R and Nos1527F/Nos1773R have a broad specificity and target the *nosZ* genes from several species including *Alcaligenes denitrificans*, *Alcaligenes faecalis*, *Blastobacter denitrificans*, *Paracoccus denitrificans*, *Pseudomonas aeruginosa*, *Pseudomonas denitrificans*, *Pseudomonas stutzeri* and *Ralstonia eutropha* [31, 51]. The primer set nosLb/nosRb has a limited specificity targeting *Pseudomonas* spp. and *Paracoccus*

Table 3
PCR primers for the detection of the nitric oxide reductase (*norB* gene) and nitrous oxide reductase (*nosZ* gene) both involved in the denitrification pathway, nitrogenase reductase (*nifH* gene) involved in nitrogen fixation and nitrite reductase (*nrfA* gene) involved in dissimilatory nitrate reduction to ammonium (DNRA)

Primers	Gene	Specificity	Sequences (5′-3′) forward/reverse	~Product length (bp)	Applications/environment primers first used/relevant to oil reservoirs[a]	Reference
norB1	*norB*	Denitrifiers	CGN GAR TTY CTS GAR CAR CC		PCR, position[b] 400–419/various pure cultures	[47]
norB2	*norB*	Denitrifiers	GAC AAR HWY TAY TGG TGG T		PCR, position[b] 592–610/various pure cultures	[47]
norB3	*norB*	Denitrifiers	CCY TCV ACC CAG ASA TGC AS		PCR, position[b] 616–635/various pure cultures	[47]
norB6	*norB*	Denitrifiers	TGC AKS ARR CCC CAB ACB CC		PCR, position[b] 967–986/various pure cultures	[47]
norB7	*norB*	Denitrifiers	CCR TGG STR WAR WAR TTS AC		PCR, position[b] 1000–1019/various pure cultures	[47]
norB8	*norB*	Denitrifiers	CRT ADG CVC CRW AGA AVG C		PCR, position[b] 1051–1069/various pure cultures	[47]
qnorB2F	*qnorB*	Denitrifiers	GGN CAY CAR GGN TAY GA		PCR, position[c] 1204–1220/freshwater sediments	[48]
qnorB5R	*qnorB*	Denitrifiers	ACC CAN AGR TGN CAN ACC CAC CA		PCR, position[c] 1466–1444/freshwater sediments	[48]
qnorB7R	*qnorB*	Denitrifiers	GGN GGR TTD ATC ADG AAN CC		PCR, position[c] 1481–1822/freshwater sediments	[48]
cnorB1F	*cnorB*	Denitrifiers	GAR TTY CTN GAR CAR CC		PCR, position[c] 364–380/freshwater sediments	[48]
cnorB2F	*cnorB*	Denitrifiers	GAC AAG NNN TAC TGG TGG T		PCR, position[c] 553–571/freshwater sediment	[48]
cnorB6R	*cnorB*	Denitrifiers	GAA NCC CCA NAC NCC NGC		PCR, position[c] 942–925/freshwater sediments	[48]
cnorB7R	*cnorB*	Denitrifiers	TGN CCR TGN GCN GCN GT		PCR, position[c] 1007–991/freshwater sediments	[48]
nosLb	*nosZ*	Denitrifiers	CCC GCT GCA CAC CRC CTT CGA		PCR, position[d] 1124–1144/soils	[49]
nosRb	*nosZ*	Denitrifiers	CGT CGC CSG GAG ATG TCG ATC A		PCR, position[d] 1405–1425/soils	[49]
Nos2230R	*nosZ*	Denitrifiers	TTC CAT GTG CAG CGC ATG G		PCR/marine sediments	[50]
Nos661F/Nos1773R	*nosZ*	Denitrifiers	CGG CTG GGG GCT GAC CAA/ATR TCG ATC ARC TGB TCG TT	1,100	PCR, RFLP/marine sediments[e]	[50]
Nos1527F/Nos1773R	*nosZ*	Denitrifiers	CGC TGT TCH TCG ACA GYC A/ATR TCG ATC ARC TGB TCG TT	250	PCR, DGGE, SYBR Green Q-PCR/marine sediments[e]	[50]

(continued)

Table 3
(continued)

