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Contribution of enrichments and resampling for sulfate reducing bacteria diversity assessment by high-throughput cultivation

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ABSTRACT

The development of new high-throughput cultivation methods aims to increase the isolation efficiency as compared to standard techniques that often require enrichment procedures to compensate the low microbial recovery. In the current study, estuarine sulfate-reducing bacteria were isolated using an anaerobic isolation procedure in 384-well microplates. Ninety-nine strains were recovered from initial sediments. Isolates were identified according to their partial 16S rRNA sequences and clustered into 13 phylotypes. Besides, the increase in species richness obtained through enrichments or resampling was investigated. Forty-four enrichment procedures were conducted and shifts in sulfate-reducing bacterial communities were investigated through *dsr*AB gene fingerprinting. Despite efforts in conducting numerous enrichment conditions only few of them were statistically different from initial sample. The cultural diversity obtained from 3 of the most divergent enrichments, as well as from resampled sediments equally contributed to raise the sulfate-reducing diversity up to 22 phylotypes. Enrichments (selection of metabolism) or resampling (transient populations and micro-heterogeneity) may still be helpful to assess new microbial phylotypes. Nevertheless, all the newly cultivated strains were all representatives of minor Operational Taxonomic Units and could eventually be recovered by maintaining high-throughput isolation effort from the initial sediments.

1. Introduction

Microbiologists always aimed to expand access to the microbial world through cultural approaches since isolates allow the investigation of the physiology of microorganisms and their potential role in ecosystems. Shortcomings that relied on culture-dependent methods explain part of the statement that 99% of bacteria observed under a microscope are not cultivated on the lab bench (Trevors, 1998). The transition from environmental to artificial conditions that mimic as much as possible the natural habitat constitutes a major challenge (Alain and Querellou, 2009). The design of a culturing medium that fulfill all required conditions for the microbial growth is a crucial step and a single medium will not sustain the development of all the microorganisms. Enrichment procedures may help to solve a part of the missing fraction. Due to the large panel of physico-chemical parameters fluctuating in situ (temperature, pH, pO2, light, …), bacteria may change from a culturable state to a temporal or permanent viable but non-culturable state (Oliver, 2005; Shigematsu et al., 2007). Sampling at different time periods could also increase the microbial recovery since different populations may dominate a community according to variable environmental conditions.

The common techniques for anaerobe isolation are based on slight modifications of the pioneering roll tube's technique (Hungate, 1950; Macy et al., 1972; Hungate and Macy, 1973; Miller and Wolin, 1974; Balch and Wolfe, 1976). Most of the sulfate-reducing bacteria (SRB), even recently isolated from various ecosystems, were recovered by these methods (Ben Ali Gam et al., 2009; Suzuki et al., 2009; van Houten et al., 2009; Alazard et al., 2010), or by using the deep agar dilution series (ADS) technique (Pfennig and Trüper, 1992). Alternatively, isolations and incubations may be carried out by plating on Petri dishes and liquid dilution series (Gittel et al., 2008; Zhang et al., 2009; Finster and Kjeldsen, 2010) under controlled atmosphere (glovebox, anaerobic jars, and Gaspak® system). Nevertheless, due to technical procedures, anaerobic culturing is time consuming and handling isolates at larger scale is not conceivable. During the last decades, continuous efforts have been conducted to improve the efficiency in microbial cultivation and high-throughput culturing methods were designed (Giovannoni et al., 2007), some of them being applied to anaerobes (Colin et al., 2013; Alain and Querellou, 2009). These techniques aimed to reduce the culturing devices, to isolate a large number of microbial strains and successfully contributed to grow new phylotypes.

In this context, the aim of the present work was to obtain the highest SRB cultural diversity from initial estuarine sediments and to evaluate the increase in species richness obtained through enrichments or resampling. All isolations were achieved through a dilution-to-extinction procedure in 384-well microplates (Colin et al., 2013). The effectiveness of culture enrichments or resampling on the microbial diversity is discussed based on phylogenetic analysis of all isolates.

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2. Methods

2.1. Study site and sample collection

Sulfate-reducing bacteria diversity was investigated in the sediments from B20 station of the Adour estuary (French South Atlantic Coast). The sampling site is located approximately 10 km upstream of the mouth estuary (Duran et al., 2008). Surface sediments (0–5 cm depth horizon) were collected in November 2009 and stored less than 48 h at 4 °C in a closed vial before analysis. SRB isolation was directly performed on initial sediments (IS sample). In parallel, enrichments were achieved in order to modify the composition of the sulfate-reducing community and 3 of them were used for isolation (Fig. 1). Finally, sediments were resampled on October 2009 (RS sample) and were used for direct SRB isolation as performed with IS sample (Fig. 1).

2.2. Enrichment procedures

All enrichment cultures were set-up as a 10% (vol/vol) sediment slurry prepared in anoxic water collected at the B20 station. The prepared slurry was dispensed in 20 mL Bellco tubes. Two tubes were immediately processed and constituted the IS sample (initial control, see Table 1). Other slurries were incubated under 44 different physico-chemical conditions, all of them in duplicates (Table 1). Once sulfidogenic growth was detected by black ferrous sulfide precipitates, enrichments were homogenized by vortexing and 18 mL of the slurry was centrifuged at 5000 *g*, 15 min at room temperature. Supernatants were discarded and pellets were stored at −80 °C for molecular analysis. The remaining 2 mL of the slurry was supplemented with 1 mL of glycerol, homogenized and placed at −80 °C for potential future SRB isolation.

