The sequence capture by hybridization: a new approach for revealing the potential of mono-aromatic hydrocarbons bioattenuation in a deep oligotrophic aquifer

Magali Ranchou-Peyruse, Cyrielle Gasc, Marion Guignard, Thomas Aüillo, David Dequidt, Pierre Peyret, Anthony Ranchou-Peyruse

To cite this version:
Magali Ranchou-Peyruse, Cyrielle Gasc, Marion Guignard, Thomas Aüillo, David Dequidt, et al.. The sequence capture by hybridization: a new approach for revealing the potential of mono-aromatic hydrocarbons bioattenuation in a deep oligotrophic aquifer. Microbial Biotechnology, Wiley, 2017, 10 (2), pp.469-479. <http://onlinelibrary.wiley.com/doi/10.1111/1751-7915.12426/epdf>. <10.1111/1751-7915.12426>. <hal-01658765>

HAL Id: hal-01658765
https://hal.archives-ouvertes.fr/hal-01658765
Submitted on 8 Dec 2017

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.
The sequence capture by hybridization: a new approach for revealing the potential of mono-aromatic hydrocarbons bioattenuation in a deep oligotrophic aquifer

Magali Ranchou-Peyruse,1 Cyrielle Gasc,2 Marion Guignard,1 Thomas Aullo,3 David Dequidt,4 Pierre Peyret2 and Anthony Ranchou-Peyruse1,*

1 Université de Pau et des Pays de l’Adour, Equipe Environnement et Microbiologie, IPREM-CNRS 5254, F-64013 Pau, France.
2 Université d’Auvergne, EA 4678 CIDAM, 63001 Clermont-Ferrand, France.
3 TIGF – Transport et Infrastructures Gaz France, 40 Avenue de l’Europe, CS20522, 64000 Pau, France.
4 STORENGY – Geosciences Department, Bois-Colombes, France.

Summary

The formation water of a deep aquifer (853 m of depth) used for geological storage of natural gas was sampled to assess the mono-aromatic hydrocarbons attenuation potential of the indigenous microbiota. The study of bacterial diversity suggests that Firmicutes and, in particular, sulphate-reducing bacteria (Peptococcaceae) predominate in this microbial community. The capacity of the microbial community to biodegrade toluene and m- and p-xylene was demonstrated using a culture-based approach after several hundreds of days of incubation. In order to reveal the potential for biodegradation of these compounds within a shorter time frame, an innovative approach named the solution hybrid selection method, which combines sequence capture by hybridization and next-generation sequencing, was applied to the same original water sample. The bssA and bssA-like genes were investigated as they are considered good biomarkers for the potential of toluene and xylene biodegradation. Unlike a PCR approach which failed to detect these genes directly from formation water, this innovative strategy demonstrated the presence of the bssA and bssA-like genes in this oligotrophic ecosystem, probably harboured by Peptococcaceae. The sequence capture by hybridization shows significant potential to reveal the presence of genes of functional interest which have low-level representation in the biosphere.

Introduction

The degradation of toluene and m, p and o-xylene (TX) under anoxic conditions has been demonstrated in numerous marine and continental environments. It can be associated with the reduction of nitrate, sulphate, iron and CO2 (Dolfing et al., 1990; Beller and Spormann, 1997; Harms et al., 1999; Kane et al., 2002; Kube et al., 2004; Morasch et al., 2004; Morasch and Meckenstock, 2005; Washer and Edwards, 2007; Aullo et al., 2016). In all cases, the initial addition of fumarate to the hydrocarbon molecule is catalysed by either benzylsuccinate synthase (toluene) or benzylsuccinate synthase-like enzymes (xylene). For about 15 years, the bssA gene, which encodes for the alpha subunit of this protein, has been used as a biomarker for the biodegradation of TX under anoxic conditions. Beller et al. (2002) were the first to design primers targeting this gene, using available sequences from isolated strains. These primers then preferentially targeted nitrate-reducing Betaproteobacteria. Subsequently, other primer sets enabled sulphate-reducing, iron-reducing and syntrophic bacteria to be targeted (Winderl et al., 2007; Beller et al., 2008; Staats et al., 2011; Fowler et al., 2014). There was a difficulty in amplifying the bssA gene of some sulphate-reducing bacteria belonging to the Clostridia which was partly solved by the subsequent design of specific primers for this class (von Netzer et al., 2013; for the latest review on bssA-like gene diversity, see von Netzer et al., 2016).

In oligotrophic and stable environments, like deep continental aquifers (~500 to ~1200 m), the input of exogenous TX represents a potential source of carbon which is likely to modify the growth and survival strategies of microorganisms. Over the last several years, the potential for biodegradation of monoaromatic hydrocarbons in...
such environments has been demonstrated through culture-based approaches (Morasch et al., 2004; Berlendis et al., 2010; Aülo et al., 2016). The low biomass present in these ecosystems makes the demonstration of biodegradation capacities difficult (i.e. detection of bssA genes) in formation water (FW). It is currently difficult, indeed impossible, to assess the degradation potential of FW sampled from a deep aquifer without having recourse to laboratory-based degradation assays. In our previous studies, amplifications of the bssA gene using the primers cited previously have unfortunately often proved to be unsuccessful, as a result of the few targets available and problems of non-specific amplification (Aülo et al., 2016). Next-generation sequencing (NGS) techniques allow a deeper analysis of genetic diversity and thus offer the possibility of dispensing with culture-based techniques. However, diversity analyses are very often unable to identify microbial populations with low-level representation. The sequence capture by hybridization (Gasc et al., 2016), therefore, constitutes an alternative for the efficient detection of rare or unknown sequences in metagenomic samples (Denonfoux et al., 2013; Bragalinì et al., 2014; Biderre-Petit et al., 2016).

In the context of this study, we sought to demonstrate the presence of bssA genes in a deep aquifer used to store natural gas, which is associated with trace amounts of other hydrocarbons, in order to reveal a potential for natural bioattenuation of TX. We hypothesized that the Clostridia, in particular members of the Peptococcaceae family, play a key role in the degradation of TX in deep aquifers (Basso et al., 2009; Berlendis et al., 2010; Aülo et al., 2016). It is known that some members of Peptococcaceae are capable of degrading mono-aromatic hydrocarbons directly or via syntrophic relationships (Morasch et al., 2004; Taubert et al., 2012). A conventional approach to amplify the bssA gene using the sets of primers available in the literature is compared with the sequence capture by hybridization approach. This study represents the first instance of its use in this type of environment and in an industrial context.

