



Gene Capture Coupled to High-Throughput Sequencing as a Strategy for Targeted Metagenome Exploration

Jérémie Denonfoux, Nicolas Parisot, Eric Dugat-Bony, Corinne Petit, Delphine Boucher, Diego Morgavi, Denis Le Paslier, Eric Peyretailade, Pierre Peyret

► To cite this version:

Jérémie Denonfoux, Nicolas Parisot, Eric Dugat-Bony, Corinne Petit, Delphine Boucher, et al.. Gene Capture Coupled to High-Throughput Sequencing as a Strategy for Targeted Metagenome Exploration. DNA Research, 2013, 20 (2), pp. 1-12. 10.1093/dnares/dst001 . hal-00783545

HAL Id: hal-00783545

<https://hal.science/hal-00783545>

Submitted on 4 Feb 2014

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Gene Capture Coupled to High-Throughput Sequencing as a Strategy for Targeted Metagenome Exploration

JÉRÉMIE Denonfoux^{1,2,3,†}, NICOLAS Parisot^{1,2,3,†}, ERIC Dugat-Bony^{1,4}, CORINNE Biderre-Petit^{3,5}, DELPHINE Boucher^{1,4}, DIEGO P. Morgavi⁶, DENIS Le Paslier^{7,8,9}, ERIC Peyretailade^{1,4}, and PIERRE Peyret^{1,4,*}

Centre de Recherche en Nutrition Humaine Auvergne, Clermont Université, Université d'Auvergne, EA 4678, Conception, Ingénierie et Développement de l'Aliment et du Médicament, BP 10448, Clermont-Ferrand 63000, France¹; Clermont Université, Université Blaise Pascal, Clermont-Ferrand 63000, France²; UMR CNRS 6023, Université Blaise Pascal, Clermont-Ferrand 63000, France³; UFR Pharmacie, Clermont Université, Université d'Auvergne, Clermont-Ferrand 63000, France⁴; Laboratoire Microorganismes: Génome et Environnement, Clermont Université, Université Blaise Pascal, BP 10448, Clermont-Ferrand 63000, France⁵; INRA, UMR1213 Herbivores, F-63122 Saint-Genès-Champanelle and Clermont Université, VetAgro Sup, UMR Herbivores, BP 10448, F-63000, Clermont-Ferrand, France⁶; CEA, DSV, Institut de Génomique, Genoscope, 2 rue Gaston Crémieux, Evry 91057, France⁷; CNRS, UMR8030, Evry 91057, France⁸ and UEVE, Université d'Evry, Evry 91057, France⁹

*To whom correspondence should be addressed. EA4678 CIDAM, 28 place Henri Dunant, 63001 Clermont-Ferrand. Tel. +33 47-3178-308. Fax. +33 47-3275-624. Email: pierre.peyret@udamail.fr

Edited by Prof. Masahira Hattori

(Received 10 October 2012; accepted 9 January 2013)

Abstract

Next-generation sequencing (NGS) allows faster acquisition of metagenomic data, but complete exploration of complex ecosystems is hindered by the extraordinary diversity of microorganisms. To reduce the environmental complexity, we created an innovative solution hybrid selection (SHS) method that is combined with NGS to characterize large DNA fragments harbouring biomarkers of interest. The quality of enrichment was evaluated after fragments containing the methyl coenzyme M reductase subunit A gene (*mcrA*), the biomarker of methanogenesis, were captured from a *Methanosarcina* strain and a metagenomic sample from a meromictic lake. The methanogen diversity was compared with direct metagenome and *mcrA*-based amplicon pyrosequencing strategies. The SHS approach resulted in the capture of DNA fragments up to 2.5 kb with an enrichment efficiency between 41 and 100%, depending on the sample complexity. Compared with direct metagenome and amplicons sequencing, SHS detected broader *mcrA* diversity, and it allowed efficient sampling of the rare biosphere and unknown sequences. In contrast to amplicon-based strategies, SHS is less biased and GC independent, and it recovered complete biomarker sequences in addition to conserved regions. Because this method can also isolate the regions flanking the target sequences, it could facilitate operon reconstructions.

Key words: α -subunit of the methyl-coenzyme M reductase; metagenomics; sequence capture; 454 pyrosequencing; microbial diversity

1. Introduction

Microorganisms are extremely diverse and crucial for healthy, functioning biospheres.^{1,2} Although studies of isolated species have produced a great deal

of information about microbial genetics, physiology, biotechnology and molecular biology, the diversity and structure of complex microbial communities are still poorly understood. This deficiency results from the inability to culture most microorganisms using standard microbiological techniques.^{1,3} Consequently, although there are most likely millions of bacterial

[†] These authors contributed equally to this study.

species on the planet, only a few thousand have been formally described.⁴

Culture-independent techniques, such as metagenomics,⁵ circumvent the problem of unculturability and transcend previous studies on individual organisms to focus on microbial communities present in an environment. Metagenomics has enriched our knowledge of environmental microbiology through the structural (gene/species richness and distribution)⁶ and functional (metabolic)⁷ profiling of complex environmental microbial communities. Based on unselective (shotgun) or targeted (activity driven and sequence driven) methods, metagenomics links genome information with structure and function relationships within microbial populations.^{8,9}

Recently developed next-generation sequencing (NGS) technologies recover genetic materials from environmental samples without the preparation of metagenomic clone libraries.¹⁰ Furthermore, they explore a greater amount of sequence information because they have higher throughput and lower costs than other methods.¹¹ Nevertheless, Quince *et al.*¹² showed that covering 90% of the species richness in some hyper-diverse environments could require 10–1000-fold increases in the current NGS sequencing efforts. In addition, the massive amount of short metagenomic sequence reads (between 20 and 700 bases depending on the platform) can be problematic for assembling and identifying complete coding DNA sequence and/or operon structure.¹³ One promising alternative is to reduce the environmental sample complexity by enriching the desired genomic target before sequencing.

Currently, several strategies of genomic-scale sequence enrichment have been reported.¹⁴ The more efficient methods rely on complementary nucleic acid capture probes that hybridize to the targeted DNA sequences. Two hybridization methods—solid phase^{15–17} and solution phase, also known as solution hybrid selection (SHS)^{18,19}—can be used to ascertain genetic variation by specifically enriching and resequencing regions from complex eukaryotic genomes.

To the best of our knowledge, only high-throughput enrichment methods based on polymerase chain reaction (PCR) have been applied to target functional genes in complex environments.²⁰ Because no current methods use oligonucleotide capture probes to specifically enrich targeted genes from a complex environmental genomic DNA (gDNA), we applied this methodology in the context of microbial ecology (Fig. 1A) to specifically capture DNA fragments harbouring known or unknown genetic biomarkers of interest (Fig. 1B). We hypothesized that the use of variant specific and explorative probes^{21,22} would more accurately define the overall biomarker diversity (including the rare biosphere and unknown sequences) and would facilitate

the discovery of genes linked to the target sequences *via* the reconstruction of adjacent DNA regions. This method should lead to better diversity coverage that is not influenced by PCR biases, as generally occurs in amplicon sequencing.^{23,24} Because it is not limited to a specific DNA region (as in PCR enrichment), this strategy will increase the sequence coverage over target regions and lower the cost per target when compared with shotgun sequencing.