Primers	Gene	Specificity	Sequences (5′-3′) forward/reverse	~Product length (bp)	Applications/environment primers first used/relevant to oil reservoirs[a]	Reference
Nos661F/ Nos1527R	*nosZ*	Denitrifiers	CGG CTG GGG GCT GAC CAA/CTG RCT GTC GAD GAA CAG	900	PCR, RFLP/marine sediments[c]	[50]
Nos1111F	*nosZ*	Denitrifiers	STA CAA CWC GGA RAA SG		Sequencing/marine sediments[c]	[50]
Nos1111R	*nosZ*	Denitrifiers	SCS TTY TCC GWG TTG TA		Sequencing/marine sediments[c]	[50]
Nos1484F	*nosZ*	Denitrifiers	CCG CTG CAC ACS RCC TTC GA		Sequencing/marine sediments[c]	[50]
Nos1484R	*nosZ*	Denitrifiers	TCG AAG GYS GTG TGC AGC GG		Sequencing/marine sediments[c]	[50]
nosZ-F/ nosZ-R	*nosZ*	Denitrifiers	CGY TGT TCM TCG ACA GCC AG/CAT GTG CAG NGC RTG GCA GAA	453	Position[d] 1169–1188/1849–1869 PCR/various pure cultures	[51]
nosZ661b	*nosZ*	Denitrifiers	CGG YTG GGG SMW KAC CAA		RT-PCR, position[d] 303–320/estuary sediments	[52]
nosZ661b/ nosZ1773b	*nosZ*	Denitrifiers	CGG YTG GGG SMW KAC CAA/ATR TCG ATC ARY TGN TCR TT	1,100	RT-PCR, position[d] 303–320/1396–1415/estuary sediments	[52]
nosZF-1181/ nosR-1880	*nosZ*	Denitrifiers	CGC TGT TCI TCG ACA GYC AG/ATG TGC AKI GCR TGG CAG AA	700	PCR, T-RFLP/soils	[53]
nosZ1622R	*nosZ*	Denitrifiers	CGC RAS GGC AAS AAG GTS CG		DGGE, position[d] 1603–1622/soils	[31]
nosZ1F/ nosZ1R	*nosZ*	Denitrifiers	WCS YTG TTC MTC GAC AGC CAG/ATG TCG ATC ARC TGV KCR TTY TC	259	SYBR Green Q-PCR/soils	[54]
nosZ2F- nosZ2R	*nosZ*	Denitrifiers	CGC RAC GGC AAS AAG GTS MSS GT	267	SYBR Green Q-PCR/soils	[54]
arc-Nos-f/ arc-Nos-r	*nosZ*	Denitrifying Archaea	TGG GGH GAC TMY CAY CAY CC/AKG TGK CCR RSG TTG TAG TK	910–1,030	PCR/lakewater	[19]
nifH2/nifH1	*nifH*	Nitrogen fixers	TGY GAY CCN AAR GCN GA/GCC ATC AITY TCN CC	330	Nested PCR with nifH32/nifH623 primers/pure culture *Trichodesmium thiebautii*	[55]

Primer	Gene	Target	Sequence	Size	PCR/application	Ref.
nifH4/nifH5	*nifH*	Nitrogen fixers	TTY TAY GGN AAR GGN GG/ATR TTR TTN GCN GCR TA	460	RT-PCR followed by nested PCR using this primer set followed by nifH2/1 [55]/*Cyanobacteria* isolated from a freshwater lake	[56]
nifH-F/nifHR	*nifH*	Nitrogen fixers	AAA GGY GGW ATC GGY AAR TCC ACC AC/TTG TTS GCS GCR TAC ATS GCC ATC AT	432	PCR/soils	[29]
nifH32/nifH623	*nifH*	Nitrogen fixers	TGA GAC AGA TAG CTA TYT AYG GHA A/GAT GTT CGC GCG GCA CGA ADT RNA TSA	600	Nested PCR with nifH2/nifH1 primers/river water	[57]
arc-Nif-f/arc-Nif-r	*nifH*	Nitrogen-fixing Archaea	TAY GGA AAR GGN GGN ATY GG/CCN CCR CAG ACR ACR TCN CC	360–415	PCR/lakewater	[19]
NrfA-F1/NrfA-7Rl	*nrfA*	Nitrite-reducing bacteria	GCN TGY TGG WSN TGY AA/TWN GGC ATR TGR CAR TC	490–570	PCR/various pure cultures	[58]
nrfA-2F/nrfA-2R	*nrfA*	Nitrite-reducing bacteria, phylotype *nrfA-2*	CAC GAC AGC AAG ACT GCC G/CCG GCA ATT TCG AGC CC	67	Q-PCR used with *Taq*Man probe *nrfA-2* (TM-MGB) TTG ACC GTC GGC A/estuary sediments	[11]
nrtfAF2aw/NrfA-7Rl	*nrfA*	Nitrite-reducing bacteria	CAR TGY CAY GTB GAR TA/TWN GGC ATR TGR CAR TC	269	PCR and SYBR Green Q-PCR, modified forward primer paired with reverse primer from Mohan et al. [58]. Primer sets used for ION Torrent PGM Sequencing [59]/soils	[60]

*TM-MGB Taq*Man minor groove binding

Ambiguity codes: N = G, A, T or C; W = A or T; Y = C or T; M = A or C; R = G or A; K = G or T; S = G or C; H = A, C or T; D = G, A or T; B = C or G or T; V = A or C or G

a If using primers for Q-PCR, it is advisable to adhere to MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines [20] (for further details, see chapter by [21])

b Numbering based on complete *norB* gene sequence from *Pseudomonas stutzeri* (Z28384)

c Primers are named by *qnorB*-targeting genes for quinol-oxidising nitric oxide reductase in *Paracoccus denitrificans* Pd1222 (U28078) nitric oxide reductase in *Ralstonia eutropha* H16 (AF002661) and *cnorB*-targeting cytochrome *c*-oxidising

d Positions in the *nosZ* gene of *Pseudomonas aeruginosa* DSM 50071 (X65277)

e Numbers in primer names are relative to the *nosZ* sequence of *Pseudomonas stutzeri* Zobell EMBL X56813

denitrificans [31]. Throbäck et al. [31] suggest that nosZF/nosZ1622R are the best primers for *nosZ* gene amplification for denitrifier community analysis, and these primers also produce a fragment size suitable for DGGE. However, many *Firmicutes* and *Bacteroidetes* have also been identified as denitrifiers, and *nosZ* genes have been found in *Geobacillus thermodenitrificans* NG80-2 [61]. The discovery of *nosZ* sequences in Gram-positive strains has led to questions arising about the specificity of previously designed *nosZ* PCR primers. Primers (arc-Nos-f,-r) targeting nitrous oxide reductase in Archaea have also been developed (Table 3) and evaluated *in silico*. These primers were found to be sensitive to most archaeal *nosZ* sequences but little complementarity to bacterial ones [19].