Materials and methods

Sampling

In 2011, water samples were obtained from a deep aquifer (853 m of depth) used for geological storage of natural gas (aquifer 1, Paris Basin, France). Several physicochemical parameters of the FW are indicated in Table 1 (IPL Santé, Environnement Durables, Ile De France). After the tubing was cleaned as described previously (Basso et al., 2005), the biomass from the FW was collected at the wellhead by filtration through 70 Sterivex® filters (EMD; Millipore, Molsheim, France), while maintaining anoxic conditions. Filters were used to collect the microbial biomass over a period of 6.5 h from 500 l of FW. At the end of sampling, the filters were placed in bags maintaining anaerobiosis (GasPak™ EZ; BD, Franklin Lakes, NJ, USA). Several litres of FW were also sampled in sterile glass bottles and degassed with nitrogen in the laboratory prior to subsequently preparing and running microbial cultures. The set of samples (filters and water) were immediately placed at 4°C and transported to the laboratory. The samples to be used for molecular analysis were then frozen at −20°C and those for the cultures used the following day.

Biodegradation assays

Two biodegradation assays of a mixture of toluene, m-, p- and o- xylene, here referred to as TX (with a final concentration of 10 ppm for each hydrocarbon; Sigma-Aldrich, Saint Quentin Fallavier, France) were carried out at the same time with FW alone (FW condition) or enriched with the concentrated biomass (FWCB condition). The concentrated biomass was obtained for the latter condition by resuspended 67 Sterivex® filters (EMD; Millipore) in 250 ml of anoxic FW under agitation. Ten per cent (v/v) of this concentrated biomass was used to inoculate the FWCB. For each condition, that is, without addition of concentrated biomass (FW+TX) and with addition of concentrated biomass (FWCB+TX), an abiotic

| Table 1. Physico-chemical parameters and constituents of the formation water sampled from a deep aquifer (aquifer 1 in this study) at 853 m of depth below groundwater and analysed at atmospheric pressure. |
|-----------------------------------------------|
| **Physico-chemical parameters**               |
| Temperature (°C)                              | 36 |
| pH                                           | 8.25 |
| Conductivity at 25°C (μS cm⁻¹)                | 6000 |
| Redox potential (mV)                         | −363 |
| Pressure (bars)                              | 93 |
| Total suspended solids (mg l⁻¹)               | 16.0 |
| **Constituents**                              | |
| Carbonates (mg l⁻¹)                          | < 20 |
| Sulphates (mg l⁻¹)                           | 2186.5 |
| Ammonium (mg l⁻¹)                            | 1.75 |
| Calcium (mg l⁻¹)                             | 25.1 |
| Magnesium (mg l⁻¹)                           | 15.9 |
| Sodium (mg l⁻¹)                              | 1400.0 |
| Potassium (mg l⁻¹)                           | 34.0 |
| Chloride (mg l⁻¹)                            | 260 |
| Silicates (mg SiO₂ l⁻¹)                      | 15.8 |
| Phosphorus (mg l⁻¹)                          | < 0.05 |
| Nitrates (mg l⁻¹)                            | < 2 |
| Fluoride (mg l⁻¹)                            | 1.51 |
| Barium (mg l⁻¹)                              | 0.013 |
| Total iron (μg l⁻¹)                          | 3200 |
| Ferrous iron (μg l⁻¹)                        | < 100 |
| Manganese (μg l⁻¹)                           | 44 |
| Organic carbon (mg l⁻¹)                      | 1.0 |

a. Arsenic, cadmium, chrome, copper, tin, mercury, lead, vanadium and zinc were also measured, but were below the limits of detection.
control was performed by the addition of 5% v/v of 1 M HCl. The four microcosms, with a final volume of 40 ml, were prepared in 100 ml Wheaton serum bottles sealed with butyl rubber stoppers (Belco Glass, Vineland, NJ). All manipulations were performed in a glovebox (Getinge La Calhene, France) in an atmosphere of 95% N₂ and 5% H₂. The hydrogen is necessary for the palladium catalyst can react to remove oxygen traces. All cultures were incubated at 37°C, in the dark without agitation. Periodically, TX degradation was monitored in the four microcosms using SPME/GC/FID as described in Aullo et al. (2016).

**Bacterial enumeration**

Eighteen millilitres of FW were fixed on site with 2 ml 10% borax-buffered formaldehyde (37%; Sigma-Aldrich) and stored at 4°C until quantification. Furthermore, after resuspension of the Sterivex™ filters in FW for the biodegradation assays, 18 ml of the concentrated biomass was sampled and fixed the same way. In all cases, 500 µl of 4',6'-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) stock solution (200 µg ml⁻¹) was added to 10 ml of fixed sample, and then vacuum filtration was performed using 0.2 µm pore-size black polycarbonate filters (Millipore). Ten fields were selected at random for each filter, and the cells were counted on an Axio Observer.Z1 inverted microscope (Zeiss, Oberkochen, Germany) equipped with a 63× oil immersion objective (Plan APO, N.A. 1.4, M27). Images were obtained with a Zeiss Axiocam 506 mono CCD camera via the Zeiss ZEN 2012 interface.

**Bacterial community analysis**

Microbial diversity was investigated on a Sterivex™ filter previously stored at −20°C. The filter was crushed in liquid nitrogen and the DNA extracted with the Powersoil DNA Isolation kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) following the manufacturers' specifications. Genomic DNA was sent to a commercial company (MR DNA, Shallowater, TX, USA). The hypervariable V4 region of the 16S rRNA gene was amplified with PCR primers 515/806 and sequenced by the MiSeq 2 × 300 bp run (Illumina, San Diego, CA, USA). The construction of DNA libraries with the amplicons and the sequencing were performed following the manufacturers’ specifications. The sequence data were subsequently processed through the analysis pipeline designed by MR DNA. At first, the sequences were demultiplexed and the barcodes and primers were removed before eliminating all sequences smaller than 150 bp. Sequences displaying ambiguous bases were also eliminated. Operational taxonomic units (OTUs) were defined by clustering sequences displaying 97% similarity. Thus, singleton sequences and chimeras were eliminated. The final OTUs were subsequently classified taxonomically using BLASTn against a curated database derived from RDPII and NCBI.