In the present study, we describe the first adaptation of the SHS capture method for the selective enrichment of a target-specific biomarker from a complex environmental metagenome. Methane (CH₄) is an important radiative trace gas responsible for the greenhouse effect, and a significant proportion (6–16%) of the global natural methane emissions are released from freshwater lakes.²⁵ We surveyed the methanogen diversity in a permanently stratified crater lake located in the French Massif Central (Lake Pavin). This original freshwater ecosystem is composed of an anoxic deep water layer (monimolimnion, ~60–90 m depth) separated from the oxygenated upper layer (mixolimnion) by an intermediate layer (mesolimnion),²⁶ where both the sediments and the anoxic water column contribute to methane production.²⁷ We targeted the gene coding for the α -subunit of the methyl coenzyme M reductase (*mcrA*) that is involved in the final step of methanogenesis. This gene is arranged in a single transcriptional unit—the *mcr* operon—that is highly conserved among all methanogens.^{28,29} To highlight the broad benefits of the gene capture approach when compared with the more classical sequencing methods, three methods were used for pyrosequencing of an environmental sample: the SHS method, a classical random-shotgun metagenomic approach and an *mcrA*-targeted amplicon sequencing survey.

2. Materials and methods

2.1. Capture probe design and synthesis

Two sets of capture probes were designed. The first set targeted the *mcrA* gene from the *Methanosarcina acetivorans* C2A genome (GenBank accession no. AE010299), and the second set targeted the *mcrA* sequences pooled from environmental samples. The first set of capture probes consisted of six high specific 50-mer probes (P1–P6) targeting six distinct regions of the *M. acetivorans* C2A *mcrA* gene (Fig. 2, Supplementary Table S1). These probes were designed with HiSpOD software.³⁰ Adaptor sequences were added at each end, resulting in 80-mer hybrid probes consisting of 5'-ATCGCACCAGCGTGT(X)₅₀C ACTGCGGCTCCTCA-3', with X₅₀ indicating the specific capture probe.

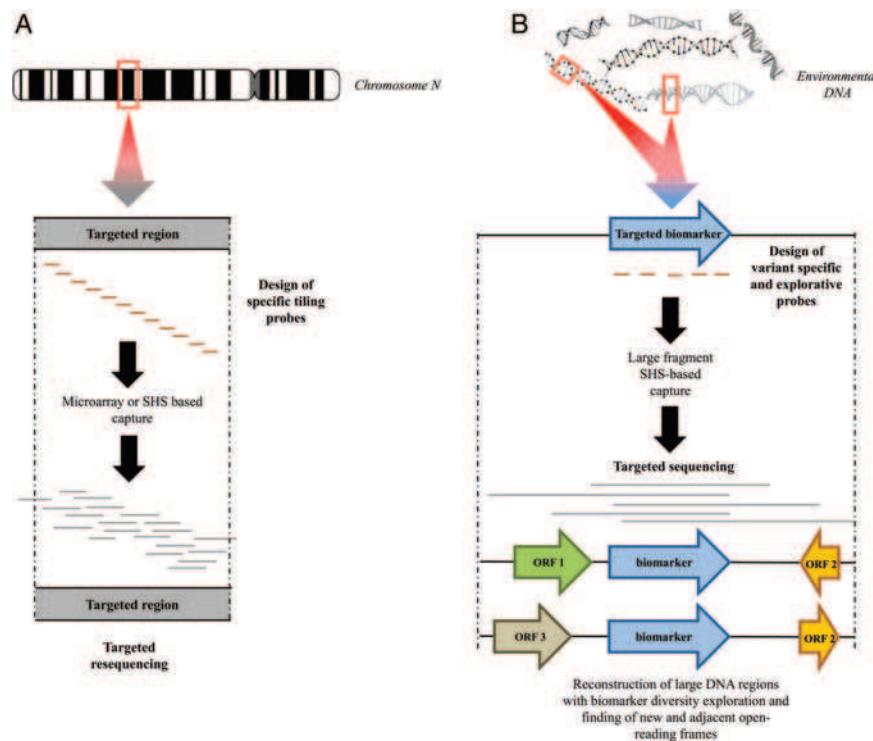


Figure 1. Schematic comparison of targeted capture methods applied to classical direct selection method of individual genomic loci (human for instance) (A) and our new approach for metagenomics targeting (B). The enrichment through microarray and the SHS of large genomic regions within complex eukaryotic genomes, as described in A, uses specific tiling probes to target resequencing genomic loci for copy number variation (CNV) and single nucleotide polymorphism detection. Our SHS method (B) uses the design of specific variants and explorative probes across a targeted biomarker to specifically enrich large DNA fragments from complex metagenomic DNA. Captured DNA fragments are sequenced to explore biomarker diversity and adjacent flanking regions. The red rectangles indicate the targeted regions.

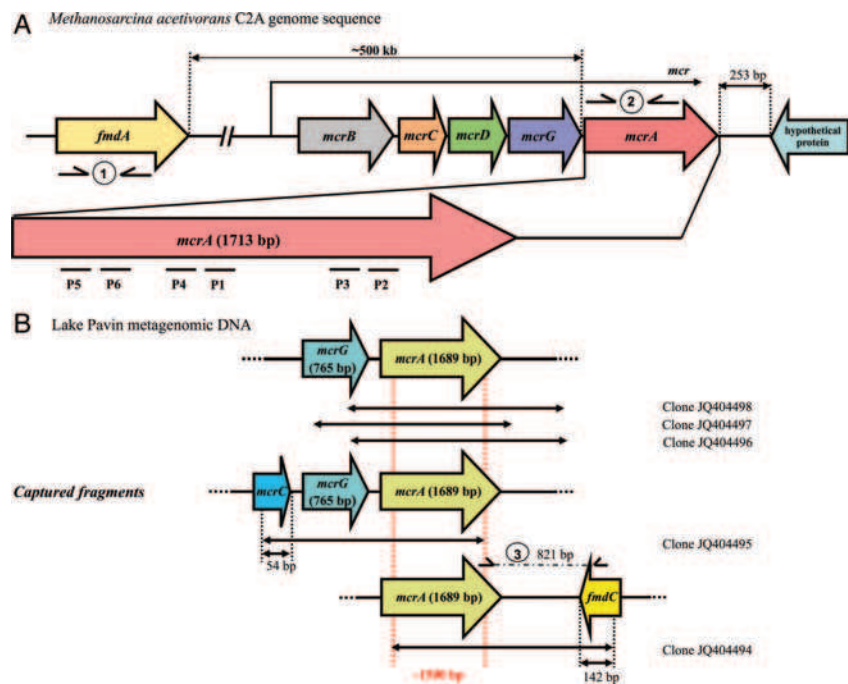


Figure 2. Schematic representation of *mcr* operon fragments on (A) *M. acetivorans* C2A gDNA and (B) Lake Pavin metagenomic DNA. Primer pairs used for *fmdA* (1) and *mcrA* (2) quantification as well as *mcrA-fmdC* region (3) amplification are symbolized. Dashed arrows indicate the sequence coverage of each of the five clones retrieved from the environmental sample (B). P1–P6: Positions of the six capture probes in the *mcrA* gene of *M. acetivorans* (see Supplementary Table S1 for probe sequences).

The second set of capture probes was 26 oligos (1 49-mer and 25 50-mers) designed to target *mcrA* and *mrtA* (encoding the α -subunit of the methyl co-enzyme M reductase isoform II), but not the *mcrA* of anaerobic methanotrophs (Supplementary Table S2, Supplementary Fig. S1).

