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MOLECULAR CHARACTERIZATION OF ACTIVATED SLUDGE FROM A SEAWATER-PROCESSING WASTEWATER TREATMENT PLANT

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Summary

The prokaryotic community composition of activated sludge from a seawater-processing wastewater treatment plant (Almeria, Spain) was investigated by using the rRNA approach, combining different molecular techniques such as denaturing gradient gel electrophoresis (DGGE), clone libraries and in situ hybridization (FISH and CARD-FISH). Most of the sequences retrieved in the DGGE and the clone libraries were similar to uncultured members of different phyla. The most abundant sequence recovered from Bacteria in the clone library corresponded to a bacterium from the *Deinococcus-Thermus* cluster (almost 77% of the clones), and the library included members from other groups such as the *Alpha, Gamma-* and *Delta-* subclasses of *Proteobacteria*, the *Bacteroidetes* and *Firmicutes*. Concerning the archaeal clone library, we basically found sequences related to different orders of methanogenic *Archaea*, in correspondence with the recovered DGGE bands.

Enumeration of DAPI (4', 6-diamidino-2-phenylindole) - stained cells from two different activated sludge samples after a mechanical flocculation disruption revealed a mean cell count of 1.6×10^9 ml^{-1}. Around 94% of DAPI counts (mean value from both samples) hybridized with a *Bacteria* specific probe. *Alphaproteobacteria* were the dominant bacterial group (36% of DAPI counts), while *Beta-*, *Delta-* and *Gammaproteobacteria*, *Bacteroidetes, Actinobacteria* and *Firmicutes* contributed to lower proportions (between 0.5 – 5,7% of DAPI counts). *Archaea* accounted only for 6% of DAPI counts. In addition, specific primers for amplification of the *amoA* (ammonia monooxygenase) gene were used to detect the presence of *Beta*, *Gamma* and archaeal nitrifiers, yielding positive amplifications only for *Betaproteobacteria*. This, together with negative in situ hybridizations with probes for well-known nitrifiying bacteria suggests that nitrification is performed by still undetected microorganisms. In summary, the combination of the three approaches provided different and complementary pictures of the real assemblage composition and allowed to get closer to the main microorganisms involved in key processes of seawater-processing activated sludge.

Introduction

Activated sludge systems are one of the most important biotechnological processes in wastewater treatment plants (wwtps). They consist of a complex mixture of microorganisms able to remove organic substances and nutrient contaminants from municipal or industrial sewage, being thus a crucial tool in environmental protection. For years, researchers have investigated the microbial communities of activated sludge in order to understand their specific biological processes (Amann *et al*., 1998; Wagner *et al*., 2002). Studies of diversity can provide insight on the correlation between microbial composition and ecosystem function, as well as knowledge about temporal and spatial variations in microbial communities. However, the vast majority of bacteria present in activated sludge cannot be isolated by conventional culture-dependent techniques; the percentage of culturable bacteria in comparison with total cell counts is estimated to range between 1 and 15% with optimized media (Wagner *et al*., 1993).

The current use of molecular methods, that do not require the isolation and cultivation of microorganisms, has allowed a more comprehensive analysis of microbial diversity in wastewater research. Sequence analysis of 16S rRNA gene clone libraries (Snaidr *et al*., 1997), fingerprinting techniques such as denaturing gradient gel electrophoresis (Boon *et al*., 2002), thermal gradient gel electrophoresis (Eichner *et al*., 1999), and terminal restriction fragment length polymorphism (T-RFLP) (Saikaly *et al*., 2005), as well as the design of group-specific rRNA-targeted oligonucleotide probes for Fluorescence In Situ Hybridization (FISH) (Wagner *et al*., 1993, 1994) have greatly expanded our understanding of wastewater microbiology. The cultivation-independent rRNA approach allows to determine the composition and dynamics of microbial communities in these systems and to identify the microbial key players for the different processes.

Considerable microbial diversity has been detected in wwtps, including bacteria involved in biological phosphorus removal (Bond *et al*., 1999; Jeon *et al*., 2003; Seviour *et al*., 2003), nitrifiers (Juretschko *et al*., 1998; Coskuner and Curtis, 2002; Otawa *et al*., 2006), and methanogens (Zheng, 2000). Sequences from *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Actinobacteria* and the *Planctomycetes* were retrieved in significant numbers in different clone libraries (Wagner *et al*., 2002). Nevertheless, all studies of microbial diversity in wwtps refer to freshwater treatment plants, either domestic or industrial. As far as we know, no studies have been done on wwtps that utilize seawater for their operation.

On the other hand, only a few works have applied the full-cycle rRNA approach for the study of microbial communities in activated sludges, which includes the establishment of a 16S rRNA gene clone library, the design of a set of clone-specific oligonucleotide probes, and the determination of the abundance of the respective bacterial populations by quantitative FISH (Snaidr *et al*., 1997; Juretschko *et al*., 1998). In this paper, the prokaryotic diversity of a wwtp from a pharmaceutical industry located in the south of Spain which has the particularity to utilize seawater has been characterized using a polyphasic approach with three molecular tools such as DGGE, clone libraries and FISH. This wastewater treatment plant is in operation since 1998 and today, very few plants of this type are running in the world; its main influent corresponds to intermediate products from amoxicillin synthesis. The use of seawater instead of freshwater responds to the defficiency in hydric resources prevailing in this location, one of the driest areas in Spain. To our knowledge, this is the first study that analyzes an activated sludge with these characteristics.