2.3. dsrAB genes T-RFLP analyses

Total DNA extraction was performed on enriched and IS samples from 0.25 g of wet weight sediment using the Powersoil DNA Isolation kit according to the manufacturer's instructions (MoBio Laboratories, Solana Beach, CA, U.S.A.). The *dsr*AB genes fragments were amplified using the FAM-labeled DSRB1F and the DRS4R primers (Wagner et al., 1998), in a final volume of 50 μL. PCR conditions were optimized for the amplification of all samples under the same conditions as follow: 0.2 μM of each primer, 0.4 mM of dNTPs, 2 mM of $MgCl_2$, and 1.25 U of Taq DNA polymerase (Eurobio) in its buffer. PCR was carried out as follow: 5 min at 94 °C for the initial denaturation, 34 cycles of 45 s at 94 °C, 45 s at 58 °C, 1 min at 72 °C; and a final extension for 10 min at 72 °C. PCR products were digested with *Sau*3AI and *Taq*αI (New England Biolabs) and were processed as described in Goñi-Urriza et al. (2007).

Electropherograms were analyzed using GeneScan software (Applied Biosystems). For each T-RFLP fingerprint generated, all the size peaks exceeding 25 units above the background fluorescence were aligned with the internal size standard using the GeneScan software. T-RFs below 35 bp and above 500 bp were excluded from the analysis in order to minimize sizing errors. In order to facilitate comparisons of fingerprints between different enrichments, the peak height for individual T-RFs was normalized to percent of the total fingerprint height. This data were used to calculate pairwise similarity values for all samples. A Canonical Correspondence Analysis (CCA) was performed to evaluate the influence of the enrichments on the structure of the sulfate-reducing bacterial community using MVSP software (Multi-Variate Statistical Package 3.12d, Kovach Computing Services, 1985–2001, UK). The Bray–Curtis quantitative similarity indexes were also calculated.

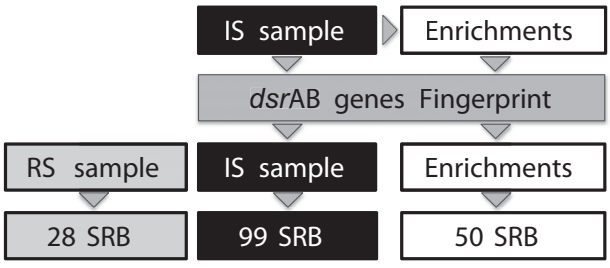

Fig. 1. Flow chart summarizing the overall procedure carried out for the sediments enrichment procedure and subsequently the SRB microplate isolation. IS: initial sediments; RS: resampled sediments.

2.4. Isolation procedure for sulfate reducing bacteria

Based on the T-RFLP fingerprint approach, sediments enriched with acetate 1 mM (Ac1-30-L sample), glycerol 20 mM (Gly-10-O sample) or pyruvate 20 mM (Pyr-30-O sample), belonged to the 3 divergent clusters as compared to initial sediments (IS sample), and were selected for isolation. Thus, initial sediments, resampled sediments as well as these 3 enrichments cultures were used for SRB isolation by using the high throughput dilution-to-extinction procedure in 384-well microplates (Colin et al., 2013). Briefly, ten fold dilution series (final volume 50 mL) were performed in Bellco flasks and the dilutions were distributed in 384-well microplates. To ensure anoxic conditions and to prevent reoxidation, all the procedure was carried out in an anoxic chamber (Bactron III) with N_2/H_2 (95%/5%) as oxygen-free gas. Microplates were sealed with an AlumaSeal CS TM film and incubated at 18 °C in anaerobic bags (BD GasPakTM EZ Gas Generating Pouch Systems) during 3 weeks. The microplates where both positive and negative wells were found (dilutions 10^{-5} to 10^{-6}) were used for strain isolation. Positive wells were detected by the black iron sulfide precipitate due to the sulfide which originated from sulfate reduction. All positive wells were recultivated in bigger volumes and served for direct partial 16S rRNA gene amplification and sequencing.

2.5. Genetic diversity analysis of isolates

One microliter of the culture was directly used for 16S rRNA gene amplification using the 63F/1387R primer set (Marchesi et al., 1998). Amplifications were made in a final volume of 50 μL, with 0.2 μM of each primer, 0.4 mM of dNTPs, 2 mM of MgCl2, and 1.25 U of Taq DNA polymerase (Eurobio) in its buffer. PCR was carried out as follow: 15 min at 94 °C for the initial denaturation and cell lysing, 34 cycles of 45 s at 94 °C, 45 s at 58 °C, 1 min at 72 °C; and a final extension for 10 min at 72 °C. PCR products were sequenced at GATC Biotech (Konstanz, Germany). Isolates of interest were recultivated and stored at −80 °C with 30% glycerol.

Sequences with similarities greater than 97% for partial 16S rRNA genes were used to assign sequences to a distinct Operational Taxonomic Units (OTU) (Stackebrandt and Goebel, 1994). Rarefaction curves (Heck et al., 1975) were produced using the DOTUR software program (Schloss and Handelsman, 2005) applying the default sequence assignment algorithm. Rarefaction diagrams were made by plotting the number of OTUs as a function of the number of individual isolates. Coverage (C) was calculated using the following formula: $C = 1 - (n1 / N)$, where n1 is the number of singletons (OTUs including a single isolate) and N is the total number of isolates examined (Mullins et al., 1995). 16S rRNA gene sequences have been submitted to NCBI and were assigned respectively with accession nos. HE600725–HE600902.

3. Results

3.1. SRB isolation from initial sediments

A SRB isolation procedure was performed in 384-well microplates. Based on a 97% sequence similarity cut-off, the 99 sulfate-reducing strains isolated from IS sample were grouped into 13 phylotypes (Fig. 2). 5 phylotypes were repeatedly isolated and represented more than 80% of the isolates. These dominant SRB were affiliated to the