Oligonucleotides were purchased from Eurogentec S.A. (Belgium). The RNA probe was prepared as described by Gnirke *et al.*¹⁹

2.2. Preparation of biological samples and libraries

The two biological models used in this study were the *M. acetivorans* C2A strain (DSM 2834) and Lake Pavin, located in the French Massif Central (45°29'74"N, 2°53'28"E). The *M. acetivorans* C2A strain was cultivated using the medium 304 (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium304.pdf) according to the manufacturer's instructions. gDNA from the strain was extracted using the Easy DNA kit (Invitrogen), whereas environmental DNA was extracted from 350 ml of freshwater collected from Lake Pavin at a 90-m depth, as described by Dugat-Bony *et al.*³⁰

Libraries were prepared using Roche's GS FLX Titanium General Library Preparation Kit (Roche Applied Science) according to the manufacturer's instructions. First, 5 μ g of DNA was sheared by nebulization. DNA fragments were size selected with AMPure beads (Beckman Coulter Genomics). After purification, fragment end polishing, adaptor ligation (A and B adapter keys; Supplementary Table S1) and fill-in reactions, the libraries were PCR amplified with the 454 Ti-A and 454 Ti-B primers (Supplementary Table S1). The cycle conditions were 3 min at 93°C followed by 20 cycles of 15 s at 93°C, 1 min at 58°C and 8 min at 68°C and a final elongation step at 68°C for 6 min. The amplified libraries were purified with AMPure beads and stored at -20°C until use.

For the amplicon library, *mcrA* fragments were PCR amplified from total community DNA with the *mcrA*-specific primer pair MM_01/MM_02³¹ (Supplementary Table S1). The amplicon was run on a 2% (wt/vol) agarose gel, and the ~500 bp product was purified with a QIAquick gel extraction kit (Qiagen) and AMPure beads. Each DNA library was quantified by fluorometry with a Quant-iT PicoGreen dsDNA assay kit (Invitrogen). The DNA quality and size distribution were assessed on an Agilent Bioanalyzer High Sensitivity DNA chip (Agilent Technologies).

2.3. Hybridization capture and elution

For each SHS-capture method library, 2.5 μ g of salmon sperm DNA (Ambion) and 500 ng of DNA library were mixed (7 μ l final volume), denatured for 5 min at 95°C, incubated for 5 min at 65°C

before adding 13 μ l of prewarmed (65°C) hybridization buffer (10X SSPE, 10X Denhardt's Solution, 10 mM EDTA and 0.2% SDS) and 6 μ l freshly prepared, prewarmed (2 min at 65°C) biotinylated RNA probes (500 ng). After 24 h at 65°C, 500 ng of washed M-280 Dynabeads coated with streptavidin (Invitrogen) were added to the hybridization mix that was incubated for 30 min at room temperature (RT). The beads were precipitated with a magnetic stand (Ambion) and washed once for 15 min at RT with 500 μ l 1X SSC/0.1% SDS and three times for 10 min at 65°C with 500 μ l prewarmed 0.1X SSC/0.1% SDS. The captured DNA was eluted with 50 μ l 0.1 M NaOH for 10 min at RT. After magnetic bead precipitation, the DNA supernatant was transferred to a sterile tube containing 70 μ l of 1 M Tris-HCl pH 7.5, purified on a QIAquick column (Qiagen) and eluted in a final volume of 20 μ l. A 2.5 μ l aliquot was subjected to 15 cycles of PCR amplification using the 454 Ti-A and Ti-B primers as described above. After purification, a second round of capture was performed from each first-round PCR product. To increase the DNA yield, a final PCR amplification consisting of 20 cycles was performed. The final product was purified on a QIAquick column (Qiagen) and quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

2.4. Sanger sequencing and data analysis

PCR products were cloned using the TOPO TA cloning kit (Invitrogen). Plasmids were screened for high-size inserts by digestion with *EcoRI*, and positive clones were Sanger sequenced at MWG DNA sequencing services (Ebersberg, Germany). Sequences were processed and joined using the Staden package program,³² and primer sequences were removed from paired-end consensus sequences. The *mcr* sequence data retrieved from Lake Pavin by the SHS method were deposited in the GenBank database under accession numbers JQ404494, JQ404495, JQ404496, JQ404497 and JQ404498, and the sequence of the *mcrA-fmd* region-spanning fragment was deposited under accession number JQ425691.

2.5. 454 GS FLX Titanium sequencing and data analysis

DNA samples were sequenced using the GS FLX Titanium system on the 'GINA' platform (part of the GENTYANE platform, labelled IBISA since 2009; BP 392, 63 011 Clermont-Ferrand, France) at the Centre Jean Perrin, according to the manufacturer's specifications. For quality filtering and de-replication of reads, sequences were trimmed with the PRINSEQ-lite PERL script³³ using the parameters described in

the preprocessing chart (http://prinseq.sourceforge.net/Preprocessing_454_SFF_chart.pdf).

Functional assignment and enrichment were assessed with a BLASTX query³⁴ against a database containing 12 603 McrA protein sequences downloaded from the Genbank database (<http://www.ncbi.nlm.nih.gov/>), using WWW-Query (http://pbil.univ-lyon1.fr/search/query_fam.php) to perform an advanced keyword search. Reads showing >40% identity over 100 or more amino acids were classified as McrA sequences. Chimaera detection was performed with the UCHIME program³⁵ with a stringent threshold score of five. Sequences containing possible frameshifts were identified with the '-w 20' BLAST option and disabled low complexity filters. Amino acid sequences without frameshifts were extracted from the BLAST results, and only the sequences that passed this filter were chosen for further phylogenetic analysis.

The sequence data were deposited in the NCBI as a Short Read Archive (SRA) project under accession no. SRA049219.

2.6. Phylogenetic analysis and tree construction

All McrA sequences derived from the SHS method and metagenomic libraries were aligned to a sequence obtained from the amplicon library. The amino acid alignment used the ClustalW2 alignment method³⁶ driven by the Seaview version 4 program³⁷ to select the reads having at least 100 amino acids in common with this reference sequence. The overlapping regions of the remaining amino acid sequences, all amplicon pyrosequences and 29 McrA sequences previously identified from the same sampling depth and downloaded from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) were fed to CD-HIT³⁸ that assigned them to operational taxonomic units (OTUs) using a complete linkage clustering method at a 91% cut-off value.^{27,39}

One representative sequence of each OTU was chosen to build a phylogenetic tree (Seaview 4)³⁷ using the neighbour-joining method^{40,41} and 1000 bootstrapped trials. Closely related sequences available from GenBank (<http://www.ncbi.nlm.nih.gov/>) were included in the phylogenetic trees to decipher the microbial community diversity. A final tree was drawn in MEGA version 5.⁴²

2.7. qPCR assays for enrichment and methanogen abundance

The assays were conducted in 20 µl with 5 µl of DNA sample or *mcrA* PCR product standards (covering a dynamic range of 5×10^7 to 50 copies), 10 µl of 2X MESA Green quantitative Polymerase Chain Reaction (qPCR) for SYBR assay mixture (Eurogentec S.A) and 0.2 µM forward and reverse primers. The

thermo cycling protocol included an initial step of 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at the melting temperature of each primer set for 15 s and elongation at 68°C for 30 s. The samples and each point of the standard curve were quantified in triplicate. The primer sets are described in Supplementary Table S3. The data were analysed with Realplex software version 1.5 (Eppendorf Inc.) and MxPro qPCR software 4.10d (Agilent technologies). Based on the $\Delta\Delta C_t$ method,⁴³ relative enrichments (*R*) were calculated according to $R = 2^{-\Delta\Delta C_t}$. The relative quantification method established a mean *Ct* value comparison (ΔC_t) between *mcrA* (target gene) and *fmdA* (non-target gene 500 kb upstream from *mcrA*). The relative capture enrichment was determined by the comparison of ΔC_t before and after capture, and this result described the fold change or $\Delta\Delta C_t$.