**Cloning and sequencing of benzylsuccinate synthase alpha-subunit (bssA) genes**

PCR amplifications of the bssA gene were performed for different sets of primers found in the literature. The primer sets 7772F/8546R (Winderl et al., 2007), 7768F/8543R (von Netzer et al., 2013), bss3F/bssAR (Staats et al., 2011), and 997F/1230R (Brow et al., 2013) were used to target, respectively, the bssA gene of Deltaproteobacteria, Clostridia, Geobacter and nitrate-reducing Betaproteobacteria (Table S1). For each positive amplification, the PCR fragment corresponding to the expected size was purified on an agarose gel and cloned using the TOPO TA Cloning kit (Fisher Scientific, Hampton, NH, USA) before being sequenced by Qiagen Genomic Services (Hilden, Germany).

**Capture probe design and synthesis**

A set of five 31- to 38-mers degenerate probes covering the bssA gene was designed from nine bssA nucleic sequences from strains belonging to Deltaproteobacteria, Firmicutes (EF123665, EF123667, EU780921, FO203503, EF123663, EF123662) and environmental sequences obtained with 7772F/8546R primers set. These three latter amplicons were obtained at the end of TX degradation from enrichment cultures with autochthonous microbiota of different deep aquifers (aquifer 1: KX576576, aquifer 2: KX576577, aquifer 3: KX576575). The deep aquifer 2 was studied by Berlendis et al. (2010) (Paris Basin). The third aquifer (aquifer 3) is located in the Aquitaine Basin (southwest of France). These sequences were processed using the KASpOD software (Parisot et al., 2012) (Table S1). Adaptor sequences were added at each extremity of the probe to enable their PCR amplification, resulting in “ATCGCAC CACCGTGT-N(31-38)GACTCGGCTCCTCA” sequences, with N(31-38) representing the bssA-specific capture probe. Biotinylated RNA capture probes were then synthesized as described by Ribière et al. (2016).

**Preparation of biological samples and libraries**

Next-generation sequencing libraries were constructed on genomic DNA extracted directly from aquifer 1 FW after concentration by means of ethanol precipitation. Libraries were prepared using the Nextera XT Kit (Illumina) using the manufacturer’s instructions (Genoscreen, Lille, France) and were PCR amplified using the GC-RICH PCR system kit (RocheDiagnostics GmbH, Mannheim,
Germany) with primers complementary to the library adapters to obtain sufficient amounts of DNA to run the sequence capture by hybridization.

**Hybridization capture and elution**

Solution hybrid selection (SHS) was conducted on FW sample according to the protocol described by Ribiére et al. (2016). Briefly, 500 ng of heat denatured libraries was hybridized to the set of biotinylated RNA probes for 24 h at 65°C. Probe/target heterodimers were trapped by streptavidin-coated paramagnetic beads (Dynabeads M-280 Steptavidin, Invitrogen, Carlsbag, CA, USA). After several washing steps, the captured targets were eluted from the beads using NaOH and purified using AMPure beads (Beckman Coulter Genomics, Takeley, Essex, United Kingdom). Enriched products were PCR amplified using primers complementary to the library adapters and purified again using AMPure beads (Beckman Coulter Genomics). To increase the enrichment, a second round of hybridization and amplification was performed using the obtained captured products.

**Illumina MiSeq sequencing and data analysis**

Captured DNA products were sequenced using a single MiSeq 2 × 300 bp run (Illumina) according to the manufacturer’s specifications (Genoscreen). All raw reads were scanned for library adapters and quality filtered using PRINSEQ-lite Perl script (Schmieder and Edwards, 2011) prior to assembly and analysis. The clean reads were assembled de novo using IDBA-UD (v1.1.1) (Peng et al., 2012). Contigs generated were combined for a second round of assembly using CAP3 to generate longer contigs (Huang and Madan, 1999). The amino acid (AA) sequences were deduced from the final assembled nucleotide contigs and then aligned with reference open-reading frames sourced from public databases using MEGA version 6 (Tamura et al., 2016). The phylogenetic tree was constructed with the same software using the neighbour-joining method. The bootstrap analysis was performed for 1000 replicates.

**Nucleotide sequence accession numbers**

Sixty-five sequences (16S rRNA and bssA genes) have been submitted to GenBank under accession numbers KX576572 to KX576636.