6   Dissimilatory Nitrate Reduction to Ammonium (DNRA) (*nrfA* Gene)

The *nrfA* gene encodes a periplasmic nitrite reductase which catalyses the conversion of NO_2^- to NH_4^+ (Fig. 1). Mohan et al. [58] designed degenerate primers targeting *nrfA* (NrfA-F1/NrfA-7R1, Table 3) from the alignment of six *nrfA* sequences from Epsilon- and Gammaproteobacteria including *Escherichia coli* and *Wolinella succinogenes*. These primers were subsequently used to retrieve *nrfA* sequences from sulphate and anammox reactors. The majority of clones containing *nrfA* genes were from *Bacteroides* [58]. These primers have also been used to retrieve *nrfA* sequences similar to Delta-, Gamma- and Epsilonproteobacteria in addition to *Chlorobium* and Bacteroides from sediments [11]. Welsh et al. [60] redesigned the *nrfA* forward primer (nrfAF2aw, Table 3) incorporating 474 putative *nrfA* gene sequences drawn from genomes, pure cultures and environmental gene sequences. Used in combination with the reverse *nrfA* primer designed by Mohan et al. [58], it amplifies a 269 bp product. This primer set has revealed diverse *nrfA* sequences from soils [60]. Furthermore, it has been used in an SYBR Green Q-PCR assay and next-generation sequencing to reveal extensive *nrfA* diversity in estuarine sediments [59].

7   Nitrogenase Reductase (*nifH* Gene)

N_2-fixing microorganisms from both bacteria and archaea are the only natural biological source that can reduce atmospheric N_2 to biologically available NH_4^+. Most microorganisms that perform biological N_2 fixation do so using an evolutionarily conserved nitrogenase protein complex that is composed of two multisubunit metalloproteins. Component I, which contains the active site for N_2 reduction, is composed of two heterodimers, encoded by the *nifD* and *nifK* genes. Component II, which couples ATP hydrolysis to inter-protein electron transfer, is composed of two identical subunits, encoded by the *nifH* gene [62]. Since several *nifH* gene

sequences are available, this is the biomarker of choice, and PCR primers have been designed primarily on this gene target [63]. Phylogenetic analysis of the *nifH* gene classified the sequences into five clusters and includes Proteobacteria, *Cyanobacteria*, *Frankia* and *Paenibacillus*. Primers that target the *nifH* gene need to be degenerate to encompass the sequence variability in this gene [55]. Currently, there are 51 universal *nifH* primers and 35 group-specific primers, all of which have been extensively evaluated elsewhere in a recent review [64].

Primers (arc-Nif-f,-r) targeting nitrogenase reductase in archaea have also been developed (Table 3). Whilst these primers were highly sensitive towards the target gene, there was little discrimination between archaeal and bacterial homologues [19]. Thus, it depends on the application of these primers whether co-detection of bacterial *nifH* sequences is desirable, if, for instance, the entire N_2-fixing communities in a given environment can be targeted using a single primer pair that gives good coverage of both domains.

8 Microbial Nitrification

Ammonia-oxidising bacteria (AOB) and ammonia-oxidising archaea (AOA) oxidise ammonia into hydroxylamine, which is further oxidised to NO_2^- by other microorganisms during nitrification (Fig. 1). AOB belong to the Beta- or Gammaproteobacteria [65], and all known AOA belong to the Thaumarchaeota [66].

9 Ammonia Monooxygenase (*amoA* Gene)

Both AOB and AOA contain the membrane-bound enzyme ammonia monooxygenase (encoded by the *amoA* gene) [67]. AOB belonging to the Betaproteobacteria generally contain multiple copies of the *amoA* gene, whilst those belonging to the Gammaproteobacteria contain one copy of the *amoA* gene [68, 69]. It is thus important to consider *amoA* gene copy numbers in AOB Q-PCR assays. Since the *amoA* gene is phylogenetically related to the *pmoA* gene in methane-oxidising bacteria (MOB), some PCR primers may amplify both AOB *amoA* and MOB *pmoA* genes (e.g. A189 (amoA151f) and A682 (amoA681r) [70]). There are also primers targeting the *amoB* and *amoC* gene homologues in bacteria and archaea [37]. Whilst *amoA* is traditionally targeted, *amoB* has a higher number of variable regions and so may be a more appropriate choice if closely related species need to be resolved [71]. An advantage of targeting *amoB* and *amoC* genes, for example, using 305F (amoC763f) and 308R (amoB506r), is that this allows amplification of the entire *amoA* region [68]. Most PCR primers target the *amoA* gene (Table 4), and there is considerable difference in their performance and specificity [37, 71].