2.8. SHS de novo read assembly

The filtered SHS reads were assembled with Newbler version 2.6 (Roche Applied Science) using stringent assembly parameters (60 bases overlap and 95% overlap identity) and the '- rip' option that forces Newbler to place each read into one unique contig. The functional assignment of contigs and singletons was performed by a BLASTX query³⁴ against our database containing 12 603 McrA protein sequences. Chimaeras were detected in the *mcrA* contigs and singletons with the UCHIME program³⁵ and a stringent threshold score of five. Prediction of the *mcrA* gene location within contigs and singletons was performed by BLASTN⁴⁴ against the reference genomes of *Candidatus Methanoregula boonei* 6A8 (*Methanomicrobiales* order, accession no. NC_009712), *Methanosaeta concilii* GP-6 (*Methanosarcinales* order, accession no. CP002565) and *Methanospaera stadtmannae* DSM 3091 (*Methanobacteriales* order, accession no. CP000102). Contigs extending at least 100 nucleotides beyond *mcrA* were segregated for BLASTX³⁴ analysis against the non-redundant (nr) protein sequences database to identify putative open-reading frames within the flanking regions.

The sequence data from homologous *mcrA* contigs (without chimaeras or frameshifts) were deposited in the GenBank database under accession no. KC184908 to KC185399.

3. Results

3.1. Development of an SHS method for genomic-scale sequence enrichment

3.1.1. Method validation: *mcrA* gene enrichment from *M. acetivorans* C2A gDNA We performed the initial validation of our enrichment

strategy by capturing the *mcrA* gene from a 1 to 3 kb fragment library of the completely sequenced methanogenic *M. acetivorans* C2A strain. The minimal probe set spanned different non-overlapping regions of the gene (Fig. 2A). The qPCR reactions revealed a 461-fold relative enrichment of *mcrA* sequences after the first cycle of capture and at least 175 365-fold enrichment after the second cycle. Furthermore, as the *M. acetivorans* C2A genome consists of 5751 kb with a single *mcrA* gene copy, the probability of randomly sequencing this gene from a 1 to 3 kb fragment size clone library is 0.02–0.05%. Using our solution-based DNA capture-enrichment method and working on an isolated species, the likelihood increased from 7.8 to 23% after the first cycle and could reach 100% after the second.

The DNA sequence of fragments retrieved after the second cycle of capture was controlled by the cloning-sequencing method. Six clones were sequenced, and all had a perfect correspondence to the *mcrA* gene from *M. acetivorans* C2A, reinforcing the efficiency of the two iterative cycles of capture. The captured fragments were assembled into a 1834-bp contig containing the nearly complete *mcrA* gene (1645 bp) and its 3' non-coding region (189 bp). After validating this approach, we further tested the performance of the method by enriching *mcrA* sequences from a complex methanogenic freshwater environment.

3.1.2. Environmental application: *mcrA* sequence enrichment from a methanogenic lacustrine environment (Lake Pavin) The freshwater sample was collected in the anoxic zone at 90 m depth, where the highest methanogen diversity was available in the lacustrine environment.²⁷ An improved *mcrA* probe set included all known *mcrA* sequences and targeted new variants with explorative probes (Supplementary Table S2). The efficiency of the *mcrA* enrichment was determined by cloning and sequencing the second capture product. Five out of the ten clones with large inserts (2041–2493 bp) included *mcrA* sequences. All positive clones had a ~1500 bp common zone corresponding to the *mcrA* gene, but they also harboured upstream or downstream regions containing other genes (Fig. 2B). BLAST analysis of the cloned sequences revealed that they are very similar (99% similarity) to *mcrA* sequences previously retrieved from this ecosystem (accession nos. GQ389949, GQ389912 and GQ389806).²⁷ The closest relative to the *mcrA*, *mcrG* and partial *mcrC* sequences were from a cultured methanogen, *Candidatus Methanoregula boonei* 6A8 (>85, 84 and 81% similarity, respectively). This hydrogenotrophic species belongs to the *Methanomicrobiales* order, and it was isolated from an acidic peat bog.⁴⁵ Furthermore, the *fmdC* gene fragment identified 821 bp downstream the target gene (Fig. 2B)

that shared 77% identity with subunit C of the formyl methanofuran dehydrogenase gene of this species. This gene has been located in the reference genome (GenBank: CP000780.1) at almost 300 kb from the *mcr* operon. It should be noted that this genome organization—with the *fmd* operon located just downstream from the *mcr* operon—has not been described previously in methanogens. To exclude the possibility of chimera formation during metagenomic library amplification, a PCR fragment spanning the *mcrA*–*fmdC* region was obtained directly from the initial metagenomic DNA sample, using two specific primers (Fig. 2B, Supplementary Table S1). The sequenced 821 bp PCR product (JQ425691) confirmed the organization revealed by the SHS method (100% identity with the captured DNA fragment).

Our results showed that the capture method not only efficiently enriched targets out of a complex environmental genomic mixture, but also recovered sequences adjacent to the targeted biomarker gene. Additionally, the SHS method was coupled with NGS technologies to assess the coverage of archaeal *mcrA* diversity in a complex ecosystem.

3.2. Metagenome exploration with genome-scale sequence enrichment and NGS

The benefit of the SHS method in terms of diversity coverage, when compared with more classical approaches, was further examined by sequencing the SHS capture products. A new random-shotgun DNA metagenomic library adapted for pyrosequencing (fragment sizes ~500 bp) was prepared for the SHS products and for direct sequencing (shotgun metagenomics approach). From the same metagenomic DNA sample, *mcrA* PCR products were also amplified with the primer set MM_01–MM_02,³¹ generating amplicons of ~500 bp. Sequencing (captured DNA fragments, metagenome and amplicons) was performed with the 454 GS FLX Titanium technology, generating a slightly different amount of raw data with an average read length of 414–471 bases. After pre-processing, sequencing datasets from all three approaches had nearly equivalent numbers of reads (Table 1).

3.2.1. Functional assignment and enrichment performance Only three reads (0.003% of total reads) from the random-shotgun sequencing approach corresponded to the *mcrA* gene. For the SHS method, 50 727 reads were identified as *mcrA* sequences (41.32%), and almost all the amplicon approach sequences were from *mcrA* (119 409 reads, 99.98%).

For *mcrA* diversity evaluation, however, we only analysed high-quality sequences (no chimeras or

Table 1. Summary statistics from 454 pyrosequencing

	Metagenome	Amplicons	SHS
Total number of raw reads	136 256	121 665	177 977
Number of reads after pre-processing	116 365	119 437	122 772
Average length of cleaned reads (bases)	471	414	454
<i>mcrA</i> homologous sequences ^a	3	119 409	50 727
Enrichment performance (%)	0.003	99.98	41.32
Number of chimaeras	0	150	30
Number of reads containing frameshifts	1	80 390	21 855
Number of high-quality <i>mcrA</i> homologous sequences (without chimaera and frameshifts)	2	38 869	28 842
<i>McrA</i> sequences used for methanogenic diversity and abundance (comparison of a common region)	1	38 807	11 442
Number of OTUs	1	40	44
<i>McrA</i> sequences related to OTUs	1	38 784 ^b	11 324 ^b
Relative abundance of <i>mcrA</i> sequences affiliated with <i>Methanomicrobiales</i> (%)	0	98.57	98.82
Relative abundance of <i>mcrA</i> sequences affiliated with <i>Methanosarcinales</i> (%)	0	0.005	0.86
Relative abundance of <i>mcrA</i> sequences affiliated with the Novel Order (%)	100	1.43	0.13
Relative abundance of <i>mcrA</i> sequences affiliated with <i>Methanobacteriales</i> (%)	0	0	0.19

^aBLASTX parameters: percentage of identity: 40%; E-value cut-off: 10.

^b*McrA* sequences related to OTUs containing more than one sequence.

frameshifts), and all the problematic reads were subsequently excluded.

3.2.2. Methanogen diversity and abundance The phylogeny of the methanogenic *McrA* protein sequences was investigated and compared for each of the three approaches. We used ClustalW2³⁶ to determine a common reference region of 143 amino acids shared by the largest number of *McrA* sequences retrieved from the 3 approaches. All *McrA* sequences that included this region were truncated so that at least 100 amino acids aligned with this reference. The resulting sequences, which included 1 read from the shotgun library, 11 442 reads from the SHS method library and 38 807 reads from the amplicon library, were used for further analysis. Furthermore, 29 additional sequences (referred to as Pavin90m) from a previous study²⁷ were included in the analysis.