Results

DGGE fingerprinting from seawater-processing activated sludge

The DGGE analysis from the two samples of activated sludge, corresponding to years 2007 and 2008, yielded a total of 20 and 17 different band positions for *Bacteria* and *Archaea* respectively (Fig. 1). Both samples showed virtually the same pattern for both set of primers, although differences in band intensity could be observed in some bands. This finding suggested that the system was rather stable along time. Bands were excised from both gels in order to determine their phylogenetic affiliation, and informative sequences were obtained from 12 (*Bacteria*) and 5 bands (*Archaea*) (Table 1). These bands accounted for 58% (*Bacteria*) and 32% (*Archaea*) of the total mean band intensity and most of them showed similarities with sequences from uncultured clones by BLAST search.

Four bands of bacterial origin affiliated to *Bacteroidetes*, with a contribution of 17% to total mean band intensity. The remaining bacterial bands belonged to different subclasses (*Alpha*-, *Gamma*- and *Delta*-) of the phylum *Proteobacteria* except two bands that affiliated to the *Deinococcus-Thermus* group.

Excision of bands from the archaeal DGGE gel yielded sequences related to methanogenic *Archaea*, although identities were relatively low.

Fig. 1
Table 1

Identification of taxonomic groups by clone libraries

We analysed 278 and 117 clones in bacterial and archaeal clone libraries constructed with a sample from the marine activated sludge. Sequences were grouped in OTUs using a similarity criteria clustering of 98.5% (Stackebrandt and Goebel, 1994). One representative sequence of each OTU is shown in Tables 2 and 3, together with the closest relatives and the frequency of the OTU. Coverage of the libraries was 95.7 and 91.5% respectively, indicating that in both cases this particular approach provided most of the measurable diversity.

Table 2
Table 3

A significant number of clones from both libraries showed similarity to uncultured sequences deposited in GenBank. The most abundant sequence recovered from Bacteria corresponded to a bacterium from the *Deinococcus-Thermus* cluster (almost 77% of the clones) with the same sequence as band DER_12. The library included also members from other groups such as the *Alpha-, Gamma-* and *Delta-* subclasses of *Proteobacteria*, the *Bacteroidetes* and *Firmicutes*. Within the *Proteobacteria*, which represented 21% of the clones, members of the *Gammaproteobacteria* predominated (14% of total clones). One clone was affiliated to the unclassified bacterium *Denitromonas indolicum*. Similarities based on sequence comparison of these clones varied between 81.7 and 99.6%. The 16S rRNA similarities were approximately at the species level (≥97%, Stackebrandt and Goebel, 1994) for 19% of the clones, while 79% were similar at the genus level (95-97%). In general, there was agreement between the different sequences retrieved by DGGE and the clone library. Inclusion of all the sequences in a phylogenetic tree indicated that most of the DGGE band sequences corresponded to several of the most abundant clones recovered from the library (tree not shown).

Concerning the archaeal clone library, we basically found sequences related to different orders of methanogenic *Archaea*, in correspondence with the recovered DGGE bands. In this case, similarities ranged between 83.2 and 99.5%. We paid especial attention to these archaeal sequences, since most of them were only moderately related to known archaea. Phylogenetic analyses were performed by several methods and summarized in a maximum-likelihood tree with Bayesian posterior probabilities and neighbor joining bootstrap values in the relevant nodes (Fig. 2). Based on the tree structure and bootstrap values, a high percentage of archaeal sequences (77%) were grouped into 3 separate clusters, named DER_1, _2 and _3, which formed three independent branches composed exclusively by environmental clones.

Other sequences clustered within three major phylogenetic groups of methanogens: Methanosarcinales, Methanomicrobiales and Methanobacteriales, while sequences belonging to Methanococcales and Methanopyrales were not retrieved.

Detection of amoA genes

Using specific primers for the amplification of the gene *amoA* from the *Beta*, *Gamma* and archaeal nitrifiers, we were able to confirm the presence of ammonia-oxidizers from the Beta-subclass of *Proteobacteria* (data not shown). The gene *amoA* encodes the catalytic α-subunit of ammonia monooxygenase, the enzyme responsible for catalyzing the rate-limiting step in ammonia oxidation, and it has been used extensively as a molecular marker for cultivation-independent studies of ammonia-oxidizing communities. However, we could not find amplification for *Gamma* and *Archaea* nitrifiers in our samples.

Quantitative analysis of marine activated sludge composition by in situ hybridization

The activated sludge samples from years 2007 and 2008 were also analyzed by DAPI staining and fluorescent in situ hybridization. A sound quantification of activated sludge samples was complicated by the heterogeneous cell distribution caused by the flocculation. Several treatments were tested for cell dispersal, e.g. sonication and vortexing at different times (5, 15, or 30 min, data not shown). No differences in hybridization signals were found in treatments at 5 and 15 min, while the fraction of hybridized cells decreased after sonicating or vortexing samples during 30 min. In any case, microscopic examination clearly showed that there were still aggregates. In order to increase accuracy we counted separately the hybridized free cells and the hybridized cells in aggregates for each probe, evaluating several thousand cells in the case of aggregates. Enumeration of DAPI-stained preparations revealed total cell counts of 1.89×10^9 ml^{-1} (year 2007) and 1.28×10^9 ml^{-1} (year 2008), being 65% (mean value from both years) in the form of aggregates and the rest as free cells. The mean number of cells per aggregate was 444. However, one has to keep in mind that microscopic enumeration of aggregated cell clusters is likely resulting in underestimations, but since this effect applies equally to DAPI and FISH counts, the conclusions drawn from the different fractions of probe positive-DAPI stained cells remain valid.