**Results and discussion**

**Characterization of the site**

In the autumn of 2011, the FW of a deep aquifer (aquifer 1) used for geological storage of natural gas was sampled after cleaning of the sampling well, to remove the microorganisms growing as a biofilm on the tubing and the wellhead (see Basso et al., 2009 for Scheme of principle of a natural gas underground storage in an aquifer). During this study, the methane bubble was located close to the well and the presence of methane, originally dissolved at reservoir depth, was observed in the sampled water. At the time of sampling, the water was at a temperature of 36°C, with a pH of 8.25, and no oxygen was detected and a redox potential of −363 mV (Table 1). This aquifer displayed a total organic carbon concentration (TOC; 1 mg l⁻¹) typical of this type of environment which has been little impacted by human activity (Pedersen, 1997; Sahl et al., 2008). Meckinstock et al., 2015 report in their review dedicated to microbial clean-up in contaminated aquifers that only 0.5–5% of this TOC is likely to be directly used as carbon source by the microbial community which makes this deep aquifer an oligotrophic environment. The analysis of the chemical composition of this water reveals a sulphate content of 2186.5 mg l⁻¹, a total iron content of 3200 µg l⁻¹ but no nitrate. These data suggest that the indigenous microbial community is probably dominated by sulphate-reducing bacteria. Sulphate reduction is indeed an important metabolic process in deep subsurface environments and particularly in aquifers (Detmers et al., 2004; Amend and Teske, 2005; Bombach et al., 2010; Itävaara et al., 2011) where it has been demonstrated that sulphate-reducing bacteria also play a key role in the degradation of mono-aromatic hydrocarbons (Basso et al., 2009; Berlendis et al., 2010; Ailón et al., 2016). The concentrated biomass derived from the filtration of FW was subjected to a diversity analysis in order to determine the predominant bacterial groups. For this purpose, the V4 region of the sequence of the 16S rRNA gene was targeted by NGS (no archaea were detected). The taxa detected in this study are regularly found in studies of deep subsurface ecosystems. Figure 1 shows that Firmicutes were clearly predominant (61.1%) among the 62 OTUs found (similarity ≥ 97%), with 29.3% of Peptococcaceae (Carboxythermus, candidatus Desulfurodus, Pelotomaculum and Desulfotomaculum) and 31.2% of sequences belonging to Thermoanaerobacteraceae (Thermoanaerobacter, Moorella, Sporotomaculum and Ammonifex). The detection thermophilic microorganisms signatures (e.g. Moorella or Thermoanaerobacter) in a mesothermic environment may be surprising. However, similar results have already been reported in the study of another mesothermic aquifer (Berlendis et al., 2010; aquifer 2 in this study). Taking into account of the diversity observed, most sulphate reduction must be carried out by Firmicutes since the Deltaproteobacteria, which include a large number of sulphate-reducing bacteria, represent less than 1% of total sequences. The
dominance of the sulphate-reducing community by Firmicutes has been shown in a 120 m deep aquifer displaying low sulphate concentrations (≤17.1 mg l⁻¹; Detmers et al., 2004). Salinity seems to play a role in the distribution of Deltaproteobacteria and sulphate-reducing Firmicutes (Leloup et al., 2005). Here, the dominance of Firmicutes can probably be further explained by their capacity to sporulate, their capacity to survive in oligotrophic environments and their metabolic versatility (Sass et al., 1997; Spring and Rosenzweig, 2006; Orsi et al., 2016). Moreover, some studies have suggested or demonstrated the key role of some Peptococcaceae, and in particular representatives of the genera Desulfotomaculum, Desulfosporosinus and Pelotomaculum, in the degradation of BTEX (Robertson et al., 2000; Liu et al., 2004; Morasch et al., 2004; Cupples, 2011; Abu Laban et al., 2015).

Biodegradation assays

The day after sampling, assays of biodegradation of toluene and the 3 xylene isomers were initiated either directly on the water obtained from the site (FW+TX) or in water supplemented with concentrated indigenous bacterial biomass (FWCB+TX). A cell-count carried out in FW using epifluorescence (DAPI) showed a cell concentration of 4.5 × 10⁵ ± 2.3 × 10⁵ cells ml⁻¹, while the concentration of microorganisms in FWCB was four times greater, 1.8 × 10⁶ cells ml⁻¹. While it is difficult to quantify microorganisms attached to the mineral matrix in deep aquifers, it is commonly accepted that the indigenous biomass growing in a biofilm is very largely dominant compared with pelagic microorganisms (Whitman et al., 1998; Griebler and Lueders, 2009). Even if the diversity between the pelagic and the attached microorganisms can differ (Röling and van Verseveld, 2002), the biomass supplemented condition tended to simulate the influence of the bacterial concentration in our biodegradation assays. In the two microcosms of this study, toluene and m- and p- xylenes (Tm, pX) were degraded simultaneously (Fig. 1). Toluene is assumed to be the most easily biodegraded aromatic hydrocarbon in anoxic conditions, which induces that it is degraded first and therefore delay the degradation of other hydrocarbons (Haag et al., 1991; Edwards et al., 1992; Phelps and Young, 1999; Meckenstock et al., 2004; Morasch et al., 2004). However, this interpretation must be tempered since it is evident that the biodegradation potential depends on the pool of key genes present. Indeed simultaneous biodegradation of toluene and xylene has already been observed (Herrmann et al., 2009; Shah, 2014). It is interesting to observe that while the m- and p-xylenes were degraded, o-xylene resisted degradation throughout the incubation period. Degradation was almost four times faster (237 days) when the biomass was concentrated fourfold, which implies that the number of microorganisms influences Tm, pX degradation. This result suggests that the in situ biodegradation process, with a supposed higher concentration of microorganisms in biofilm structures, could be faster than revealed by biodegradation studies in the laboratory (Botton and Parsons, 2006; Berlendis et al., 2010; Higashioka et al.,

Fig. 1. Composition of the bacterial community in the formation water collected from a deep aquifer (∼853 m). The pie chart represents the percentage of each taxon within the whole microbial species analysed.

Sequence capture by hybridization applied for bssA 473
BssA detection assay by PCR

Numerous studies have shown that the bssA and bssA-like genes can serve as biomarkers for the in situ anaerobic degradation of toluene and xylenes (Krieger et al., 1999; Andreoni and Gianfreda, 2007; Kazý et al., 2010; Cury et al., 2015). These studies are most frequently conducted in surface environments or in shallow aquifers. Even though the diversity study based on 16S rRNA gene indicates a predominance of Firmicutes and the water composition suggests that sulphate reduction could play a major role in this aquifer, the choice was made to be as exhaustive as possible regarding the metabolisms involved in bioattenuation. For this purpose, four primer pairs designed to target nitrate-reducing bacteria (Brow et al., 2013), iron-reducing bacteria (Staats et al., 2011), sulphate-reducing bacteria belonging to the Proteobacteria or Clostridiales (Winderl et al., 2007; von Netzer et al., 2013) were tested as part of this study (Table S1). The bssA gene was not amplified by PCR in the DNA extracted from FW samples whether supplemented or not supplemented with concentrated biomass, although the degradation results obtained subsequently in the microcosms demonstrated the capacity to degrade the Tm, pX.

Our team has been trying to demonstrate the presence of this gene directly in FW for some years without success. Three times only this gene was amplified by the primers of Winderl et al. (2007) in microcosms from three different aquifers (aquifers 1, 2 and 3), after incubation periods of more than 100 days (data not shown). It should be noted that the amplicons corresponding to the expected size had to be systematically purified on an agarose gel in order to eliminate numerous non-specific amplifications before sequencing. Although this amplification approach has proven its value in surface environments and shallow aquifers, it did not seem appropriate for the constraints of our study sites (trace mono-aromatic hydrocarbons, low biomass and impossibility of recovering biofilms in this industrial context). Therefore, we had to develop a new molecular biological approach enabling detection of the bssA gene directly in FW while conducting time-consuming biodegradation assays.

