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Influence of primer mismatch and microdiversity on DGGE results: a case study with SAR11

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ABSTRACT: Although SAR11 is usually the dominant bacterial group in most marine ecosystems when analyzed with clone libraries and fluorescence *in situ* hybridization, it is often not retrieved in studies where denaturing gradient gel electrophoresis (DGGE) has been used. We analyzed the microdiversity of SAR11 in Blanes Bay (NW Mediterranean) and we suggest that the high evenness of multiple microdiverse phylotypes, none of which being particularly dominant, is the probable reason for this methodological discrepancy. We used seeding experiments in which different amounts of 2 SAR11-affiliated clones were mixed with DNA from an environmental sample obtained from the Blanes Bay Microbial Observatory. Two primer sets differing at 2 base positions produced DGGE images that varied in their SAR11 detection threshold concentration. Our results show that primer mismatches and/or the presence of faint bands due to microdiversity could explain why SAR11 is frequently not retrieved from DGGE gels.

KEY WORDS: DGGE · SAR11 · Microdiversity · Primers · Methodology

INTRODUCTION

Denaturing gradient gel electrophoresis (DGGE) is often used to describe bacterioplankton community structure because it is thought to offer a fair representation of the most abundant groups in natural communities, allowing the comparison of a large number of samples along spatial, temporal or experimental gradients (Riemann et al. 1999, Casamayor et al. 2000, Schauer et al. 2003).

However, the literature abounds with reported discrepancies in microbial assemblage composition found when DGGE results are compared to other molecular techniques such as clone libraries or fluorescence *in situ* hybridization (FISH) (Castle & Kirchman 2004, Alonso-Sáez et al. 2007). An example of such discrepancies occurs with SAR11, an alphaproteobacterial clade, which is considered to be the most abundant phylogenetic group in the sea (Morris et al. 2002), often contributing 35% of total prokaryotes in the surface ocean. Members of the SAR11 clade consistently dominate 16S rDNA clone libraries (Bano & Hollibaugh 2002, Alonso-Sáez et al. 2007, Crump et al. 2007), and usually account for a large proportion of the bacterial assemblage in FISH studies (Morris et al. 2002, Alonso-Sáez et al. 2007). Functional studies also suggest that they are active and play a significant role in carbon, nitrogen, and sulfur cycling in the ocean (Alonso-Sáez & Gasol 2007, Alonso-Sáez et al. 2008). However, in many studies where DGGE has been used, this bacterial group was not retrieved (e.g. Fandino et al. 2001, Schauer et al. 2003, Pinhassi et al. 2004, Sala et al. 2005, Kan et al. 2006, Alonso-Sáez et al. 2007, Celusi & Cataletto 2007, Sapp et al. 2007).

Discrepancies between molecular methods have also been found for other bacterial groups (Kong et al. 2001, Castle & Kirchman 2004). These studies concluded that DGGE could be expected to identify the most abundant phylogenetic groups even if only a few bands were analyzed, unless all representatives of a particu-

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lar phylogenetic group were rare. Other authors have reported difficulties in detecting many rare ribotypes with DGGE because they were yielding many faint, hardly detectable DGGE bands (Torsvik et al. 1998, Kisand & Wikner 2003).

Substantial microdiversity is one of the factors that could lead to an underestimation of a dominant bacterial group by DGGE. In this case, instead of an intense band of a nondiverse phylotype, different closely related (microdiverse) sequences would share the biomass of the group and none would be dominant in abundance. In a DGGE gel, such a group would appear split in different faint bands, which would escape sequencing efforts. The SAR11 group is known to show a high degree of microdiversity in natural populations (García-Martínez & Rodríguez-Valera 2000, Acinas et al. 2004), and it could be hypothesized that the different DGGE studies that have failed to detect this abundant clade did so for this reason. Alternatively, it could be hypothesized that lack of detection is due to inappropriate primer choice, with the commonly used primers showing biases against that group.

Here we tested these 2 hypotheses for the SAR11 group in Blanes Bay (NW Mediterranean) samples. This is one of the places where the group is abundant, as clone libraries and catalyzed reporter deposition (CARD)-FISH reveal, but where it has been rarely retrieved using DGGE (Schauer et al. 2003, Alonso-Sáez et al. 2007). We analyzed the level of microdiversity within the group and compared it to other groups abundant at the site. We further tested the effect of small differences in the primers used (357fGC-907r and 357fGC-907rM) on the detectability of variable amounts of SAR11 over a background formed by a natural community. These primer sets have been frequently described in the literature and, in addition, we recently showed that primer set 357fGC-907rM was the most adequate for the routine use of polymerase chain reaction (PCR)-DGGE analyses of bacterioplankton samples among 5 different primer pairs commonly used in molecular microbial ecology studies (Sánchez et al. 2007).