Following the clustering method, 127 distinct OTUs (longer than 300 bp) were observed, and the 58 OTUs that contained more than 1 sequence were included in a more detailed phylogenetic analysis. The shotgun library sequence, which contained a single final read, was also included. Among these 58 OTUs, 44 were detected from the SHS method, 40 from the amplicon approach, 1 from the metagenomic shotgun library and 3 from Pavin90m sequences. The SHS method and amplicons shared 27 OTUs, including 3 from the Pavin90m sequences (Fig. 3). The remaining 31 OTUs were specific to a single method, with 1 for the metagenome, 17 for the SHS and 13 for the amplicons (Fig. 3).

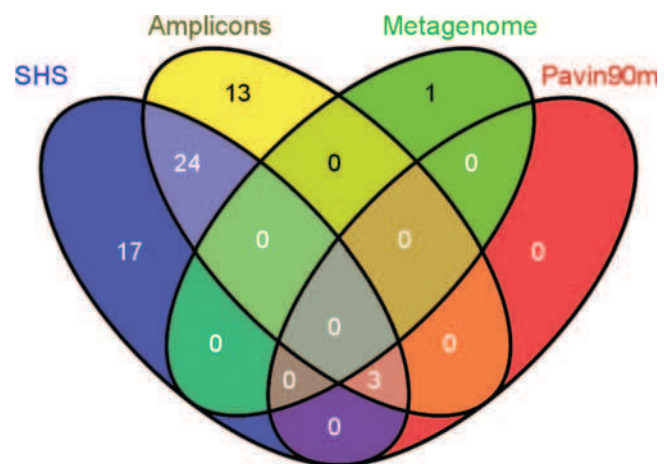


Figure 3. Venn diagram showing the number of unique and shared OTUs for the in-solution capture method (SHS), PCR-based strategy (Amplicons) and sequences isolated at 90 m depth from a previous PCR-based study of Lake Pavin (Pavin90m).²⁷ The Venn diagram was generated with Venny (<http://bioinfogp.cnb.csic.es/tools/venny/index.html>).

The 58 OTUs covered four lineages including *Methanobacteriales*, *Methanomicrobiales*, *Methanosarcinales* and a putative fourth lineage called 'Novel Order'. Most OTUs were closely related to the *Methanomicrobiales* order (48 OTUs, 98.6% of the total input sequences). OTU3, OTU10 and OTU17 formed a distinct branch within this cluster (Fig. 4A), and they were closely related to cultured methanogenic species that also have an insertion in their *McrA* protein sequence (Supplementary Fig. S2). Both the SHS and amplicon

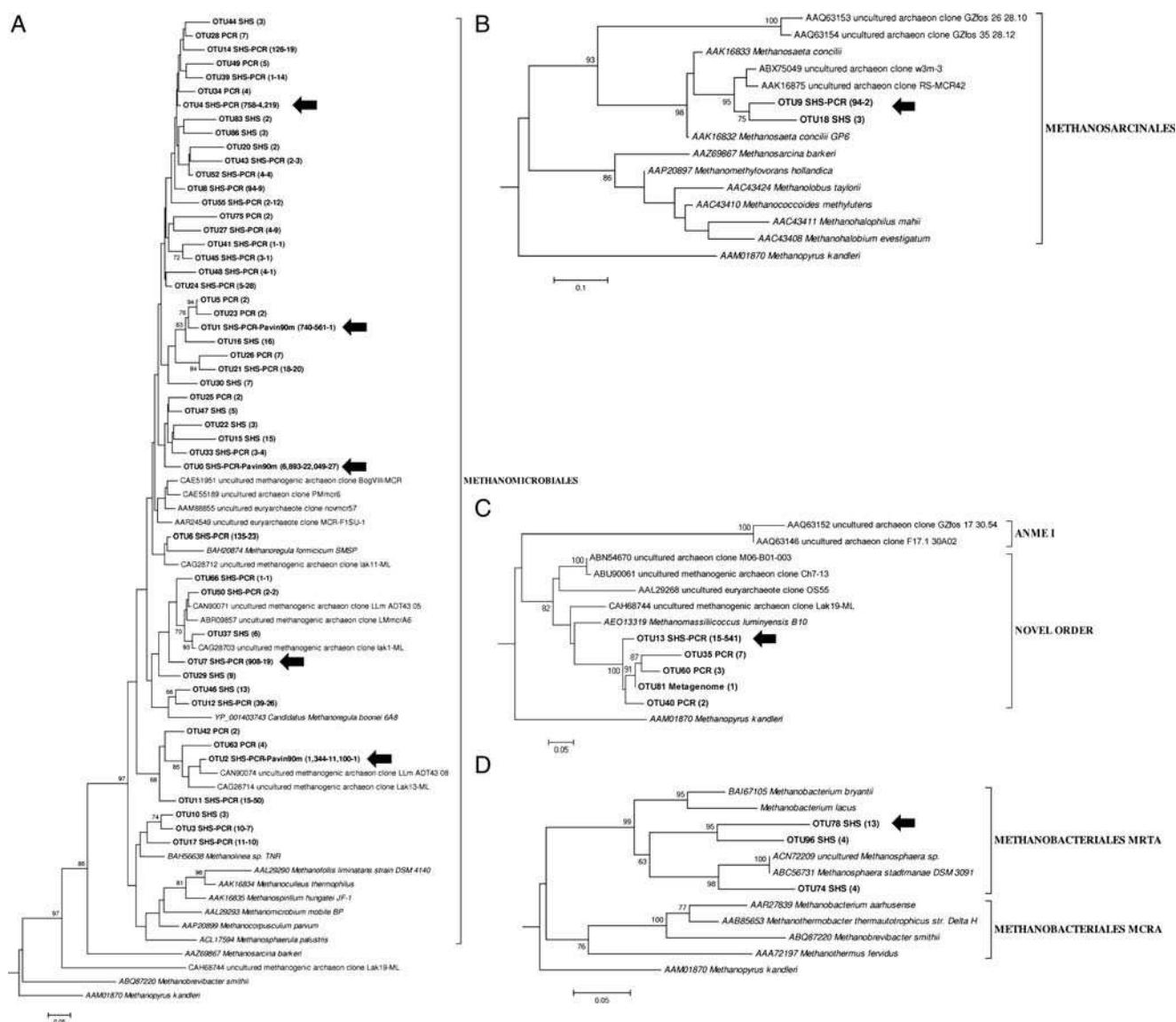


Figure 4. Phylogenetic analysis of deduced McrA amino acid sequences obtained from the PCR, SHS and Pavin90m datasets showing evolutionary distances within the orders *Methanomicrobiales* (A), *Methanosarcinales* (B), Novel Order (C) and *Methanobacteriales* (D). Evolutionary history was inferred using the neighbour-joining method^{40,41} (Poisson distance model) using Seaview software.³⁷ The final tree was drawn in MEGA 5.⁴² The bars represent a 5% sequence divergence. Numbers at the nodes represent bootstrap values >60% (1000 resamplings). The number of amino acid sequences assigned to each OTU is given in brackets, together with the name of the strategies for obtaining them. McrA amino acid sequence from *Methanosarcina barkeri* (AAZ69867), uncultured methanogenic archaeon clone Lak19-ML (CAH68744) and *Methanobrevibacter smithii* (ABQ87220) were used as outgroups, and *Methanopyrus kandleri* (AAM01870) was an outgroup for rooting the tree. Bold arrows indicate dominant OTUs.