BssA detection assay using sequence capture by hybridization approach

In 2013, Denonfoux and co-workers successfully combined a SHS method with next-generation sequencing for the first time, in order to capture a biomarker gene in a complex metagenome. The methodology was used to explore the methanogenic communities present in a lacustrine environment by targeting the methyl coenzyme M reductase subunit A (mcrA) gene with a set of non-overlapping probes, which targeted both known sequences and potential undescribed variants of the mcrA gene. The mcrA sequences represented more than 40% of the obtained sequences after two cycles of capture, revealing enrichment compared with shotgun sequencing, in which only 0.003% of the sequences corresponded to the target gene. In addition, because mcrA and 16S rRNA gene phylogenies are congruent, this approach allowed the methanogen community to be described and revealed higher diversity than previously observed with other methods. Indeed, hybridization capture recovered sequences from the Methanobacteria order, belonging to the rare biosphere, which were not detected through direct sample sequencing due to the sequencing depth, or through PCR amplification, due to possible primer bias. This method appeared to be appropriate for our constraints given its sensitivity, and the fact that it does not need amplification step, which is often unfruitful in this type of study. The SHS probes (Table S1) were designed from bssA sequences of sulphate-reducing bacteria belonging to the Deltaproteobacteria and the Firmicutes, as well as the three sequences previously amplified with the primers of Winderl et al. (2007) in enrichments with mono-aromatic hydrocarbons (KX576575, KX576576, KX576577). After analysis of reads (Table S2), 498 500 reads proved close to bssA and bssA-like genes and were grouped into three bssA homologous contigs. The contig sequence_10944 alone includes 98% of the bssA reads indicating that such bssA gene dominates in the ecosystem. Sequence capture by hybridization gives quantitative results close to that obtained by qPCR as demonstrates by Denonfoux et al. (2013). The three contigs obtained by sequence capture and the three bssA amplicons obtained in this study were compared with sequences from pure strains and environmental samples deposited in international databases after having been translated into AA sequences. The results are presented as a phylogenetic tree constructed from the comparison of a 90AA region. The main contig, contig sequence_10944 (aquifer 1) and the amplicons obtained from the sulphate-reducing enrichments from FWs (aquifers 2 and 3) are closed to bssA sequences defined as sensu stricto by Acosta-González et al. (2013). The dominant bssA gene obtained by SHS is close to the BF clone obtained from an enrichment described as degrading benzene and dominated by Peptococcaceae-related Gram-positive microorganisms (87% identity, 276AA). In our current state of knowledge, the reason for the presence of this gene in this enrichment described by Abu Laban et al. (2010) cannot be explained since the initial benzene
biodegradation step does not seem to involve the addition of a fumarate molecule (enabled by the benzylsuccinate synthase) but a carboxylation as described by the authors. In our case, the procurement of a bssA gene associated with the Peptococcaceae family is consistent with the diversity data obtained in this study (Fig. 1) and tends to confirm the supposed role of Peptococcaceae in the degradation of Tm, pX in this deep aquifer. In referring to the broad dominance of Firmicutes over Deltaproteobacteria, we can hypothesize that these are the principal sulphate-reducing bacteria in this deep aquifer. Several studies have provided evidence for a positive correlation between the dominance of sulphate-reducing bacteria affiliated to Peptococcaceae in subsurface environments and depth (Moser et al., 2005; Chivian et al., 2008; Itäväära et al., 2011; Guan et al., 2013). These microorganisms play a major role in the carbon cycle in deep environments via the recycling of organic material, which is, in our context, the degradation of mono-aromatic hydrocarbons. To date, only two strains of Peptococcaceae (Desulfotomaculum sp. Ox39 and Desulfoisporosinus meridiei) have been described as being able to degrade toluene and/or xylenes (Liu and Peptococcaceae, 2012, 2014). The key role of the bssA gene was shown to initiate toluene degradation (Fowler et al., 2013). How-ever, several studies using non culture-based approaches tend to demonstrate that these microorganisms are often playing a key role in the degradation of mono-aromatic hydrocarbons. In the case of a gas condensate-contaminated aquifer, Desulfoisporosinus sp. was shown to initiate toluene degradation (Fowler et al., 2012, 2014). The key role of the Peptococcaceae has also been demonstrated in other environments and in sulphate-reducing conditions and/or methanogenesis (Abu Laban et al., 2009; Winderl et al., 2010; Pilloni et al., 2011; Sun and Cupples, 2012; Sun et al., 2014; Abu Laban et al., 2015; Tan et al., 2015). It is interesting to note that the bssA amplicon obtained for the enrichment culture with FW from the aquifer 1 at the end of degradation (Fig. 2) is phylogenetically located in the OX39-homologues cluster as described by von Netzer et al. (2013). The genes present in this cluster could be involved in the degradation of xylenes (Herrmann et al., 2009; Bragalin et al., 2014). This sequence is close to the Desulfotomaculum sp. Ox39 bssA-like gene (74% similarity, 227AA) and to environmental sequences obtained from enrichments derived from contaminated aquifers (Herrmann et al., 2009; von Netzer et al., 2013). Finally, the two last contigs that represent only 2% of the bssA homologous sequences obtained by SHS (Table S2), contig sequence_48572 and contig sequence_31410, form a separate cluster located between the assA and the bssA genes. The existence of deeply branching bacteria was also found in the DNA-SIP study performed on a sample from the Testfeld Süd aquifer contaminated by hydrocarbons (Winderl et al., 2010), suggesting that a large part of the diversity of bssA sequences sensu stricto and sensu lato, and bssA homologues (assA, nmsA, hbsA) is still to be discovered. The contig sequence_48572 is close to the Desulfovbac ula toluolica bssA sequence (42% similarity, 115AA; ABM92935). As regard the contig sequence_31410 is close to a Desulfotomaculum sp. 46_20 alkylsuccinate synthase obtained from an oil reservoir in Alaska (46%/138AA; KUK63464), but also to a pyruvate-formate lyase derived from a strain affiliated to Peptococcaceae from a Opalinus Clay rock porewater BRC-3 borehole (39%/142AA; KJS47223) and to a glycy radical enzyme of the strain Desulfoisporosinus sp. BRH_c37, also obtained from the BRC-3 site (38%/143AA; KUO70645).