The results obtained from CARD-FISH analyses for both years (Fig. 3) indicated that the microbial assemblage had virtually the same composition, and that the percentage of the

hybridized groups remained very similar, confirming that the system was stable along time. Hybridization with the universal set of probes EUB (Eub+) detected a mean value from both years of 86% of DAPI-stained free cells and 97% of cells in aggregates (Eub+), while *Archaea* reached only 4 and 3% of mean DAPI counts for free and aggregated cells respectively, indicating that the majority of fixed cells were *Bacteria*. A considerable amount of total Eub+ free cells (54%) were identified with probes for broad phylogenetic groups (*Alpha-, Beta-, Gamma-,* and *Deltaproteobacteria, Bacteroidetes, Firmicutes* and *Actinobacteria*). This value was similar for aggregates (62%). *Alphaproteobacteria* were the dominant bacterial group [27% (free cells) and 44% (aggregates) of mean DAPI counts], while *Gammaproteobacteria* and *Bacteroidetes* contributed to lower and similar proportions in the free-cells fraction (around 8% of mean DAPI counts). These values were lower in the case of aggregated cells (around 4% of mean DAPI counts). Other groups, such as *Beta* and *Deltaproteobacteria,* Firmicutes and Actinobacteria were present even at lower numbers (1-4% of mean DAPI counts in free cells, and 0-1% in aggregates). A specific probe (DT01) corresponding to a sequence that accounted for 77% of the clones in the bacterial clone library was designed in this work. This sequence had approximately 96% similarity with an uncultured bacterium from the *Deinococcus-Thermus* phylum (Table 2). However, CARD-FISH results showed that this microorganism was not particularly abundant (3 and 7% of mean DAPI counts for free and aggregated cells respectively), denoting a large positive bias for this bacterium in the clone library.

Fig. 3

In situ hybridization with a set of well-known hierarchical 16S rRNA-targeted probes for ammonia-oxidizing bacteria (including the genera *Nitrobacter* and most of the betaproteobacterial ammonia-oxidizers, such as members of the genera *Nitrosomonas*, *Nitrosococcus mobilis* and *Nitrosospira*), usually used for activated sludge (Juretschko *et al.,* 1998) showed no signal in our samples, in accordance with the results observed in the DGGE and the bacterial clone library for these particular microorganisms.

Quantitative comparison between DGGE, CARD-FISH and clone libraries

In the case of *Bacteria*, we quantitatively compared the results obtained by the three different molecular methods in order to test the strong and weak points of each approach, and how they affect the overall picture of activated sludge diversity (Fig. 4). For FISH representation, we took into account the contribution of free and aggregated cells for every probe. The most remarkable trend in this figure is the overrepresentation of a sequence corresponding to the

Deinococcus-Thermus group in the clone library as compared with CARD-FISH and DGGE. *Alphaproteobacteria* and *Bacteroidetes*, by contrast, seemed to be underrepresented in the clone library, while *Alphaproteobacteria* were overrepresented with CARD-FISH. On the other hand, the detection of *Gamma* and *Deltaproteobacteria*, was more proportionate by the three methods. Other groups could only be detected by CARD-FISH, such as *Betaproteobacteria*, and *Actinobacteria*, although at very low relative abundance.

Discussion

The advent of molecular techniques in the past two decades has provided many insights into the diversity and functions of predominantly uncultured wastewater microorganisms. However, relatively few works have studied activated sludge microorganisms by a full-cycle rRNA approach, and never before the diversity of a marine activated sludge has been detailed. In our study, the objective was to obtain a comprehensive picture of the diversity of the prokaryotic assemblage in a seawater-processing wwtp, combining and comparing different molecular approaches (DGGE, clone library and FISH).

In this particular wwtp, we predict that the influent composition becomes crucial in order to understand the composition of the microbial community. The presence of amoxicillin, together with a high salt concentration, will surely affect microbial diversity. Actually, concern is growing over environmental contamination with pharmaceuticals because of their widespread use and incomplete removal during wastewater treatment. Thus, Kraigher et al. (2008) investigated the influence of pharmaceutical residues on the structure of activated sludge bacterial communities in wastewater treatment bioreactors and observed a minor but consistent shift in the community structure in bioreactors supplied with pharmaceuticals, as well as a reduction in diversity.