MATERIALS AND METHODS

Estimation of microdiversity. A subset of sequences of SAR11 (20 clones) and *Roseobacter* (20 clones), randomly selected from 4 clone libraries from the Blanes Bay Microbial Observatory (described in Alonso-Sáez et al. 2007), were aligned with Clustal X (Thompson et al. 1997) and grouped into similarity clusters using the Clusterer bioinformatics tool (Klepac-Ceraj et al. 2006; http://web.mit.edu/polz/clusterer/). This allows grouping of sequences into percentage similarity clusters (100, 99, 98%, and so on) by the neighbor-joining method.

Samples. Two clones (BL03-AUT03 [A3] and BL03-SUM03 [S3], accession numbers DQ778230 and DQ77814, respectively) retrieved from two 16S rRNA clone libraries from the Blanes Bay Microbial Observatory were selected. These two clones contained recombinant plasmids with an insert affiliated with the SAR11 cluster and had a similarity value of 95.2%. *Escherichia coli* was grown in Luria-Bertani medium and plasmid DNA was extracted using the NucleoSpin Plasmid Quick Pure kit (Macherey-Nagel). Equal concentrations of plasmid DNA from each clone (30 ng μl^{-1}) were mixed, and different amounts of this mixture (Table 1) were combined with 1 µl of DNA extract from 1 environmental sample of surface sea-

Table 1. Contribution (in %) of DNA from mixed clones and DNA from an environmental sample (March 2003) to total DNA in each PCR product. Numbers from 1 to 12 correspond to samples amplified with primer set 357fGC-907r, whereas numbers from 1′ to 12′ refer to primer set 357fGC-907rM. 16S rRNA gene copy number has also been roughly estimated. Grey shading indicates the lowest target concentration where the 2 clones appear in the DGGE gel (see Fig. 1) for each primer set

Sample	% of DNA from the added clones (16S rRNA copy number)[a]	% of DNA from environmental sample (16S rRNA copy number)[b]
1, 1′	0 (0)	100 (3.7×10^6)
2, 2′	0.01 (5.2×10^5)	99.9 (3.7×10^6)
3, 3′	0.2 (1.0×10^6)	99.8 (3.7×10^6)
4, 4′	0.5 (2.6×10^6)	99.5 (3.7×10^6)
5, 5′	1 (5.2×10^6)	99.0 (3.7×10^6)
6, 6′	9.1 (5.2×10^7)	90.9 (3.7×10^6)
7, 7′	16.7 (1.0×10^8)	83.3 (3.7×10^6)
8, 8′	33.3 (2.6×10^8)	66.7 (3.7×10^6)
9, 9′	50 (5.2×10^8)	50 (3.7×10^6)
10, 10′	90.9 (5.2×10^9)	9.1 (3.7×10^6)
11, 11′	95.2 (1.0×10^{10})	4.8 (3.7×10^6)
12, 12′	98 (2.6×10^{10})	2 (3.7×10^6)

[a]Numbers calculated taking into consideration a plasmid plus 16S rRNA gene size of 5396 bases
[b]Based on a mean genome size of 1600 kb (Raes et al. 2007) and a mean 16S rRNA gene copy number of 2.1 $cell^{-1}$ (Moran et al. 2004)

water from Blanes Bay collected in March 2003 (3 ng μl^{-1}). A rough indication of the 16S rRNA gene copy number was determined for the mixture of plasmidic DNA and the environmental sample. For environmental DNA, calculations were done with a mean genome size of 1600 kb (Raes et al. 2007) and a mean 16S rRNA copy number of 2.1 (Moran et al. 2004). The environmental sample was processed as in Sánchez et al. (2007), and DNA extraction was performed as described by Massana et al. (1997).