Table 4

PCR primers for the detection of the ammonia monooxygenase (*amoA* gene) and hydroxylamine oxidoreductase (*hao* gene), both involved in the oxidation of NH_4^+ to NO_2^-, and hydrazine oxidoreductase (*hzo* gene) involved in anaerobic ammonia oxidation

Primers	Gene	Specificity	Sequences (5′-3′)forward/reverse	~Product length (bp)	Applications/environment primers first used/relevant to oil reservoirs[a]	Reference
AMO-F (amoA21f)/ amoA686r (AMO-R)	*amoA*	*Nitrosomonas europaea* cluster	AGA AAT CCT GAA AGC GGC/GAT ACG AAC GCA GAG AAG	665	PCR, sequencing/seawater	[72]
A189 (amoA151f)/ A682 (amoA681r)	*amoA*, *pmoA*	AOB, MOB	GGN GAC TGG GAC TTC TGG/GAA SGC NGA GAA GAA SGC	525	PCR/various pure cultures	[70]
amoA-1F (amoA332f)/ amoA-2R (amoA822r)	*amoA*	AOB, Betaproteobacteria	GGG GTT TCT ACT GGT GGT/CCC CTC KGS AAA GCC TTC TTC	453	PCR, cloning, sequencing/soils, plant roots	[69]
AMO-F2 (amoA40f)/ AMO-R2 (amoA665r)	*amoA*	AOB (*Nitrosomonas* spp. *and Nitrosospira* spp.)	AAG ATG CCG CCG GAA GC/GCT GCA ATA ACT GTG GTA	640	PCR/activated sludge	[73]
amoA1Fmod (amoA332fH)	*amoA*	AOB	GGG GHT TYT ACT GGT GGT		PCR/soils	[74]
amoA-1Fmod (amoA332fHY)*	*amoA*	AOB	GGG GHT TYT ACT GGT GGT		PCR/soils	[74]
A682mod (amoA680r)	*amoA*	AOB	AAV GCV GAG AAG AAW GC		PCR, sequencing/marine sediments	[75]
amoA-3F (amoA310f)/ amoB-4R (amoB44r)	*amoA*	AOB Gammaproteobacteria	CGT GAG TGG GYT AAC MG/GCT AGC CAC TTT CTG G	281	PCR, cloning, sequencing/wastewater treatment biofilms	[76]
AmoA154fs	*amoA*	AOB	GAC TGG GAC TTC TGG		PCR/biofilm	[77]

Primer	Gene	Group	Sequence	Size	Application/source	Ref.
amoA-1FF (AmoA187f)	*amoA*	AOB (selected *Nitrosomonas* spp. and *Nitrosospira* spp.)	CAA TGG TGG CCG GTT GT		PCR/biofilm	[77]
amoA822rTC (amoA-2R-TC)	*amoA*	AOB	CCC CTC TGC AAA GCC TTC TTC		DGGE/estuary	[78]
amoA-2F/amoA-5R	*amoA*	AOB	AAR GCG GCS AAG ATG CCG CC/TTA TTT GAT CCC CTC	786	DGGE/soils	[79]
amoA-3F	*amoA*	AOB	ACC TAC CAC ATG CAC TT		DGGE/soils	[79]
amoA-4R	*amoA*	AOB	GGG TAG TGC GAC CAC CAG TA		DGGE/soils	[79]
amoA154f (301F)/amoA828r (302R)	*amoA*	AOB	GAC TGG GAC TTC TGG CTG GAC TGG AA/TTT GAT CCC CTC TGG AAA GCC TTC TTC	675	PCR	[68]
305F (amoC763f)/304R (amoA60r)	*amoA*	AOB (selected *Nitrosomonas* spp. and *Nitrosospira* spp.)	GTG GTT TGG AAC RGI CAR AGC AAA/ TAY CGC TTC CGG CGG CAT TTT CGC CGC	665	PCR/soils	[68]
amoA822rTG (amoA-2R-TG)	*amoA*	AOB	CCC CTC TGG AAA GCC TTC TTC		DGGE (with amoA-1F)/soils	[80]
A337 (amoA337p)	*amoA*	AOB Betaproteobacteria	TTC TAC TGG TGG TCR CAC TAC CCC ATC AAC T		Q-PCR, *Taq*Man probe/soils	[80]
AmoA-2R' (amoA822r)	*amoA*	AOB	CCT CKG SAA AGC CTT CTT C		SYBR Green Q-PCR with forward primer amoA-1F/soils	[80]
amoArNEW	*amoA*	AOB	CCC CTC BGS AAA VCC TTC TTC		DGGE/wastewater treatment plant	[81]
amoA34f	*amoA*	AOB	GCG GCR AAA ATG CCG CCG GAA GCG		PCR, cloning, sequencing/seawater	[82]
amoA49f	*amoA*	AOB	GAG GAA GCT GCT AAA GTC		Database sequences	[71]
amoA627r	*amoA*	AOB	CGT ACC TTT TTC AAC CAT CC		Database sequences	[71]
amoA349r	*amoA*	AOB	ACC ACC AGT AGA AAC CCC		Database sequences	[71]
amoA664r	*amoA*	AOB	GCS TTC TTC TCN GCS TTT C		Database sequences	[71]
amoA802f	*amoA*	AOB	GAA GAA GGC TTT SCM GAG GGG		Database sequences	[71]

(continued)

Table 4
(continued)