Diversity in seawater-utilizing activated sludge

Different authors have addressed the study of activated sludge diversity either in wwtp or laboratory-scale reactors by means of molecular techniques (Bond *et al*., 1995; Kämpfer *et al*., 1996; Snaidr *et al*., 1997; Christensson *et al*., 1998; Dabert *et al*., 2001; Daims *et al*., 2001; Liu and Seviour, 2001; Juretschko *et al*., 2002; Eschenhagen *et al*., 2003, Sanapareddy *et al*., 2009). These studies indicated considerable microbial diversity in wwtps and the dominance of the *Beta* subclass of the class *Proteobacteria*. Apart from the *Proteobacteria*, other groups such as the *Bacteroidetes*, the *Chloroflexi*, the *Actinobacteria* and the *Planctomycetes* could

be detected either in clone libraries or using FISH with group-specific probes. The composition of the bacterial community of the seawater activated sludge described here differed strongly from those previously reported, since *Betaproteobacteria* did not seem to be the predominant group. It was even not detected in the clone library and represented only a 3% of the total hybridized bacteria. This is most likely due to the fact that our wwtp is fed with seawater, which is known to contain very few *Betaproteobacteria* (Rappé and Giovannoni, 2003). In contrast, other subclasses of *Proteobacteria*, such as *Alpha-*, *Gamma-* or *Deltaproteobacteria* were detected with the three methodologies, although in the case of *Alphaproteobacteria*, at different proportions. On the other hand, the *Bacteroidetes* group seemed also to be represented by the three methods.

The most remarkable feature of clone libraries is the severe overrepresentation of one sequence belonging to the *Deinococcus/Thermus* group (around 77% of total bacterial clones). Its closest match in GenBank was an uncultured bacterium, and its closest cultured match was *Truepera radiovictrix*, although at low similarities (less than 90%, Table 1). The phylum *Deinococcus/Thermus* includes extremely radiation resistant bacteria, as well as slightly thermophilic or thermophilic members, and it also comprises a number of environmental 16S rRNA gene sequences, several of which are not closely related to any cultured strains and form distinct lineages. Nevertheless, when comparing to DGGE and CARD-FISH data, this sequence turned out to be less abundant and discrepancies with the clone library become clear.

Most of the sequences retrieved in the bacterial clone library were similar to uncultured members of different phyla, but in some cases, cultured closest matches were related at the genus or even at the species level (Table 1). For example, one clone of *Alphaproteobacteria* (BACDER07_1D12) was similar at the genus level to *Sneathiella chinensis*, a marine chemoheterotrophic bacterium, and another clone (BACDER07_2B7) to *Methylocystis* sp., a methanotrophic bacterium. On the other hand, one clone (BACDER07_2G10) was similar at the species level to *Sphingomonas* sp., a genus recognized by its capability to degrade a wide variety of refractory environmental pollutants and to carry out diverse other biotechnologically useful activities, such as the biosynthesis of valuable biopolymers (Laskin and White, 1999); Sphingomonads have been identified in situ by FISH in activated sludge samples and turned out to be rather abundant, accounting for about 5-10% of the total cells (Neef *et al.*, 1999). Since exopolysaccharides are a significant part of the polymeric extracellular matrix material of flocs, and members of the genus *Sphingomonas* are known to be able to produce slimes and/or capsules, the authors suggested that they could be involved in the formation process of sludge flocs. Finally, another clone was similar at the genus level to *Denitromonas indolicum*,

an unclassified bacterium. This genus was found to be able to grow with perchlorate as the sole electron acceptor (Zuo *et al*., 2009).

Concerning the diversity of *Archaea*, less attention has been paid to their role in wastewater treatment processes, since it seems clear that *Bacteria* are responsible for the majority of carbon removal in the activated sludge process (Gray *et al*., 2002). In our samples, *Archaea* represented only a small fraction of total mean DAPI counts from the two samples (4% in free cells and 3% in aggregates). Virtually all sequences retrieved in our archaeal clone library were related to methanogenic bacteria (Fig. 2), consistent with previous reports of the existence of anoxic microenvironments in the flocs, in which methanogens might be active (Schramm *et al*., 1999). All methanogens are strictly anaerobic *Archaea* pertaining to the *Euryarchaeota*. Although they are very diverse phylogenetically, they can only utilize a restricted number of substrates of three major types: CO_2, methyl-group containing compounds, and acetate. Their common habitats include marine and freshwater sediments, flooded soils, human and animal gastrointestinal tracts, termites, anaerobic digestors, landfill, geothermal systems and heartwood of trees. Nevertheless, it has been demonstrated the presence of methanogenic bacteria in aerated activated sludge. Thus, Gray *et al*. (2002) retrieved archaeal 16S rRNA gene sequences related to Methanosarcinales, Methanomicrobiales and Methanobacteriales. However, the relatively low rates of methanogenesis measured by these authors indicated that, although active, the methanogens played a minor role in carbon turnover in activated sludge.

In our study, the recovered sequences affiliated also within the orders Methanosarcinales, Methanomicrobiales, and Methanobacteriales with the same proportion in the clone library (8% each). However, most of the sequences clustered into novel branches (DER_1, _2 and _3), which were closely related to environmental clones. One of these sequences (representative clone: ARCHDER07_1A5), belonging to DER_2, accounted for 40% of the total clones and was related to a sequence from Guerrero Negro hypersaline microbial mats (Robertson *et al*., 2009).

On the other hand, *Crenarchaeota* were also detected by CARD-FISH (2% of total DAPI counts from free and aggregated cells), but this group was not represented in the archaeal clone library or in the retrieved sequences from the DGGE. PCR bias against *Crenarchaeota* could explain this disagreement.