PCR-DGGE fingerprinting. PCR was carried out using 2 primer sets separately: 357f (5′-CCT ACG GGA GGC AGC AGC AG-3′)-907r (5′-CCG TCA ATT CCT TTR AGT TT-3′) and 357f-907rM (5′-CCG TCA ATT CMT TTG AGT TT-3′). Primers 907r and 907rM differ at 2 base positions, and the latter is the most widely used today. Primer 357f carried a 40 bp GC clamp on the 5′ end. PCR conditions were the same for both primer sets and have been described previously (Sánchez et al. 2007).

The PCR mixtures contained 1 µl template DNA, each deoxynucleoside triphosphate at a concentration of 200 µM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.5 µM, 1.25 U *Taq* DNA polymerase (Invitrogen), and PCR buffer supplied by the manufacturer. Bovine Serum Albumin (BSA) at a final concentration of 600 µg ml^{-1} was added to minimize the inhibitory effect of humic substances. The volume of reactions was 50 µl. PCR products were verified and quantified by agarose gel electrophoresis with a low DNA mass ladder standard (Invitrogen).

The PCR products obtained from these mixtures were run in a DGGE gel at 60°C with a CBS Scientific system as previously described by Muyzer et al. (1998) using a 40 to 80% gradient (6% acrylamide) at 100 V (17 h). The gel was stained with SybrGold (Molecular Probes) for 45 min, rinsed with 1× Tris-acetate-EDTA buffer, removed from the glass plate to a UV-transparent gel scoop, and visualized with UV in a Chemi Doc system (Bio-Rad). DGGE images were analyzed using the Quantity One software (Bio-Rad) in order to detect the different bands present in the gels.

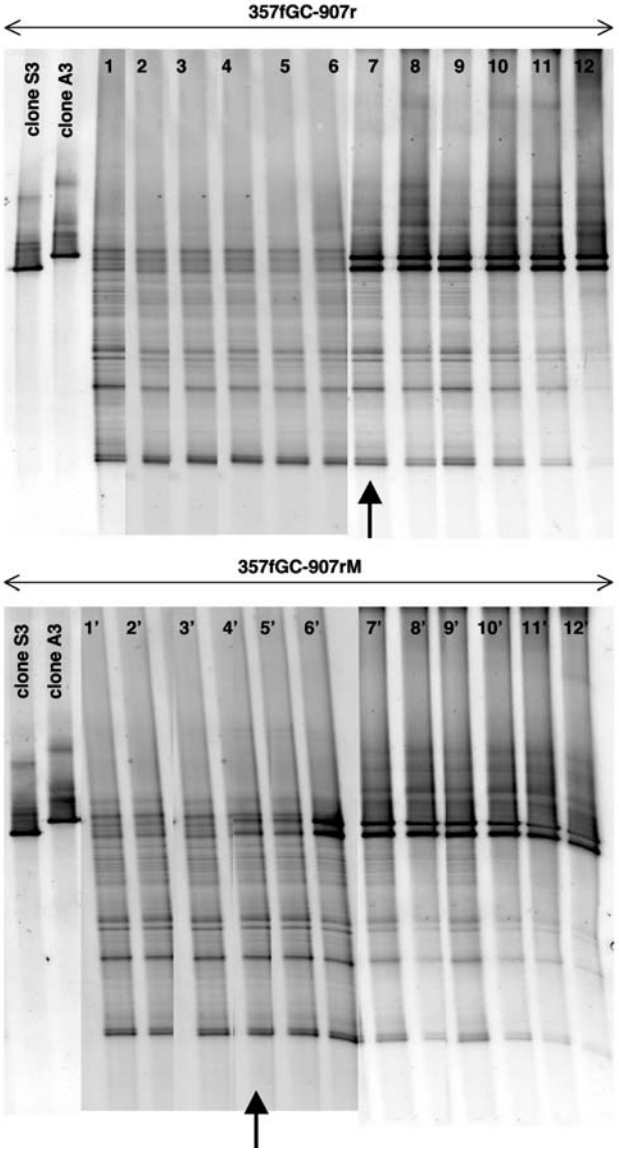

Fig. 1. DGGE fingerprints obtained with 2 different primer sets showing the influence of primer mismatching on SAR11 detection. Samples 1 to 12 correspond to PCR products obtained by combining different increasing amounts of plasmidic DNA (mix of clones S3 + A3) and DNA from a Blanes Bay sample (see Table 1). Arrows at the bottom of each gel indicate the lowest amount of plasmidic DNA mix where the 2 clones appear