Conclusions

The study of the potential for TX degradation in oligotrophic environments such as deep aquifers is very

Fig. 2. Degradation of mono-aromatic hydrocarbons (toluene, m- and p-xylenes) during incubation of formation water collected anoxically to protect autochthonous microbiota, FW (A) or with the formation water supplemented with concentrated biomass, FWCB (B). Filled circles: o-xylene; filled triangles: toluene; filled diamonds: p-xylene; filled squares: m-xylene. Arrow indicates at day 447 the addition of toluene, m- and p-xylenes (10 ppm). Start levels of mono-aromatic hydrocarbons were 10 ppm.

© 2016 The Authors. Microbial Biotechnology published by John Wiley & Sons Ltd and Society for Applied Microbiology, Microbial Biotechnology, 10. 469–479
difficult using conventional cultural approaches, as they require long incubation periods (several months to several years) and/or biomass. Biomarker detection allowing the evaluation of the biodegradation potential of an ecosystem or the monitoring of bioremediation operations is necessary tools for environmental engineering. Currently, the *bssA* and *bssA*-like genes represent excellent biomarkers for the degradation of some mono-aromatic hydrocarbons. However, no primer sets tested in the study enabled demonstration of the presence of *bssA* genes directly in FW, while enrichments subsequently showed that the metabolic potential was present.

Direct sequencing of metagenomic samples in recent years has allowed for increased precision in microbial diversity analyses but only dominant taxa could be revealed. The sequence capture by hybridization approach used in this study proved its efficiency for the specific capture of targeted *bssA* sequences. Indeed, this was the only method that enabled *bssA* and...
**Sequence capture by hybridization applied for bssA** 477

Beller, H.R., Kane, S.R., Legler, T.C., and Alvarez, P.J.J. (2002) A real-time polymerase chain reaction method for monitoring anaerobic, hydrocarbon-degrading bacteria based on a catabolic gene. *Environ Sci Technol* 36: 3977–3984.

Beller, H.R., Kane, S.R., Legler, T.C., McKelvie, J.R., Lollar, B.S., Pearson, F., *et al.* (2008) Comparative assessments of benzene, toluene, and xylene natural attenuation by quantitative polymerase chain reaction analysis of a catabolic gene, signature metabolites, and compound-specific isotope analysis. *Environ Sci Technol* 42: 6065–6072.

Berlendis, S., Lascourrèges, J.-F., Schraauwers, B., Sivadon, P., and Magot, M. (2010) Anaerobic biodegradation of BTEx by original bacterial communities from an underground gas storage aquifer. *Environ Sci Technol* 44: 3621–3628.

Bidderre-Pettit, C., Dugat-Bony, E., Mege, M., Parisot, N., Adrian, L., Moné, A., *et al.* (2016) Distribution of *Dehalococcoides* derived reductive dehalogenase homologous genes. *PLoS ONE* 11: e0145558. doi:10.1371/journal.pone.0145558.

Bombach, P., Chatzinotas, A., Neu, T.R., Kästner, M., Lueders, T., and Vogt, C. (2010) Enrichment and characterization of a sulfate-reducing toluene-degrading microbial consortium by combining in situ microcosms and stable isotope probing techniques. *FEMS Microbiol Ecol* 71: 237–246.

Botton, S., and Parson, J.R. (2006) Degradation of BTEx compounds under iron-reducing conditions in contaminated aquifer microcosms. *Environ Chem* 25: 2630–2638.

Bozinozovski, D., Taubert, M., Kleinstueber, S., Richnow, H.-H., von Bergen, M., Vogt, C., and Seifert, J. (2014) Metaproteogenomic analysis of a sulfate-reducing enrichment culture reveals genomic organization of key enzymes in the *m*-xylene degradation pathway and metabolic activity of proteobacteria. *Syst Appl Microbiol* 37: 488–501.

Brugali, C., Ribière, C., Parisot, N., Vallon, L., Prudent, E., Peyretaille, E., *et al.* (2014) Solution hybrid selection capture for the recovery of functional full-length eukaryotic cDNAs from complex environmental samples. *DNA Res* 21: 685–694.

Brow, C.N., O’Brien, J.R., Johnson, R.L., and Simon, H.M. (2013) Assessment of anaerobic toluene biodegradation activity by bssA transcript/gene ratios. *Appl Environ Microb* 79: 5338–5344.

Chivian, D., Brodie, E.L., Alm, E., Culley, D.E., Dehal, P.S., DeSantis, T.Z., *et al.* (2008) Environmental genomics reveals a single-species ecosystem deep within earth. *Science* 322: 275–278.

Cuppers, A.M. (2011) The use of nucleic acid based stable isotope probing to identify the microorganisms responsible for anaerobic benzene and toluene biodegradation. *J Microbiol Meth* 85: 83–91.

Cuy, J.C., Jurelevicius, D.A., Villela, H.D.M., Jesus, H.E., Peixoto, R.S., Schaefer, C.E.G.R., *et al.* (2015) Microbial diversity and hydrocarbon depletion in low and high

**Conflict of interest**

None declared.

**References**

Abu Laban, N.A., Selesi, D., Jobelius, C., and Meckenstock, R.U. (2009) Anaerobic benzene degradation by Gram-positive sulfate-reducing bacteria. *FEMS Microbiol Ecol* 68: 300–311.

Abu Laban, N.A., Selesi, D., Rattel, T., Tischler, P., and Meckenstock, R.U. (2010) Identification of enzymes involved in anaerobic benzene degradation by a strictly anaerobic iron-reducing enrichment culture. *Environ Microbiol* 12: 2783–2796.

Abu Laban, N.A., Dao, A., and Foght, J. (2015) DNA stable-isotope probing of oil sands tailing pond enrichment cultures reveals different key players for toluene degradation under methanogenic and sulfidogenic conditions. *FEMS Microbiol Ecol* 91: fiv039.

Acosta-González, A., Rosselló-Móra, R., and Marqués, S. (2013) Diversity of benzylsuccinate synthase-like (bssA) genes in hydrocarbon-polluted marine sediments suggests substrate-dependent clustering. *Appl Environ Microb* 79: 3657–3676.