strategies clustered sequences in the most abundant OTUs (Fig. 5). These abundant OTUs represented 94 and 98%, respectively, of the total sequences for each approach. The *Methanosarcinales* (two OTUs; Fig. 4B) grouped into two distinct branches were related to the reference acetoclastic species *M. concilii* GP6 (85 and 87% similarity with OTU9 and OTU18, respectively). The most abundant cluster was OTU9 that represented 0.83% of the total SHS reads and 0.005% for the total amplicon reads (Fig. 5). In contrast, the putative Novel Order (five OTUs; Fig. 4C) was dominated by OTU13 clustering with 1.39% of the

total amplicons sequences, but only 0.13% of the total SHS reads (Fig. 5). Even if we did not include the more recently described sequences of *Methanomassiliicoccus luminyensis*⁴⁶ and *Candidatus Methanomethylophilus alvus*⁴⁷ belonging to the novel order for the probe design, distant sequences could be captured with probes by a mismatched nucleotide pairing. We cannot exclude that the sequences captured by specific probes allow indirect hybridization of other *mcrA* sequences as described for DNA microarrays experiments and referred to as ‘hitchhiking’.⁴⁸ Despite the substantial sequencing effort for amplicons, no

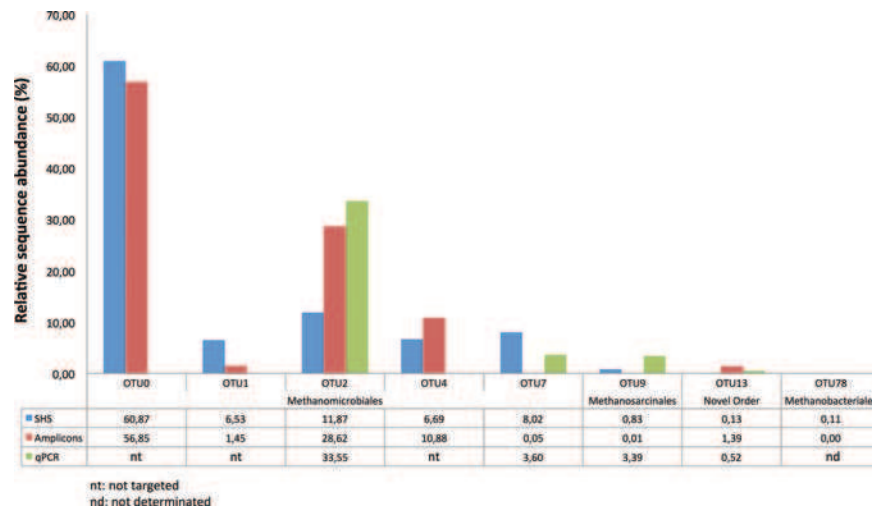


Figure 5. The relative abundances of dominant OTUs from four methanogenic bacterial orders identified by the targeted capture method (SHS), PCR-based strategy (amplicons) and qPCR experiments (qPCR). The relative abundances calculated by qPCR were computed using *mcrA* copy number as reference obtained using a primers pair targeting all OTUs (Supplementary Table S3).

sequences belonging to the *Methanobacteriales* order were recovered from this approach. These sequences were obtained only from the SHS sample (Fig. 4D), and they were clustered in three OTUs such that one was 90% similar to MrtA (MCR isoenzyme encoded by the *mrt* operon) from *M. stadtmanae* DSM 3091⁴⁹ and the remaining two were 77 and 79% identical to MrtA sequences from *Methanobacterium lacus* that is in the *Methanobacteriales* order and has been isolated from Lake Pavin sediments.⁵⁰ These sequences represented 0.19% of total SHS *mcrA*-related sequences, with the most abundant OTU78 clustering 0.11% of the total SHS reads (Fig. 5).

The GC content of the *mcrA* genes ranged from 50.4 to 61.1% for amplicons and from 37 to 63.2% for SHS. In the *mcrA* database, the GC content ranges from 36.2 to 67.2%, indicating that the SHS method is most likely less affected by GC composition than PCR approaches. Furthermore, we evaluated the presence of mismatch residues between PCR primers and probes on *mcrA* genes in both SHS and amplicon approaches. We identified 99.10, 0.77 and 0.13% of *mcrA* sequences for amplicons versus 37.68, 50.22 and 12.10% for SHS with 0, 1 and 2 mismatch residues, respectively, between probes (or primers) and sequences. This trend highlights the potential advantage of the SHS approach with long capture probes that tolerate more mismatches, allowing access to new *mcrA* gene variants.

In parallel, qPCR was used to precisely describe the methanogen abundance in Lake Pavin with regard to the most abundant OTUs and bacterial orders (primers are listed in Supplementary Table S3). The results were compared with the relative sequence abundance calculated previously for the selected OTUs with amplicons and SHS (Fig. 5). The abundance

of OTU2, which included the *Methanomicrobiales* order, was similar in qPCR and amplicons (33.5 and 28.62%), but not SHS (11.87%). In contrast, the second *Methanomicrobiales* OTU (OTU7) was more abundant in SHS (8.02%) and qPCR (3.6%), but not amplicons (0.05%). The same trend was observed for OTU9 (*Methanosarcinales*). No significant difference was observed for OTU13 (Novel Order). Finally, no qPCR amplification of OTU78 (*Methanobacteriales*) occurred. However, we validated the presence of this OTU in Lake Pavin by successive PCR cycles, cloning and sequencing (100% identity). This result indicates that *Methanobacteriales* are rare in this ecosystem.

3.2.3. De novo assembly of SHS reads To reconstruct contigs with sequences flanking the targeted *mcrA* gene, *de novo* assembly was performed using the pyrosequencing reads obtained by the SHS method (Table 2). We identified 691 contigs (301–1639 bases) with *mcrA* sequences. By mapping these sequences to complete reference genomes for the *Methanomicrobiales*, *Methanosarcinales* and *Methanobacteriales* orders (no genome was available for the Novel Order), we identified contigs extending into the *mcrA* flanking regions. The upstream sequences were all part of the *mcrG* gene. We also characterized two adjacent ORFs located at 200 bases downstream from the *mcrA* gene and in the same orientation; these ORFs encoded a DtxR family iron (metal)-dependent repressor and a DOMON domain-containing protein. The DtxR sequences were closely related (76–83% identity) to *Methanosphaerula palustris* E1-9C (accession no. ACL16981) of the *Methanomicrobiales* order. In the reference genome of this species, the gene is located ~700 kb downstream of the *mcr* operon. The

Table 2. Summary statistics from *de novo* assembly

Newbler version 2.6	SHS
No. of reads used for assembly	122 772
No. of reads assembled into contigs	53 307
No. of singletons	56 834
Outliers ^a	12 631
No. of contigs assembled	1916
N ₅₀ contig size (bases)	820
No. of <i>mcrA</i> homologous contigs	693
No. of <i>mcrA</i> homologous singletons	1142
Number of chimaeras	5
Number of high-quality <i>mcrA</i> homologous contigs (without chimaeras)	691
Number of high-quality <i>mcrA</i> homologous singletons (without chimaeras)	1139
Average <i>mcrA</i> homologous contig length (bases)	589
Largest <i>mcrA</i> homologous contig length (bases)	1639

^aReads were discarded due to quality control by Newbler.

sequences of DOMON domain-containing protein are closely related (74–80% identity) to *M. concilii* GP-6 (accession no. AEB67518) that belongs to the *Methanosarcinales* order. In the reference genome of this species, the gene is located ~50 kb downstream of the *mcr* operon.