Primers	Gene	Specificity	Sequences (5′-3′)forward/reverse	~Product length (bp)	Applications/environment primers first used/ relevant to oil reservoirs[a]	Reference
Arch-amoAF/ Arch-amoAR	*amoA*	AOA	STA ATG GTC TGG CTT AGA CG/GCG GCC ATC CAT CTG TAT GT	635	SYBR Green Q-PCR/seawater	[83]
amo196F	*amoA*	AOA	GGW GTK CCR GGR ACW GCM		PCR, SYBR Green Q-PCR/soils	[36]
amo227R	*amoA*	AOA	CRA TGA AGT CRT AHG GRT ADC C		PCR, SYBR Green Q-PCR/soils	[36]
amo247	*amoA*	AOA	CCA ACC AWG CWC CYT TKG CDA CCC		PCR, SYBR Green Q-PCR/soils	[36]
CrenAmo1F/ CrenAmo1R	*amoA*	AOA	AAT GGT CTG GCT WAG ACG C/GAC CAR GCG GCC ATC CA	640	PCR, sequencing/seawater	[67]
crenAMO_F	*amoA*	AOA	ATG GTC TGG CTA AGA CGM TGT A		PCR, sequencing/sponge symbiont	[84]
Arch-amoA-for/ Arch-amoA-rev	*amoA*	AOA	CTG AYT GGG CYT GGA CAT C/TTC TTC TTT GTT GCC CAG TA	256	PCR, sequencing/seawater	[85]
amoA19F/ amo643R	*amoA*	AOA	ATG GTC TGG CTW AGA CG/TCC CAC TTW GAC CAR GCG GCC ATC CA	624	PCR, SYBR Green Q-PCR/soils	[36, 86]
AOA-amoA-f/ AOA-amoA-r	*amoA*	AOA	CTG AYT GGG CYT GGA CAT C/TTC TTC TTT GTT GCC CAG TA	256	SYBR Green Q-PCR, DGGE/seawater	[87]
CrenamoA-23F/ CrenamoA-616R	*amoA*	AOA	ATG GTC TGG CTW AGA CG/GCC ATC CAT CTG TAT GTC CA	593	RT-PCR, DGGE/soils	[88]
Arch-amoAFA	*amoA*	Group A AOA	ACA CCA GTT TGG YTA CCW TCD GC		SYBR Green Q-PCR/seawater	[89]
Arch-amoAFB	*amoA*	Group B AOA	CAT CCR ATG TGG ATT CCA TCD TG		SYBR Green Q-PCR/seawater	[89]
Arch-amoA26F/ Arch-amoA417R	*amoA*	AOA	GAC TAC ATM TTC TAY ACW GAY TGG GC/GGK GTC ATR TAT GGW GGY AAY GTT GG	415	PCR, DGGE/freshwater and marine sediments	[90]
CG I.1b-amoAF/ CG I.1b-amoAR	*amoA*	AOA	ATA GTT GTA GTT GCT GTA AAT AG/ CTC TAG AGG GTC TCT GAC CAG	416	PCR, DGGE/freshwater and marine sediments	[90]

Primer	Gene	Target	Sequence	Size	Application/position/source	Ref
Arch-amoAF/Arch-amoAR	amoA	AOA	GCT CTA AAT ATG ACA GTA TAC/AYC ATG TTG AAY AAT GGT AAT GAC	205	PCR, DGGE/freshwater and marine sediments	[90]
Arch-amoA-479R	amoA	AOA	TAT GGT GGY AAY GTD GGT C		PCR, SYBR Green Q-PCR, sequencing/aquarium biofilters	[91]
nxrB14f/nxrB1239r	nxrB	*Nitrospira* lineages I, II and V[b]	ATA ACT GGC AAC TGG GAC GG/TGT AGA TCG GCT CTT CGA CC	1,245	PCR, position[c] 14–33/1239–1258/pure cultures, soils, marine sponge, wastewater treatment plants	[92]
nxrB19f/nxrB1237r	nxrB	All *Nitrospira* lineages[b,d]	TGG CAA CTG GGA CGG AAG ATG/GTA GAT CGG CTC TTC GAC CTG	1,239	PCR, position[c] 19–39/1237–1257/pure cultures, soils, marine sponge, wastewater treatment plants	[92]
nxrB169f/nxrB638r	nxrB	All *Nitrospira* lineages	TAC ATG TGG TGG AAC A/CGG TTC TGG TCR ATC A	485	PCR, position[c] 169–184/638–653/pure cultures, soils, marine sponge, wastewater treatment plants	[92]
haoF1/haoR3	hao	Betaproteobacterial AOB	TGC GTG GAR TGY CAC/AGR TAR GAK YSG GCA AA	992	FISH probes, position[e] 307–321/1174–1189/trickling filter biofilm	[93]
haoF4/haoR2	hao	Proteobacterial AOB	AAY CTK CGC TCR ATG GG/GGT TGG TYT TCT GKC CGG	738	FISH probes, position[e] 451–467/1283–1299/trickling filter biofilm	[93]
hzocl1F1/hzocl1R2	hzo	*hzo cluster 1*	TGY AAG ACY TGY CAY TGG/ACT CCA GAT RTG CTG ACC	470	PCR, cloning, FISH probes, position[f] 739–758/1192–1209/trickling filter biofilm	[93]
hzocl1F11/hzocl1R2	hzo	*hzo cluster 1*	TGY AAG ACY TGY CAY TGG G/ACT CCA GAT RTG CTG ACC	470	PCR, cloning, FISH probes, PCR, cloning, position[f] 739–757/1192–1209/trickling filter biofilm	[93]
hzocl2aF1/hzocl2aR1	hzo	*hzo cluster 2a*	GGT TGY CAC ACA AGG C/TYW ACC TGG AAC ATA CCC	289	FISH probes, PCR, cloning, position[f] 685–700/957–974/trickling filter biofilm	[93]
hzocl2aF1/hzocl2aR2	hzo	*hzo cluster 2a*	GGT TGY CAC ACA AGG C/ATA TTC ACC ATG YTT CCA G	525	FISH probes, PCR, cloning, position[f] 685–700/1506–1523/trickling filter biofilm	[93]
hzocl2aF2/hzocl2aR1	hzo	*hzo cluster 2a*	GTT GTG MTG MWT GTC ATG G/TYW ACC TGG AAC ATA CCC	838	FISH probes, PCR, cloning, position[f] 449–467/957–974/trickling filter biofilm	[93]