Linking diversity and function

The functional assignment of detected microorganisms is complicated by the fact that 16S rRNA sequence-based identification does generally not allow to infer their functional properties. Phylogenetically closely related microorganisms may possess different metabolic traits while on the other hand several physiological features like the ability to denitrify are dispersed in different phylogenetic lineages. Therefore, the full-cycle rRNA-approach needs to be supplemented with other techniques that allow a functional assignment of the detected microorganisms. In this particular sense, we included in our study the amplification of the functional gene coding for the active-site polypeptide of ammonia monooxygenase (*amoA*) as a physiological marker, as well as the use of specific rRNA-targeted probes for the detection of ammonia oxidizers in order to link diversity with function, in particular those aspects referred to nitrogen removal.

In engineered systems such as wwtps, the coupled nitrification and denitrification processes are considered the major mechanisms of nitrogen removal. The nitrifiers encompass two groups of microorganisms, the ammonia and the nitrite-oxidizing bacteria, which catalyze the oxidation of ammonia to nitrite and of nitrite to nitrate respectively. Many wwtps harbor diverse *Beta* and *Gammaproteobacteria* ammonia-oxidizers, such as the *Nitrosomonas europaea/Nitrosomonas eutropha*-lineage (*Beta*), the *Nitrosococcus mobilis*-lineage (*Beta*), the *Nitrosomonas marina* cluster (Beta), and the *Nitrosococcus* group (*Gamma)*. Also, heterotrophic microorganisms have been reported to oxidize nitrogen compounds under very specific conditions (Kim *et al*., 2005). In addition, *Nitrobacter* and *Nitrospira* have been recognized as nitrite-oxidizers (Wagner *et al*., 2002). Recently, ammonia-oxidizing organisms belonging to the archaeal domain have also been described (You *et al*., 2009). The denitrification process, i.e., the removal of nitrate to the atmosphere, seems to be mainly done by members of the genera *Alcaligenes*, *Pseudomonas*, *Methylobacterium*, *Bacillus*, *Paracoccus, Hyphomicrobium*, as well as by many members of the betaproteobacterial order Rhodocyclales (Wagner *et al*., 2002; Hosselhoe *et al.,* 2009).

In our study, the seawater-processing wwtp was known to have a nitrogen sludge load of 150-170 kg/h, a nitrification fraction of 98% and a total nitrogen removal over 80% (M. I. Maldonado, personal communication). Thus, nitrification and denitrification are important processes in this system. Although amplification from beta ammonia-oxidizers was detected in the two samples, we have not been able to find sequences corresponding to recognized microorganisms known to catalyze the oxidation of ammonia to nitrite or of nitrite to nitrate in freshwater wwtps. Sequences from *Betaproteobacteria* (to which a diversity of nitrifiers from freshwater wwtps belong) have not been recovered from the DGGE gels and the clone library,

although CARD-FISH analyses showed a certain amount of bacteria belonging to this group (3% of total DAPI-stained cells). It is possible that the DNA extraction technique applied was not sufficient rigorous to lyse the cells of ammonia–oxidizers in the activated sludge, or that PCR or cloning biases occurred. However, hybridization with specific probes for beta ammonia-oxidizers showed no signal, suggesting that the microorganisms carrying out this function in our samples could not be assigned to any of the well-known lineages from freshwater activated sludges. Thus, nitrifiers in this specific seawater-processing wwtp correspond to different genera. In fact, it has been shown that several heterotrophic *Bacillus* strains can carry out aerobic nitrification, as well as denitrification (Kim *et al*., 2005), and we have found sequences of *Bacillus* in the clone library (although with a low similarity) and also by culture-dependent techniques (data not shown). Actually, *Bacillus* strains are able to remove nitrogen and phosphorus as well as organic matter. On the other hand, it is also possible that, although being crucial for nitrogen removal, the well-known nitrifiers could not be detected due to their low abundance. Concerning the *Gammaproteobacteria*, no amplification of the *amoA* gene was found and most of the sequences recovered in this study corresponded to unidentified clones.

In contrast, sequences of *Nitratireductor* sp., able to reduce nitrate to nitrite, have been retrieved from the DGGE and the bacterial clone library. However, most candidates for denitrifying bacteria in this work have been found by culture-dependent approaches (data not shown). Thus, members of the genera *Alcaligenes*, *Pseudomonas*, *Bacillus*, *Paracoccus, Halomonas* and *Marinobacter* have been isolated in rich media, although we don't know whether these genera are representative for the *in situ* active denitrifiers of this system.

Recently, it has been shown that autotrophic oxidation of ammonia is not restricted to the domain *Bacteria*. Könneke *et al.* (2005) isolated an ammonia-oxidizing crenarchaeon named *Nitrosopumilus maritimus* able to oxidize ammonia to nitrite under mesophilic conditions, and Park *et al*. (2006) reported molecular evidence that ammonia-oxidizing archaea occur in activated sludge bioreactors used to remove ammonia from wastewater. However, amplification of the archaeal *amoA* was not found in our samples . Nevertheless, it is important to note that significant diversity exists in each of these functional groups of organisms and that a detailed knowledge of their biology needs to be gained.

Correspondence between DGGE, clone library and FISH

The comparison between clone library, DGGE and FISH results is not straightforward because of the different levels of phylogenetic resolution of each technique. There is general agreement

regarding the limitations of each methodology (Amann *et al*., 1995; Wintzingerode *et al*., 1997), but few studies have compared these techniques in activated sludge systems (Snaidr *et al*., 1997; Juretschko *et al*., 2002; Eschenhagen *et al*., 2003), and none has compared them in seawater-processing activated sludge.