4. Discussion

We captured specific target DNA from a complex environmental metagenome using a novel SHS capture method and NGS. We showed that the relative enrichment of the target sequence was increased to 175 365-fold with 2 cycles of capture, and this result was superior to previous studies using a single cycle^{18,19} and microarray-based capture.⁵¹ We applied this strategy to the anoxic layer of Lake Pavin, where *Archaea* account for 17% of 4,6-diamidino-2-phenylindole-stained cells⁵² and only a fraction of these microbes are methanogens. Our SHS strategy specifically enriched *mcrA* sequences from the environmental sample. In comparison with the random-shotgun metagenomic approach (0.003% recovery of *mcrA* sequences), the SHS method was superior (41.32% *mcrA* sequence enrichment). However, the capture efficiency is also likely influenced by the number of probes used per region and the mismatched residues between the probes and their targets. Consequently, two rounds of capture and multiple long RNA probes are advantageous for efficient enrichment.

With a random-shotgun metagenomics approach, many hundreds of thousands of additional single reads would have been necessary to estimate the biodiversity of the methanogen community in this

environment. The SHS experiment contained much more *mcrA* data and provided a solid taxonomic basis for studying methanogens diversity. Finally, PCR was the most effective enrichment approach; with ~100% of the amplicons corresponding to the biomarker, the primers used were very specific and efficient.³¹

The SHS and amplicon strategies both revealed similar patterns in methanogen communities such as the high abundance and diversity of *Methanomicrobiales* sequences (more than 98% of the total sequences representing 48 OTUs). These data confirm a previous study by Biderre-Petit *et al.*²⁷ High-throughput sequencing, however, reveals that methanogen diversity is much higher than previously estimated by amplicon libraries and Sanger sequencing.²⁷ Importantly, the amplicon sequencing approach missed all the *Methanobacteriales* taxonomic groups and some *Methanosarcinales*, possibly due to *mcrA* primer bias. PCR undersampling often leads to significant underestimation of true community diversity.^{24,53} SHS efficiently targets rare sequences, as demonstrated for *Methanobacteriales*, and does not appear to be influenced by GC content. As previously demonstrated for microarray approaches,^{21,22,54} more extensive explorative capture probe sets could recover rare sequences, leading to the detection of many uncharacterized microbial populations. Moreover, the SHS and amplicon library results were correlated by qPCR.

We also used *de novo* assembly of SHS sequence reads to explore the regions flanking the target gene, and we identified two ORFs (*dtxR* and DOMON domain) at previously unknown positions downstream of *mcrA*. Because this genomic organization may link methanogenesis to electron transfer and Fe homeostasis in organisms living in the anoxic layer of the Lake Pavin, it could reflect adaptation to this particular environment. More experiments are needed, however, to validate this hypothesis.

In this study, we present a novel enrichment method that, when coupled to NGS, expands our knowledge of the diversity of a target gene within a complex microbial community. The method was successfully applied to a lacustrine environment using the *mcrA* gene, and it revealed higher methanogen community diversity than observed with other methods. To some extent, this method could be applied to phylogenetic studies to explore the diversity of commonly conserved genes such as the 16S rRNA biomarker. The main limitation is the design of high quality probes sets to expect a full coverage of 16S rDNA sequences as complete as possible. New algorithms, such as KASpOD,⁵⁵ can be used to design highly specific and explorative probes (i.e. targeting sequences not already included in databases) based on oligonucleotide *k-mer* signatures. These probe designs would be extremely suitable and beneficial to the SHS approach.

With the emergence of third generation sequencing platforms and the capability to sequence longer DNA sequences without library construction,^{56,57} the SHS strategy could link genomic structure and function in microbial communities.

Acknowledgements: We would like to thank Yannick Bidet and Maud Privat from the Centre Jean Perrin for their help with sample processing on the 454 GS FLX pyrosequencing platform. We also thank Sarah Orlhac and Nicolas Gallois for their efficient technical assistance and David Tottey for reviewing the English version of the manuscript.

Supplementary data: Supplementary data are available at www.dnaresearch.oxfordjournals.org.

Funding

This work was supported by the ANR-09-EBIO-009 project (Agence Nationale de la Recherche). J.D. was supported by a studentship from the Centre National de la Recherche Scientifique (CNRS, grant number 163588) and the Région Auvergne. N.P. was funded by Direction Générale de l'Armement (DGA).

References

- Whitman, W.B., Coleman, D.C. and Wiebe, W.J. 1998, Prokaryotes: the unseen majority, *Proc. Natl. Acad. Sci. USA*, **95**, 6578–83.
- Curtis, T.P., Head, I.M., Lunn, M., Woodcock, S., Schloss, P.D. and Sloan, W.T. 2006, What is the extent of prokaryotic diversity? *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, **361**, 2023–37.
- Amann, R., Ludwig, W. and Schleifer, K.-H. 1995, Phylogenetic identification and in situ detection of individual microbial cells without cultivation, *Microbiol. Rev.*, **59**, 143–69.
- Eisen, J.A. 2007, Environmental shotgun sequencing: its potential and challenges for studying the hidden world of microbes, *PLoS Biol.*, **5**, e82.
- Handelsman, J., Rondon, M.R., Brady, S.F., Clardy, J. and Goodman, R.M. 1998, Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products, *Chem. Biol.*, **5**, R245–249.
- Biddle, J.F., Fitz-Gibbon, S., Schuster, S.C., Brenchley, J.E. and House, C.H. 2008, Metagenomic signatures of the Peru Margin seafloor biosphere show a genetically distinct environment, *Proc. Natl. Acad. Sci. USA*, **105**, 10583–88.
- Tringe, S.G., von Mering, C., Kobayashi, A., et al. 2005, Comparative metagenomics of microbial communities, *Science*, **308**, 554–7.
- Riesenfeld, C.S., Schloss, P.D. and Handelsman, J. 2004, Metagenomics: genomic analysis of microbial communities, *Annu. Rev. Genet.*, **38**, 525–52.
- Suenaga, H. 2011, Targeted metagenomics: a high-resolution metagenomics approach for specific gene clusters in complex microbial communities, *Environ. Microbiol.*, **14**, 13–22.
- Edwards, R.A., Rodriguez-Brito, B., Wegley, L., et al. 2006, Using pyrosequencing to shed light on deep mine microbial ecology, *BMC Genomics*, **7**, 57.
- Mardis, E.R. 2008, The impact of next-generation sequencing technology on genetics, *Trends Genet.*, **24**, 133–41.
- Quince, C., Curtis, T.P. and Sloan, W.T. 2008, The rational exploration of microbial diversity, *ISME J.*, **2**, 997–1006.
- Hoff, K.J. 2009, The effect of sequencing errors on metagenomic gene prediction, *BMC Genomics*, **10**, 520.
- Summerer, D. 2009, Enabling technologies of genomic-scale sequence enrichment for targeted high-throughput sequencing, *Genomics*, **94**, 363–8.
- Albert, T.J., Molla, M.N., Muzny, D.M., et al. 2007, Direct selection of human genomic loci by microarray hybridization, *Nat. Methods*, **4**, 903–5.
- Okou, D.T., Steinberg, K.M., Middle, C., Cutler, D.J., Albert, T.J. and Zwick, M.E. 2007, Microarray-based genomic selection for high-throughput resequencing, *Nat. Methods*, **4**, 907–9.
- Mokry, M., Feitsma, H., Nijman, I.J., et al. 2010, Accurate SNP and mutation detection by targeted custom microarray-based genomic enrichment of short-fragment sequencing libraries, *Nucleic Acids Res.*, **38**, e116.
- Tewhey, R., Nakano, M., Wang, X., et al. 2009, Enrichment of sequencing targets from the human genome by solution hybridization, *Genome Biol.*, **10**, R116.
- Gnirke, A., Melnikov, A., Maguire, J., et al. 2009, Solution hybrid selection with ultra-long oligonucleotides for massively parallel targeted sequencing, *Nat. Biotechnol.*, **27**, 182–9.
- Iwai, S., Chai, B., Sul, W.J., Cole, J.R., Hashsham, S.A. and Tiedje, J.M. 2010, Gene-targeted-metagenomics reveals extensive diversity of aromatic dioxygenase genes in the environment, *ISME J.*, **4**, 279–85.
- Terrat, S., Peyretailade, E., Goncalves, O., et al. 2010, Detecting variants with Metabolic Design, a new software tool to design probes for explorative functional DNA microarray development, *BMC Bioinformatics*, **11**, 478.
- Dugat-Bony, E., Peyretailade, E., Parisot, N., et al. 2011, Detecting unknown sequences with DNA microarrays: explorative probe design strategies, *Environ. Microbiol.*, **14**, 356–371.
- Suzuki, M. and Giovannoni, S. 1996, Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR, *Appl. Environ. Microbiol.*, **62**, 625–30.
- Hong, S., Bunge, J., Leslin, C., Jeon, S. and Epstein, S.S. 2009, Polymerase chain reaction primers miss half of rRNA microbial diversity, *ISME J.*, **3**, 1365–73.
- Bastviken, D., Cole, J., Pace, M. and Tranvik, L. 2004, Methane emissions from lakes: dependence of lake characteristics, two regional assessments, and a global estimate, *Global Biogeochem. Cycles*, **18**, GB4009, doi:10.1029/2004GB002238.