Amend, J.P., and Teske, A. (2005) Expanding frontiers in deep subsurface microbiology, *Palaeogeogr Palaeoclimatol Palaeoecol* 219: 131–155.

Andreoni, V., and Gianfreda, L. (2007) Bioremediation and monitoring of aromatic-polluted habitats. *Appl Microbiol Biotechnol* 76: 287–308.

Aülio, T., Berlendis, S., Lascourrèges, J.-F., Dessort, D., Duclerc, D., Saint-Laurent, S., *et al.* (2016) New bio-indicators for long term natural attenuation of monoaromatic compounds in deep terrestrial aquifers. *Front Microbiol* 7: 122.

Basso, O., Lascourrèges, J.-F., Jarry, M., and Magot, M. (2005) The effect of cleaning and disinfecting the sampling well on the microbial communities of deep subsurface water samples. *Environ Microbiol* 7: 13–21.

Basso, O., Lascourrèges, J.-F., Le Borgne, F., Le Goff, C., and Magot, M. (2009) Characterization by culture and molecular analysis of the microbial diversity of a deep subsurface gas storage aquifer. *Res Microbiol* 160: 107–116.

Beller, H.R., and Spormann, A.M. (1997) Benzylsuccinate formation as a means of anaerobic toluene activation by sulfate-reducing strain PRTOL1. *Appl Environ Microb* 63: 3729–3731.

© 2016 The Authors. *Microbial Biotechnology* published by John Wiley & Sons Ltd and Society for Applied Microbiology, *Microbial Biotechnology*, 10, 469–479.
diesel-polluted soil samples from Keller Peninsula, South Shetland Islands. *Antarct Sci* 27: 263–273.

Denonfoux, J., Parisot, N., Dugat-Bony, E., Biderre-Petit, C., Boucher, D., Morgavi, D.P., *et al.* (2013) Gene capture coupled to high-throughput sequencing as a strategy for targeted metagenome exploration. *DNA Res* 20: 185–196.

Detmers, J., Strauss, H., Schulte, U., Bergmann, A., Knittel, K., and Kuever, J. (2004) FISH shows that *Desulfitomaculum* spp. are the dominating sulfate-reducing bacteria in a pristine aquifer. *Microbiol Ecol* 47: 236–242.

Dolfing, J., Zeyer, J., Binder-Eicher, P., and Schwarzenbach, R. (1990) Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. *Arch Microbiol* 154: 336–341.

Edwards, E.A., Wills, L.E., Reinhard, M., and Grbić-Galić, D. (1992) Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate-reducing conditions. *Appl Environ Microbiol* 58: 794–800.

Fowler, S.J., Dong, X., Sensen, C.W., Sulfita, J.M., and Gieg, L.M. (2012) Methanogenic toluene metabolism: community structure and intermediates. *Environ Microbiol* 14: 754–764.

Fowler, S.J., Gutierrez-Zamora, M.L., Manefield, M., and Gieg, L.M. (2014) Identification of toluene degraders in a methanogenic enrichment culture. *FEMS Microbiol Ecol* 89: 625–636.

Gasc, C., Peyretaille, E., and Peyret, P. (2016) Sequence capture by hybridization to explore modern and ancient genomic diversity in model and nonmodel organisms. *Nucleic Acids Res* 44: 4504–4518.

Griebler, C., and Lueders, T. (2009) Microbial biodiversity in groundwater ecosystems. *Freshwat Biol* 54: 649–677.

Guan, J., Xia, L.P., Wang, L.Y., Liu, J.F., Gu, J.D., and Mu, B.Z. (2013) Diversity and distribution of sulfate-reducing bacteria in four petroleum reservoirs detected by using 16s rRNA and dsrAB genes. *Int Biodeterior Biodegr* 76: 58–66.

Haag, F., Reinhard, M., and McCarty, P.L. (1991) Degradation of toluene and *p*-xylene in anaerobic microcosms: evidence for sulfate as terminal electron acceptor. *Environ Toxicol Chem* 10: 1379–1389.

Harms, G., Zengler, K., Rabus, R., Aeckersberg, F., Minz, D., Rosselló-Mora, R., and Widell, F. (1999) Anaerobic oxidation of *o*-xylene, *m*-xylene, and homologous alkyl-benzenes by new types of sulfate-reducing bacteria. *Appl Environ Microbiol* 65: 999–1004.

Her mann, S., Vogt, C., Fischer, A., Kuppatz, A., and Richnow, H.H. (2009) Characterization of anaerobic xylene biodegradation by two-dimensional isotope fractionation analysis. *Environ Microbiol Rep* 1: 535–544.

Higashioka, Y., Kojima, H., and Fukui, M. (2012) Isolation and characterization of novel sulfate-reducing bacterium capable of anaerobic degradation of *p*-xylene. *Microbes environ* 27: 273–277.

Huang, X., and Madan, A. (1999) CAP3: a DNA sequence assembly program. *Genome Res* 9: 868–877.

Itävaara, M., Nyysönen, M., Kapanen, A., Nousiainen, A., Ahonen, L., and Kukkonen, I. (2011) Characterization of bacterial diversity to a depth of 1500 m in the Outokumpu deep borehole, Fennoscandian Shield. *FEMS Microbiol Ecol* 77: 295–309.

Kane, S.R., Beller, H.R., Legler, T.C., and Anderson, R.T. (2002) Biochemical and genetic evidence of benzylsuccinate synthase in toluene-degrading, ferric iron-reducing *Geobacter metallireducens*. *Biodegradation* 13: 149–154.

Kazy, S.K., Monier, A.L., and Alvarez, P.J.J. (2010) Assessing the correlation between anaerobic toluene degradation activity and bssA concentrations in hydrocarbon-contaminated aquifer material. *Biodegradation* 21: 793–800.

Krieger, C.J., Beller, H.R., Reinhard, M., and Spormann, A.M. (1999) Initial reactions in anaerobic oxidation of *m*-xylene by the denitrifying bacterium *Azooococcus* sp strain T. *J Bacteriol* 181: 6403–6410.

Kube, M., Heider, J., Amann, J., Hufnagel, P., Kuhner, S., Beck, A., *et al.* (2004) Genes involved in the anaerobic degradation of toluene in a denitrifying bacterium, strain Ebn1. *Arch Microbiol* 181: 182–194.