RESULTS AND DISCUSSION

DGGE gels (Fig. 1) indicated that both sets of primers could amplify the SAR11 clones when alone in the DNA template. When mixed with environmental DNA (samples 1/1′ to 12/12′), the 2 bands corresponding to these clones appeared at lower target concentrations in the gel where primer pair 357fGC-907rM was used (40 times less of the mix of plasmidic DNA was needed for their detection, as indicated with arrows in Fig. 1). We estimated the 16S rRNA gene copy number for the plasmidic mixture and the environmental sample (Table 1). Based on this estimation, primer set 357fGC-907rM significantly amplified the SAR11 clones when the gene copy number was the same order of magnitude as the gene copy number of the environmental sample (corresponding to sample 4′), in contrast to primer set 357fGC-907r, which clearly amplified both clones when the gene copy number was 2 orders of magnitude above that of the environmental sample (sample 7). This indicates that a larger number

of copies would be needed in order to significantly detect SAR11 in this last case (around 41% of the total copy number in the case of primer set 357fGC-907rM, and 96% for primer set 357fGC-907r).

Besides the clear and expected effect of primer mismatches, we hypothesized that another reason for the discrepancy between DGGE and other molecular techniques could be the presence of multiple low-abundance microdiverse phylotypes within the SAR11 group in the Blanes Bay Microbial Observatory.

The concept of microdiversity, i.e. the genetic diversity within species-like phylogenetic groups, has been recently addressed by different authors. It has been well documented for marine and freshwater habitats, for specific populations such as sulfate-reducing bacteria and populations of *Vibrio* and *Polynucleobacter* (Acinas et al. 2004, Klepac-Ceraj et al. 2004, Hahn & Pöckl 2005, Zo et al. 2008). However, and as far as we know, little research has been done concerning microdiversity of the SAR11 group. Acinas et al. (2004) studied fine-scale phylogenetic relationships in a bacterioplankton sample and constructed phylogenetic trees with the relationships between SAR11 clusters; they observed that the number of operational taxonomic units (OTUs) plotted against changing degrees of cut-offs decreased significantly from 100 to 99% sequence similarity, and the SAR11 group formed the most sequence-rich microdiverse clusters. Other studies, such as the one of García-Martínez & Rodríguez-Valera (2000), showed a very large group of SAR11 clones with high within-cluster similarity (<1% of nucleotide differences).

In order to estimate the microdiversity of the SAR11 group present in Blanes Bay, we analyzed 20 randomly selected SAR11 clones obtained from different libraries from this environment. Similarity clustering showed a remarkable decline in the number of OTUs as cluster cut-off values were decreased from 98 to 91% (Fig. 2). In stark contrast, the number of OTUs greatly exceeded this decline for values above 98%, which suggests decreased removal of diversity within microdiverse clusters. For the sake of comparison, 20 phylotypes of the same clone libraries corresponding to the *Roseobacter* clade were also analyzed. *Roseobacter*, in contrast to SAR11, does appear frequently in DGGE gels of Blanes Bay, despite the fact that it is much less abundant than SAR11, as detected by clone libraries and CARD-FISH (Alonso-Sáez et al. 2007). For this group, there was a gradual and continuous decline in the number of OTUs between cut-off values of 100 and 82% of cluster similarity (Fig. 2). Therefore, in the case of SAR11, 50% of the ribotypes fall into discrete clusters containing <2% of divergence (between 100 and 98% similarity), while for *Roseobacter* this value was between 100 and 93%.

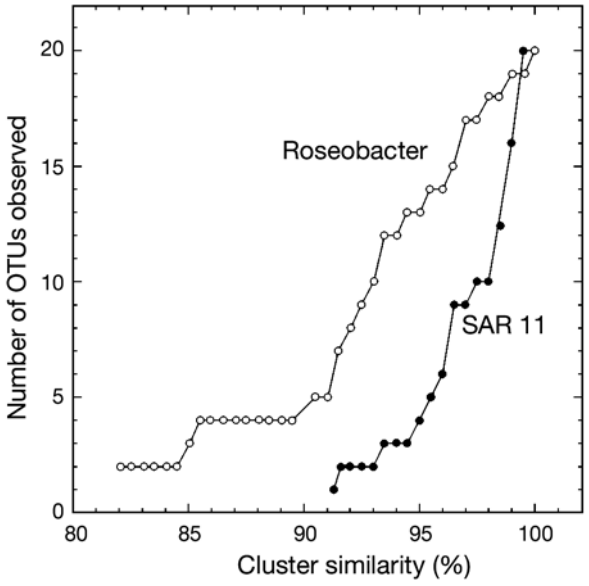

Fig. 2. Microdiversity analysis from SAR11 and *Roseobacter* sequences obtained from different clone libraries from the Blanes Bay Microbial Observatory. Number of operational taxonomic units (OTUs) is plotted against changing degrees of cut-offs in 0.5% increments for grouping of sequences into similarity clusters

Thus, according to these results, microdiversity seems to be a feasible explanation for the frequent absence of significant bands of SAR11 in DGGE gels.