26. Aeschbach-Hertig, W., Hofer, M., Kipfer, R., Imboden, D.M. and Wieler, R., 1999, Accumulation of mantle gases in a permanently stratified volcanic lake (Lake Pavin, France), *Geochim. Cosmochim. Acta*, **63**, 3357–72.
27. Biderre-Petit, C., Jezequel, D., Dugat-Bony, E., et al. 2011, Identification of microbial communities involved in the methane cycle of a freshwater meromictic lake, *FEMS Microbiol. Ecol.*, **77**, 533–45.
28. Reeve, J.N. 1992, Molecular biology of methanogens, *Annu. Rev. Microbiol.*, **46**, 165–91.
29. Klein, A., Allmansberger, R., Bokranz, M., Knaub, S., Müller, B. and Muth, E. 1988, Comparative analysis of genes encoding methyl coenzyme M reductase in methanogenic bacteria, *Mol. Gen. Genet.*, **213**, 409–20.
30. Dugat-Bony, E., Missaoui, M., Peyretailade, E., et al. 2011, HiSpOD: probe design for functional DNA microarrays, *Bioinformatics*, **27**, 641–8.
31. Mihajlovski, A., Alric, M. and Brugere, J.F. 2008, A putative new order of methanogenic Archaea inhabiting the human gut, as revealed by molecular analyses of the mcrA gene, *Res. Microbiol.*, **159**, 516–21.
32. Staden, R. 1996, The Staden sequence analysis package, *Mol. Biotechnol.*, **5**, 233–41.
33. Schmieder, R. and Edwards, R. 2011, Quality control and preprocessing of metagenomic datasets, *Bioinformatics*, **27**, 863–4.
34. Altschul, S.F., Madden, T.L., Schäffer, A.A., et al. 1997, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, *Nucleic Acids Res.*, **25**, 3389–402.
35. Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C. and Knight, R. 2011, UCHIME improves sensitivity and speed of chimera detection, *Bioinformatics*, **27**, 2194–200.
36. Larkin, M.A., Blackshields, G., Brown, N.P., et al. 2007, Clustal W and Clustal X version 2.0, *Bioinformatics*, **23**, 2947–8.
37. Gouy, M., Guindon, S. and Gascuel, O. 2009, SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building, *Mol. Biol. Evol.*, **27**, 221–4.
38. Li, W. and Godzik, A. 2006, Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences, *Bioinformatics*, **22**, 1658–9.
39. Luton, P.E., Wayne, J.M., Sharp, R.J. and Riley, P.W. 2002, The mcrA gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations in landfill, *Microbiology*, **148**, 3521–30.
40. Studier, J. and Keppler, K. 1988, A note on the neighbor-joining algorithm of Saitou and Nei, *Mol. Biol. Evol.*, **5**, 729–31.
41. Saitou, N. and Nei, M. 1987, The neighbor-joining method: a new method for reconstructing phylogenetic trees, *Mol. Biol. Evol.*, **4**, 406–25.
42. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011, MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, *Mol. Biol. Evol.*, **28**, 2731–9.
43. Livak, K.J. and Schmittgen, T.D. 2001, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method, *Methods*, **25**, 402–8.
44. Altschul, S., Gish, W., Miller, W., Myers, E. and Lipman, D. 1990, Basic local alignment search tool, *J. Mol. Biol.*, **215**, 403–10.
45. Brauer, S.L., Cadillo-Quiroz, H., Yashiro, E., Yavitt, J.B. and Zinder, S.H. 2006, Isolation of a novel acidiphilic methanogen from an acidic peat bog, *Nature*, **442**, 192–4.
46. Dridi, B., Fardeau, M.L., Ollivier, B., Raoult, D. and Drancourt, M. 2012, *Methanomassiliicoccus luminyensis* gen. nov. sp. nov. a methanogenic archaeon isolated from human faeces, *Int. J. Syst. Evol. Microbiol.*, **62**, 1902–7.
47. Borrel, G., Harris, H.M.B., Tottey, W., et al. 2012, Genome sequence of 'Candidatus Methanomethylophilus alvus' Mx1201, a methanogenic archaeon from the human gut belonging to a seventh order of methanogens, *J. Bacteriol.*, **194**, 6944–5.
48. Palmer, C. 2006, Rapid quantitative profiling of complex microbial populations, *Nucleic Acids Res.*, **34**, e5.
49. Fricke, W.F., Sedorf, H., Henne, A., et al. 2005, The genome sequence of *Methanosphaera stadtmanae* reveals why this human intestinal archaeon is restricted to methanol and H₂ for methane formation and ATP synthesis, *J. Bacteriol.*, **188**, 642–58.
50. Borrel, G., Joblin, K., Guedon, A., et al. 2011, *Methanobacterium lacus* sp. nov., a novel hydrogenotrophic methanogen from the deep cold sediment of a meromictic lake, *Int. J. Syst. Evol. Microbiol.*, **62**, 1625–1629.
51. Summerer, D., Wu, H., Haase, B., et al. 2009, Microarray-based multicycle-enrichment of genomic subsets for targeted next-generation sequencing, *Genome Res.*, **19**, 1616–21.
52. Lehours, A.C., Bardot, C., Thenot, A., Debaoas, D. and Fonty, G. 2005, Anaerobic microbial communities in Lake Pavin, a unique meromictic lake in France, *Appl. Environ. Microbiol.*, **71**, 7389–400.
53. Jeon, S., Bunge, J., Leslin, C., Stoeck, T., Hong, S. and Epstein, S.S. 2008, Environmental rRNA inventories miss over half of protistan diversity, *BMC Microbiol.*, **8**, 222.
54. Miltou, C., Rimour, S., Missaoui, M., et al. 2007, PhylArray: phylogenetic probe design algorithm for microarray, *Bioinformatics*, **23**, 2550–7.
55. Parisot, N., Denonfoux, J., Dugat-Bony, E., Peyret, P. and Peyretailade, E. 2012, KASpOD – a web service for highly specific and explorative oligonucleotide design, *Bioinformatics*, **28**, 3161–3162.
56. McCarthy, A. 2010, Third generation DNA sequencing: pacific biosciences' single molecule real time technology, *Chem. Biol.*, **17**, 675–6.
57. Schadt, E.E., Turner, S. and Kasarskis, A. 2010, A window into third-generation sequencing, *Hum. Mol. Genet.*, **19**, R227–240.