(continued)

Table 4
(continued)

Primers	Gene	Specificity	Sequences (5′-3′)forward/reverse	~Product length (bp)	Applications/environment primers first used/relevant to oil reservoirs[a]	Reference
Ana-hzo1F/Ana-hzo-2R	*bzo*	Anammox microorganisms	TGT GCA TGG TCA AIT GAA AG/ACC TCT TCW GCA GGT GCA	1,000	PCR, SYBR Green Q-PCR, cloning, position[g] 826–1859/753–1786/sludge	[94]
hzoF1/hzoR1	*bzo*		TGT GCA TGG TCA AIT GAA AG/CAA CCT CTT CWG CAG GTG CAT G	1,000	PCR, cloning, SYBR Green Q-PCR/petroleum reservoir	[95]

Ambiguity codes: N = G, A, T or C; W = A or T; Y = C or T; M = A or C; R = G or A; K = G or T; S = G or C; H = A, C or T; D = G, A or T; B = C or G or T; V = A or C or G

[a]If using primers for Q-PCR, it is advisable to adhere to MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines [20] (for further details, see chapter by 21)

[b]Different annealing temperatures were required for nxrB amplification from the tested *Nitrospira* cultures

[c]Positions relative to the *nxrB* (NIDE3256) gene of *N. defluvii* [96]

[d]Did not amplify nxrB of *Hyrtios proteus* (marine sponge) symbionts

[e]Positions relative to the *hao* gene in *Nitrosomonas europaea*

[f]Positions relative to the *bzo* gene in CAJ72085

[g]Position based on *bzo* gene of *Candidatus Kuenenia stuttgartiensis* and anammox KSI-1 enrichment culture

In Junier et al. [71], a standardised naming system was devised to give more consistent information on the position and orientation of primers. The original designation of the primer is typically given first with the new designation afterwards in parentheses (Table 4). The most widely used *amoA* primer pair is amoA-1F (amoA332F)/amoA-2R (amoA822r), due to the fact that it successfully amplifies the *amoA* region from a range of betaproteobacterial AOB [69]. A disadvantage with this primer pair is the large difference in the optimum annealing temperature of each primer [71]. Stephen et al. [74] modified amoA1F to produce the primer amoA1Fmod (amoA332fHY) designed to increase specificity towards cultured betaproteobacterial AOB. Variants of amoA-2R have since been proposed for DGGE such as amoA-2R-TC (amoA822rTC) [78] and amoA-2R-TG (amoA822rTG) [80]. In addition, a FISH probe A337 (amoA337p) was designed to target betaproteobacterial AOB [80]. A nested PCR approach using AMO-F/AMO-R (first round) and AMO-F2 (amoA40f) and AMO-R2 (amoA665r) (second round) has also been applied as general betaproteobacterial AOB primers [73]. However, given that the primers AMO-F/AMO-R used in the first round of PCR amplification have a high specificity towards *Nitrosomonas europaea*, this nested PCR approach seems to have a limited applicability. The primer pair AMO-F (amoA21f)/AMO-R (amoA686r) is highly specific for the *N. europaea* cluster under stringent PCR conditions [72]. Although the primers 301F (amoA154f)/302R (amoA828r) were designed to amplify a 675 bp *amoA* gene fragment from AOB [68], the forward primer also targets the *pmoA* gene in MOB. However, when used in combination with the reverse primer, specificity is increased and only the *amoA* gene is amplified [68]. Both 301F (amoA154f) and 302R (amoA828r) have a high melting temperature (T_m) [71], and so amoA154fs was designed as a modification of 301F with a lower T_m [77]. Primers amoA-1FF (amoA187f) and amoA-2R (amoA822r) designed to amplify *amoA* genes from *N. europaea* [77] also match several AOB [71]. In addition to amplifying betaproteobacterial AOB, primers amoA-310f (amoA-3F)/amoB-4R (amoB44r) also amplify the *amoA* gene from Gammaproteobacteria [76]. Many *amoA* primer pairs give short amplicons, which limits their use for phylogenetic inference but makes them applicable for Q-PCR analysis. The primer amoA34f, which anneals close to the 5′ end of the *amoA* gene, allows amplification of a larger proportion of the *amoA* region from betaproteobacterial AOB when paired with existing reverse primers [71].