DGGE allowed an assessment of the composition of the prokaryotic assemblage of the activated sludge sample with sufficient resolution. However, a failure to obtain sequences from faint bands prevents the use of DGGE for describing bacterial diversity accurately (Sánchez *et al*., 2009). Because not all bands were sequenced, it cannot be discarded that differences between community composition shown by DGGE and by other techniques are due to insufficient sequencing. On the other hand, the clone library provided the highest phylogenetic resolution and a detailed picture of the species within each phylogenetic group. However, PCR bias and the varying copy number of the rRNA operon in different organisms produced severe overestimations (*Deinococcus-Thermus*) or underestimations (*Alphaproteobacteria*) of specific groups compared with the direct quantification obtained by CARD-FISH. On the other hand, the picture of the bacterial assemblage composition provided by CARD-FISH was limited by the number and phylogenetic resolution of the probes. A substantial proportion of the Eub338-II-III positive cells remained unidentified by the general probes used, while no cell remained undetectable.

Although DGGE is also subject to PCR bias, in our study the group proportion with this technique was more similar to what was found for CARD-FISH than to clone libraries. This discrepancy was also shown by Massana *et al*. (2006), who observed that clone libraries obtained with a primer set amplifying one-third of the 18S rRNA gene from eukaryotes (the set that is regularly used in DGGE studies) provided very good correlation between clonal representation and cell abundance determined by FISH. In contrast, the primer set amplifying the complete 18S rRNA gene gave a very biased view of the phylogenetic groups under study when compared to FISH abundance, with some phylotypes being severely overestimated and others underestimated.

Snaidr *et al*. (1997), however, despite using a primer set which amplified almost-full-length 16S rRNA gene fragments, found a general agreement between clone library and FISH when analyzing the bacterial community structure of activated sludge from a municipal wwtp, although discrepancies became clear when using more specific probes. In their study, almost 20% of DAPI cells remained undetected.

In summary, the combination of the three techniques was very useful for assessing a comprehensive appraisal of prokaryotic diversity, and thus a polyphasic approach is essential to have a complete picture of the prokaryotic assemblage. These methods also showed that

this particular activated sludge can contain significant hidden diversity of unknown and uncultured marine-related microorganisms that can contribute to its functioning. Therefore, further attempts to isolate the key microorganisms involved will be essential in order to understand their specific biological processes.

Experimental procedures

Sampling

Samples of aerated mixed activated sludge from a seawater processing wastewater treatment plant located in Almeria (southeast Spain) were collected in December 2007 and November 2008 in a 1 L sterile bottle and stored at 4ºC until processing. The plant treats wastewater from a pharmaceutical industry and the performance of the reactor is constant in function of time, with a continuous entrance of intermediate products from amoxicillin synthesis (between 0-250 mg/L). The mean influent flow of the plant is 300 m^3/h and has a treatment volume of 32000 m^3. Nitrogen and chemical oxygen demand (COD) sludge loads were about 150-170 kg/h and 900-1000 kg/h respectively. Ionic concentrations in the influent were as follows: NH_4^+: 0.6-1.1 g/L, K^+: 121.1 mg/L, Mg^{2+}: 97.3 mg/L, Ca^{2+}: 386.6 mg/L, NO_3^-: 0 g/L, NO_2^-: 0 mg/L, SO_4^{2-}: 4-6.5 g/L and PO_4^{3-}: 3.5-5.4 g/L. In the efluent, the ionic concentrations of nitrogen compounds were: NH_4^+: 0-40 mg/L, NO_3^-: 200-600 mg/L, NO_2^-: 0-20 mg/L, total nitrogen: 100-250 mg/L, being the total nitrogen and COD removals above 80 and 90% respectively.

DNA extraction and PCR amplification

Fifty ml samples were centrifuged and the pellets were stored at –20ºC until use. Upon thawing, community DNA was extracted using the DNA Power Soil kit from MOBIO (12888-50).

Fragments of the bacterial 16S rRNA gene suitable for DGGE analysis were obtained by using the specific primer 358F with a 40-bp GC clamp, and the universal primer 907RM (Sánchez *et al*., 2007). Polymerase chain reaction (PCR) was carried out with a Biometra thermocycler using the following program: initial denaturation at 94ºC for 5 min; 10 touchdown cycles of denaturation (at 94ºC for 1 min), annealing (at 63.5-53.5ºC for 1 min, decreasing 1ºC each cycle), and extension (at 72ºC for 3 min); 20 standard cycles (annealing at 53.5ºC, 1 min) and a final extension at 72ºC for 5 min.

Primers 344f-GC and 915R were used for archaeal 16S rRNA amplification (Casamayor *et al*., 2002). The PCR protocol included an initial denaturation step at 94ºC for 5 min, followed by 20 touchdown cycles of denaturation (at 94ºC for 1 min), annealing (at 71 to 61ºC for 1 min, decreasing 1ºC each cycle), and extension (at 72ºC for 3 min); 20 standard cycles (annealing at 55ºC, 1 min) and a final extension at 72ºC for 5 min.

PCR mixtures for 16S rRNA amplification contained 1-10 ng of template DNA, each deoxynucleoside triphosphate at a concentration of 200 μM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.3 μM, 2.5 U *Taq* DNA polymerase (Invitrogen) and PCR buffer supplied by the manufacturer. BSA (Bovine Serum Albumin) at a final concentration of 600 μg ml^{-1} was added to minimize the inhibitory effect of humic substances (Kreader, 1996). 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).