Apart from the problems in DGGE analyses caused by faint bands due to microdiversity and primer specificity, an additional problem was the position of SAR11 bands in DGGE gels. When several SAR11 clones from Blanes Bay clone libraries were run in a DGGE gel, it was apparent that they migrated within quite a narrow region of the gel, which hampered the attempts to obtain clean sequences from these bands (Alonso-Sáez et al. 2007).

Kan et al. (2006) also used bacterial seeding experiments to determine the detection thresholds for PCR-DGGE, demonstrating that concentrations ranging from 2.5×10^3 to 1×10^4 cells ml^{-1} (0.1 to 0.4% of total cell counts, depending on the rRNA operon copy number considered) were below the detection limit, and consequently these bacteria would be absent from a DGGE gel. If we assume that all the different SAR11 clones obtained from diverse clone libraries of Blanes Bay made the same contribution to total abundance, and taking into account from CARD-FISH data the overall contribution of SAR11 to total DAPI counts in every clone library (Alonso-Sáez et al. 2007), we can estimate a concentration range of 1.6×10^3 to 8.2×10^3 cells ml^{-1} for each SAR11 phylotype. These values are below the detection limit of DGGE proposed by Kan et al. (2006).

Table 2. Presence of SAR11 in some studies in which the marine bacterioplankton community has been analyzed by DGGE. CARD-FISH: catalyzed reporter deposition-fluorescence *in situ* hybridization

Sampling site	Detection of SAR11 by DGGE	Other molecular methods used in the work/ detection of SAR11	Source
Arabian Sea	+		Riemann et al. (1999)
Southern California	–		Fandino et al. (2001)
California Bight, Skagerak, Baltic Sea	± (depending on the site)	Quantitative PCR / +	Simu & Hagström (2001)
Arctic Ocean	+	Clone libraries / +	Bano & Hollibaugh (2002)
NW Mediterranean	–		Schauer et al. (2003)
Weser estuary	+		Selje & Simon (2003)
Estuarine waters (Massachusetts)	+		Crump et al. (2004)
NW Mediterranean	–		Sala et al. (2005)
Baltimore inner harbor	–		Kan et al. (2006)
NW Mediterranean	–	Clone libraries / + CARD-FISH / +	Alonso-Sáez et al. (2007)
Gulf of Trieste	–		Celussi & Cataletto (2007)
Chesapeake Bay	+	Clone libraries / +	Crump et al. (2007)
North Sea	–		Sapp et al. (2007)

The experiments carried out here using 2 clones of SAR11 as internal standards confirmed, first, that primer 907rM is more suitable for SAR11 amplification than primer 907r and, second, that there are no specific biases against SAR11 that could prevent their appearance in the DGGE gels. Instead, large evenness of closely related, equally abundant microdiverse organisms could generate several separate bands that are too faint for further sequencing. SAR11 was indeed present in the environmental sample used as a control, based on CARD-FISH and clone libraries (Alonso-Sáez et al. 2007). In a previous study, Schauer et al. (2003) also used the primer pairs 357fGC-907r and 357fGC-907rM to characterize the diversity of this microbial assemblage. They obtained almost identical fingerprints with both primer sets, but they could not retrieve SAR11 phylotypes from the DGGE gels, probably due to the presence of faint bands that were not intense enough for sequencing.

Nevertheless, SAR11 bands have been detected from marine samples in some studies where DGGE has been used as a fingerprinting technique (Table 2), indicating that not all representatives of this particular phylogenetic group are always rare and below the detection limits of the DGGE technique. In view of our results, a possible explanation could be that microdiversity was lower in these cases.

In conclusion, although DGGE constitutes a useful fingerprinting method, care must be taken with the groups showing significant microdiversity, and primer specificity has to be taken into account. The consequences of missing a dominant group are detrimental to the description of the role of specific bacterial groups in marine environments.

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