Since the known diversity of thaumarchaeal lineages (based on 16S rRNA gene sequences) are greater than that derived from

amoA gene sequences, it is possible that current *amoA* gene primer sets target a restricted AOA diversity. Initial AOA *amoA* primers, Arch-amoAF/Arch-amoAR [83], CrenAmo1F/CrenAmo1R [67] and amo196F/amo227R [36], were based on a limited number of sequences and hybridise at similar positions, amplifying a large proportion of the *amoA* gene and giving PCR products of ~600 bp. Since then, new sequences have become available, and forward primers specific to the A and B group AOA (Arch-amoAFA and Arch-amoAFB, respectively) have been designed for Q-PCR [89]. These primers allow differentiation of the A and B groups, but Arch-amoAFA has been found to amplify *N. maritimus*-like sequences in some marine samples [89]. Despite the recent increase in the number of archaeal *amoA* sequences available, there has been a lack of studies comparing the specificity and effectiveness of different archaeal *amoA* primer sets.

10 Hydroxylamine Oxidoreductase (*hao* Gene)

In AOB, the second step of ammonium oxidation, the dehydrogenation of hydroxylamine (NH_2OH) to NO_2^-, is performed by hydroxylamine oxidoreductase (HAO) [97]. Multiple copies of the *hao* gene have been found in AOB [98]. So far a homologue of this enzyme (and essential cytochrome proteins found in AOB) has not been found in AOA [84], which may rely solely on multi-copper oxidases to relay electrons to the quinone pool during the conversion of ammonium to nitrite [99]. Phylogenetic analyses of *hao* gene sequences were highly congruent with the 16S rRNA and *amoA* gene [93], suggesting the *hao* gene may be a suitable biomarker for AOB in environmental studies. Degenerate primers (haoF1-haoR3 and haoF4-haoR2) suitable for detecting *hao* gene homologues in a variety of AOB have been designed [93] (Table 3). Analysis of the degenerate primers suggests that they are specific for AOB under stringent PCR conditions but under lower stringency may also target the *hao* genes in non-AOB such as MOB and the sulphur oxidiser *Silicibacter pomeroyi* [37, 100].

11 Nitrite Oxidoreductase (*nxrA* and *nxrB* Genes)

The second step of nitrification involves the oxidation of NO_2^- to NO_3^- catalysed by the enzyme nitrite oxidoreductase (NXR) and carried out by nitrite-oxidising bacteria (NOB). The enzyme NXR which comprises of alpha (NxrA), beta (NxrB) and gamma (NxrC) subunits occurs in two phylogenetically distinct forms, one

cytoplasmic type found in *Nitrobacter*, *Nitrococcus* and *Nitrolance-tus* and one periplasmic type found in *Nitrospina* and *Nitrospira* [92]. PCR primers have been developed to target the *nxrA* and *nxrB* genes and differentiated closely related *Nitrobacter* strains in pure cultures and soils [101, 102]. In addition, genome sequencing of a *Nitrospira* has enabled the development of PCR primers targeting the *nxrB* gene of *Nitrospira*, which cover all known lineages of this genus [92].

12   Hydrazine Oxidoreductase (*hzo* Gene)

Anaerobic ammonia-oxidising bacteria (AnAOB) also oxidise ammonia in anoxic environments such as in the case of petroleum reservoirs but use a different metabolic pathway to AOB and AOA. AnAOB use NO_2^- as the electron acceptor to produce dinitrogen gas (anammox, ANaerobic AMMonia OXidation) (Fig. 1). AnAOB contain several proteins assumed to be involved in the oxidation of hydrazine to dinitrogen gas and are called hydrazine oxidoreductase (HZO) [103]. The hydrazine oxidoreductase (*hzo*) gene has been used with high specificity to detect AnAOB bacteria in oilfields [95], and phylogenetic analysis shows that the *hzo* gene has good congruence with 16S rRNA gene sequences for detecting AnAOB bacteria in oil reservoirs [95]. Since the *hzo* gene is highly conserved, it is a suitable alternative target to the 16S rRNA gene for PCR primers for use in environmental samples (Table 4).

AnAOB belong to the order *Planctomycetales*, and to date five genera and 16 species [104] have been found to perform anammox reactions: *Candidatus* 'Brocadia' [105], *Candidatus* 'Scalindua' [106], *Candidatus* 'Anammoxoglobus' [107], *Kuenenia* [93] and *Jettenia* [94]. In addition, some ammonia oxidisers belonging to the Betaproteobacteria such as *Nitrosomonas eutropha* have also been found to have anaerobic ammonium-oxidising activity using nitrogen dioxide as the electron acceptor [108]. Although anammox has been found to occur in petroleum reservoirs, the diversity of AnAOB bacteria found depends on the oil production processes and physiological conditions in situ [95].

13   Perspective

Microbial communities found in the subsurface are important in the biogeochemical cycling of nitrogen, and changes in their functional structure might affect petroleum reservoir stability. In petroleum reservoirs, where in situ conditions are predominantly anoxic, denitrification is a major process. Microorganisms may also decompose organic N to ammonium (NH_4^+) by ammonification,

which can subsequently be oxidised by nitrification whereby ammonia oxidation may occur aerobically via AOB and AOA or anaerobically by AnAOB bacteria.