Primers amoA-1F and amoA-2R were used for amplification of ammonia oxidizers of the beta-subclass of *Proteobacteria* (Rotthauwe *et al.,* 1997). The PCR protocol included an initial denaturation step at 94ºC for 5 min, followed by 42 cycles of denaturation (at 94ºC for 60 s), annealing (at 60ºC for 90 s), and extension (at 72ºC for 90 s), and a final step consisting of 90 s at 60ºC and 10 min at 72ºC.

For detection of ammonia oxidizers of the gamma-subclass of *Proteobacteria*, primers amoA-3F and amoB-4R were utilized (Purkhold *et al.,* 2000). Thermal cycling was carried out by an initial denaturation step at 94ºC for 30 s, followed by 35 cycles of denaturation at 94ºC for 15 s, annealing at 48ºC for 20 s, and elongation at 72ºC for 40 s. Cycling was completed by a final elongation step at 72ºC for 10 min.

The presence of archaeal *amo*A fragments was checked by using the primers Arch-amoAF and Arch-amoAR (Francis *et al.,* 2005) with the following protocol: 95ºC for 4 min, 30 cycles consisting of 94ºC for 30 s, 56ºC for 30 s, and 72 ºC for 60s, and a final step of 72ºC for 10 min.

PCR mixtures for amplification of the *amoA* gene contained 1 μl of template DNA, each deoxynucleoside triphosphate at a concentration of 200 μM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.3 μM, 1.25 U *Taq* DNA polymerase (Promega) and PCR buffer supplied by the manufacturer. For archaeal *amo*A amplification, BSA at a final concentration of 150 μg ml^{-1} was added. The volume of reactions was 25 μl. PCR products were verified and quantified by agarose gel electrophoresis with a low DNA mass ladder standard (Invitrogen).

DGGE fingerprinting

DGGEs were run in a DCode system (Bio-Rad) as described by Muyzer *et al*. (1998). A 6% polyacrylamide gel with a gradient of 30-70% (*Bacteria*) or 40-80% (*Archaea*) DNA-denaturant agent was cast by mixing solutions of 0% and 80% denaturant agent (100% denaturant agent is 7 M urea and 40% deionized formamide). Seven hundred ng of PCR product were loaded for each sample and the gels were run at 100 V for 18 h at 60°C in 1xTAE buffer (40 mM Tris [pH 7.4], 20 mM sodium acetate, 1 mM EDTA). The gel was stained with SybrGold (Molecular Probes) for 45 min, rinsed with 1xTAE buffer, removed from the glass plate to a UV-transparent gel scoop, and visualized with UV in a Gel Doc EQ (Bio-Rad). Prominent bands were excised from the gels, resuspended in milli-q water overnight and reamplified for its sequencing.

Clone libraries

Bacterial 16S rRNA was amplified using universal primers 27F and 1492R (Lane, 1991). Reactions were carried out in an automated thermocycler (Biometra) with the following cycle: an initial denaturation step at 94ºC for 5 min, followed by 30 cycles of 1 min at 94ºC, 1 min at 55ºC and 2 min at 72ºC, and a final extension step of 10 min at 72ºC.

Primers 109F and 915R were used for Archaeal amplification (Großkopf *et al*., 1998). The cycle was as follows: 5 min at 94ºC, 38 cycles consisting of primer annealing at 52ºC for 1 min, DNA elongation at 72ºC for 90 s, and denaturation at 94ºC for 1 min, and a final cycle of 52ºC for 1 min and 72ºC for 6 min.

PCR mixtures contained 1-10 ng of template DNA, each deoxynucleoside triphosphate at a concentration of 200 μM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.3 μM, 2.5 U *Taq* DNA polymerase (Invitrogen) and PCR buffer supplied by the manufacturer.

PCR products were cloned with the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Putative colonies were picked, transferred to a multiwell plate containing Luria-Bertani medium and 7% glicerol, and stored at –80ºC for further amplification and sequencing.

rRNA sequencing

Purification of PCR products and sequencing reactions from DGGE bands and clones were performed by Macrogen (South Korea) with primers 907rM (DGGE), 27F (bacterial clone library) and 109F (archaeal clone library). Macrogen utilized the Big Dye Terminator version 3.1 sequencing kit and reactions were run in an automatic ABI 3730XL Analyzer-96 capillary

type. Gene sequences were deposited in Genbank under accession numbers FN597722-FN597999 and FN598017-FN598150.

Sequences were subjected to a BLAST search (Altschul *et al.*, 1997) to obtain an indication of the phylogenetic affiliation, and to the Bellerophon program (Huber *et al.*, 2004) to determine potential chimeric artifacts. Sequences sharing similarities over 98.5% were considered similar phylogenetic entities (OTU). The coverage of the clone libraries was calculated according to the following equation: C=1-(n/N), where *n* is the number of unique clones and *N* is the total number of clones examined. Seventeen sequences (Accession numbers: FN598000-FN598016), corresponding to three OTU from the Deinoccoccus-Thermus group (representative clones: BACDER07_1B5, BACDER07_1G6 and BACDER07_1F5) were fully sequenced using primers 27F and 1492R for probe design purposes.