Leloup, J., Petit, F., Boust, D., Deloffre, J., Bally, G., Clarisse, O., and Quillet, L. (2005) Dynamics of sulfate-reducing microorganisms (dsrAB genes) in two contrasting microflats of the Seine Estuary (France). *Microb Ecol* 50: 307–314.

Liu, A., Garcia-Dominguez, E., Rhine, E.D. and Young, L.Y. (2004) A novel arsenate respiring isolate that can utilize aromatic substrates. *FEMS Microbiol Ecol* 48: 323–332.

Meckenstock, R.U., Safinowski, M., and Griebler, C. (2004) Anaerobic degradation of polycyclic aromatic hydrocarbons. *FEMS Microbiol Ecol* 47: 381–386.

Meckenstock, R.U., Elsner, M., Griebler, C., Lueders, T., Stumpf, C., Aamand, J., *et al.* (2015) Biodegradation: updating the concepts of control for microbial cleanup in contaminated aquifers. *Environ Sci Technol Lett* 4: 7073–7081.

Morasch, B., and Meckenstock, R.U. (2005) Anaerobic degradation of *p*-xylene by a sulfate-reducing enrichment culture. *Curr Microbiol* 51: 127–130.

Morasch, B., Schink, B., Tebbe, C.C., and Meckenstock, R.U. (2004) Degradation of *o*-xylene and *m*-xylene by a novel sulfate-reducer belonging to the genus *Desulfothermus*. *Arch Microbiol* 181: 407–417.

Moser, D.P., Gihring, T.M., Brockman, F.J., Fredrickson, J.K., Balkwill, D.L., Dolhopf, M.E., *et al.* (2005) Desulfothermus and *Methanobacterium* spp. dominate a 4- to 5-kilometer-deep fault. *Appl Environ Microbiol* 71: 8773–8783.

von Netzer, F., Pil lion, G., Kleindienst, S., Kruger, M., Kni t tel, K., Grundger, F., and Lueders, T. (2013) Enhanced gene detection assays for fumarate-adding enzymes allow uncovering of anaerobic hydrocarbon degraders in terrestrial and marine systems. *Appl Environ Microbiol* 79: 543–552.

von Netzer, F., Kuntze, K., Vogt, C., Richnow, H.H., Boll, M., and Lueders, T. (2016) Functional gene markers for fumarate-adding and deaeromatizing key enzymes in anaerobic aromatic hydrocarbon degradation in terrestrial environments. *J Mol Microbiol Biotechnol* 26: 180–194.

Orsi, W.D., Jørgensen, B.B., and Biddle, J.F. (2016) Transcriptional analysis of sulfate reducing and chemolithotrophic sulfur oxidizing bacteria in the deep subsea floor. *Environ Microbiol Rep* 8: 452–460.

Parisot, N., Denonfoux, J., Dugat-Bony, E., Peyret, P., and Peyretaille, E. (2012) KASPQD-a web service for highly
specific and explorative oligonucleotide design. Bioinformatics 28: 3161–3162.
Pedersen, K. (1997) Microbial life in deep granitic rock. FEMS Microbiol Rev 20: 399–414.
Peng, Y., Leung, H.C.M., Yiu, S.M., and Chin, F.Y.L. (2012) IDBA-UD: a de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth. Bioinformatics 28: 1420–1428.
Phelps, C.D., and Young, L. (1999) Anaerobic biodegradation of BTEX and gasoline in various aquatic sediments. Biodegradation 10: 15–25.
Sahl, J.W., Schmidt, R., Swanner, E.D., Mandernack, K.W., Ribi, P., Pilloni, G., von Netzer, F., Engel, M., and Lueders, T. (2016) The Authors. Bioinformatics 28: 1827–1836.
Shah, M. (2014) An application of sequencing batch reactors in microbial degradation of benzene, toluene, & xylene under anoxic and micro aerobic condition. J Appl Environ Microbiol 2: 231–236.
Spring, S., and Rosenzweig, F. (2006) The genera Desulfitobacterium and Desulfovosporinus: taxonomy. Prokaryotes 4: 771–786.
Staats, M., Braster, M., and Röling, W.F.M. (2011) Molecular diversity and distribution of aromatic hydrocarbon-degrading anaerobes across a landfill leachate plume. Environ Microbiol 13: 1216–1227.
Sun, W., and Cupples, A.M. (2012) Diversity of five anaerobic toluene degrading microbial communities investigated using stable isotope probing (SIP). Appl Environ Microbiol 78: 972–980.
Sun, W., Sun, X., and Cupples, A.M. (2014) Identification of Desulfovosporinus as toluene-assimilating microorganisms from a methanogenic consortium. Int Biodeter Biodegr 88: 13–19.
Tan, B., Jane Fowler, S., Abu Laben, N., Dong, X., Sensen, C.W., Foght, J. et al. (2015) Comparative analysis of metagenomes from three methanogenic hydrocarbon-degrading enrichment cultures with 41 environmental samples. ISME J 9: 2028–2045.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30: 2725–2729.
Tauber, M., Vogt, C., Wubet, T., Kleinsteuber, S., Tarkka, M.T., Harms, H., et al. (2012) Protein-SIP enables time-resolved analysis of the carbon flux in a sulfate-reducing, benzene-degrading microbial consortium. ISME J 6: 2291–2301.
Washer, C.E., and Edwards, E.A. (2007) Identification and expression of benzylsuccinate synthase genes in a toluene-degrading methanogenic consortium. Appl Environ Microbiol 73: 1367–1369.
Whitman, W.B., Coleman, D.C., and Wiebe, W.J. (1998) Prokaryotes: the unseen majority. Proc Natl Acad Sci 95: 6578–6583.
Winderl, C., Schaefer, S., and Lueders, T. (2007) Detection of anaerobic toluene and hydrocarbon degraders in contaminated aquifers using benzylsuccinate synthase (bssA) genes as a functional marker. Environ Microbiol 9: 1035–1046.
Winderl, C., Penning, H., von Netzer, F., Meckenstock, R.U., and Lueders, T. (2010) DNA-SIP identifies sulfate-reducing Clostridia as important toluene degraders in tar-oil-contaminated aquifer sediment. ISME J 4: 1314–1325.