Since many of the key functional groups of microbes involved in N transformations are taxonomically diverse, 16S rRNA-based methods are generally not suitable. Primers and probes targeting functional genes may be more useful targets as biomarkers as they can often give greater taxonomic resolution. However, the paucity of suitable molecular tools may hamper culture-independent investigations of reservoir environments. In addition to detection, primer sensitivity is important when assessing diversity and primer coverage poses a continuing problem [19]. Furthermore, successful primers often double as probes for quantifying and localising gene expression. Thus, it depends on the application of the primers whether co-detection of functional gene sequences across microbial guilds is tolerable or even desirable with a single primer pair [19]. Consequently, for highly divergent functional genes such as *nirK*, single primer pairs may be unsuitable, and either multiple primer sets and/or at other conserved regions of the gene might need to be applied [24, 41].

Here, the common PCR primers used to target the major functional genes involved in N cycling and the environmental setting from which the primers were first used are described. The key N transformations likely to occur in petroleum reservoirs have been highlighted throughout, and for each functional gene, we have summarised the recommended applications for the most commonly used primer pairs (Table 5). We have also highlighted in bold in Table 5 those primers that may be the most suitable for petroleum reservoirs. Thus, when designing and evaluating primer pairs targeting metabolic genes, it is important to consider the curated reference database as well as the environment of interest, since not all N transformation processes may be occurring. In conclusion, by using well-defined conditions and constant well-performing PCR primers, the functional genes involved in N cycling in petroleum reservoirs may provide alternative tools for analysing subsurface microorganisms.

Table 5
Summary of PCR primers and recommended applications

	Applications				
Gene[a]	General PCR	Q-PCR[b]	DGGE	FISH	Reference
napA	For nitrate-reducing bacteria V16/V17	For nitrate-reducing bacteria, phylotype *napA*-1[c] *napA*-1F/*napA*-1R / For nitrate-reducing bacteria, phylotype *napA*-2[c] *napA*-2F/*napA*-2R / For nitrate-reducing bacteria, phylotype *napA*-3[c] *napA*-3F/*napA*-3R / For Alpha-, Beta- and Gammaproteobacteria[d] V17m/napA4r			[10, 11, 12]
narG	For nitrate-reducing bacteria T37/T39 / For Proteobacteria, Gram-positive bacteria and Archaea narG1960f/narG2650r	Nitrate-reducing Proteobacteria narG328F/narG497R / For nitrate-reducing bacteria[c], phylotype 1 *narG*-1*narG*-1F/*narG*-1R / For nitrate-reducing bacteria[c], phylotype 2 *narG*-2			[16, 8, 11, 18]
nirS	Nitrite-reducing bacteria nirS3F/nirS5R	Nitrite-reducing bacteria nirS3F/nirS5R / nirSCd3aF (modified)/nirSR3cd (modified) / Nitrite-reducing bacteria[c], phylotype *nirS-e* *nirS*-eF/*nirS*-efR / Nitrite-reducing bacteria[c], phylotype *nirS-m* *nirS*-mF/*nirS*-mR	Nitrite-reducing bacteria nirS1F/nirS6R		[42], (modified from [31]), [44], (modified from [23]), [11, 23]
nirK		nirK1F/nirK5R, nirK876/nirK1040 / nirKC1F/nirKC1R / nirKC2F/nirKC2R / nirKC3F/nirKC3R / nirKC3F/nirKC3R	nirK1F/nirK5R		[23, 41, 45]
norB	cnorB1F/cnorB7R		qnorB2F/qnorB7R		[48]
nosZ	Denitrifiers nosZ661b/nosZ1773b	Nos1527F/Nos1773R / nosZ1F/nosZ1R	Nos1527F/ Nos1773R		[50, 52, 54]

(continued)

Table 5
(continued)

| | Applications | | | | |
Gene[a]	General PCR	Q-PCR[b]	DGGE	FISH	Reference
nifH	Nitrogen fixers nifH2/nifH1 nifH32/nifH623				[55, 57]
nrfA	NrfA-F1/NrfA-7R1	*nrfA*-2F/*nrfA*-2R[c] nrfAF2aw/NrfA-7R1[d]			[11, 58, 60]
amoA	For AOB, Betaproteobacteria amoA-1F (amoA332f)/amoA-2R (amoA822r); for AOB Gammaproteobacteria amoA-3F (amoA310f)/amoB-4R (amoB44r)	For AOB, Betaproteobacteria amoA-1F (amoA332f)/amoA-2R (amoA822r) Or amoA-1F (amoA332f)/amoa-2R'(amoA822r)	amoA-3F/amoA-4R	A337 (amoA337p)	[69, 70, 79, 80]
amoA, pmoA	For AOB/MOB A189 (amoA151f)/A682 (amoA681r) or A189 (amoA151f)/A682 (amoA681r)				[70]
amoA	For AOA amoA19F/amo643R				[36, 86]
amoA	AOA	Arch-amoAF/Arch-amoAR	Arch-amoA26F/Arch-amoA417R AOA-amoA-f/AOA-amoA-r		[83, 87, 90]
hao	For betaproteobacterial AOB haoF1/haoR3 Or for proteobacterial AOB haoF4/haoR2				[93]
hzo	hzoF1/hzoR1	hzoF1/hzoR1			[95]

[a]Highlighted in bold are the main functional genes that are likely to be detected in petroleum reservoirs
[b]If using primers for Q-PCR, it is advisable to adhere to MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines [20] (for further details, see chapter by [21])
[c]Used with appropriate *Taq*Man probes (see [11])
[d]SYBR Green Q-PCR assay

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