Phylogenetic analyses

Partial 16S rDNA sequences from each OTU of the archaeal clone library were aligned by using MAFFT version 6 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) with a selection of euryarchaeal sequences from databases (including the closest matches obtained by BLAST search) and three crenarchaeal sequences as an outgroup. Very variable regions of the alignment were automatically removed with Gblocks (Castresana, 2000), using parameters optimized for rDNA alignments (minimum length of a block of 5; allowing gaps in half positions), leaving 695 informative positions. Maximum-likelihood analysis was carried out with PAUP 4.0b10 (Swofford, 2002), with the general time-reversible model assuming a discrete gamma distribution with six rate categories and a proportion of invariable sites. Parameters were estimated from an initial neighbor-joining tree. Bayesian analysis was carried out with MrBayes v3.0B (Huelsenbeck and Ronquist, 2001), using the same model described above but with four rate categories in the gamma distribution. Bayesian posterior probabilities were computed by running 2.000.000 generations by using the program default priors on model parameters. Trees were sampled every 100 generations. 3.000 trees were discarded as “burn-in” upon examination of the log likelihood curve of the sampled trees, so only the stationary phase was considered in the final tree. Neighbor-joining bootstrap values from 1000 replicates were calculated with PAUP following the same model used for the maximum-likelihood analysis.

In situ hybridization

For separation of the sludge flocs, the original sample was vortexed during 5 min and subsequently diluted, fixed with formaldehyde and filtered on a 0.2 μm pore-size

polycarbonate filter. CARD-FISH of prokayotic populations was carried out following the protocol described by Pernthaler *et al*. (2004). Several horseradish peroxidase probes were used to characterize the composition of the prokaryotic assemblage in activated sludge: CREN554 (Massana *et al*., 1997), EURY806 (Teira *et al*., 2004), EUB 338-II-III (Amann *et al*., 1990; Daims *et al*., 1999), ALF968 (Neef, 1997), GAM42a (Manz *et al*., 1992), CF319 (Manz *et al*., 1996), BET42a (Manz *et al*., 1992), DELTA495a (Loy *et al*., 2002), LGC354B (Meier *et al*., 1999) and HGC69a (Roller *et al*., 1994). The EUB antisense probe NON338 (Wallner *et al*., 1993) was used as a negative control. The probe DT01 (5'-ACCAAGCGCATCACACCG-3') targeting the clones BACDER07_1B5 and BACDER07_1G6 from the *Deinococcus-Thermus* phylum, was newly designed in this study by using the PROBE_DESIGN tool of the ARB software package (http://www.arb-home.de), and optimized following the protocol described in Pernthaler *et al*. (2001). This probe does not target the clone BACDER07_1F5.

FISH of nitrifying bacteria was carried out following the protocol detailed by Pernthaler *et al.* (2001). The 16S rRNA-targeted oligonucleotide probes used were: NEU, complementary to a signature region of most halophilic and halotolerant ammonia oxidizers, Nso190 and Nso1225, specific for ammonia oxidizers in the beta subclass of *Proteobacteria*, NIT3, complementary to a region of *Nitrobacter* species, and Nsv443, specific for the *Nitrosospira* cluster (Juretschko *et al*., 1998)

All probes were purchased from Thermo Fisher Scientific (Ulm, Germany). Filters were permeabilized with lysozyme (10 mg ml^{-1}, 37ºC, 1 h) and achromopeptidase (60 U ml^{-1}, 37ºC, 0.5 h) before hybridization. Hybridizations were carried out at 35ºC overnight and specific hybridization conditions were established by addition of formamide to the hybridization buffers (20% formamide for NON338 and EURY806 probes, 30% for Nsv443, 35% for Nso1225, 40% for NEU and NIT3, 45% for ALF968 and LGC354B, 50% for Delta495a and HGC69a, and 55% for the other probes). The optimal hybridization conditions (30% formamide) of the newly designed probe DT01 were experimentally determined. Counterstaining of CARD-FISH preparations was done with DAPI (1 μg ml^{-1}). Free cells and aggregates were counted separately in each field. Also, several transects were inspected and mean numbers of aggregates were calculated. Between 500 and 1000 free DAPI-positive cells were counted manually in a minimum of 10 fields, while several thousands of cells (between 4000 and 10000) were counted in aggregates.

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Figure legends

Fig. 1. Negative images of DGGE gels with PCR products amplified with bacterial and archaeal primer sets from samples of activated sludge corresponding to years 2007 and 2008. Bands excised and sequenced are numbered and their affiliations are shown in Table 1.

Fig. 2. Maximum-likelihood phylogenetic tree with partial 16S rDNA archaeal sequences (695 informative positions). Clones in bold are from this study. Posterior probabilty values and neigbor-joining bootstrap values (1000 replicates) are shown in the relevant nodes. The scale bar indicates 0.1 subtitutions per position.

Fig. 3. Proportions of bacterial groups detected by CARD-FISH with HRP probes in free cells and in aggregates from samples of activated sludge corresponding to years 2007 and 2008.

Fig. 4. Percentage of relative intensity of DGGE bands, proportions of clones (Library) and probe positive cells scaled to Eub probes (FISH) affiliated to different phylogenetic groups [*Alphaproteobacteria* (Alpha), *Betaproteobacteria* (Beta), *Gammaproteobacteria* (Gam), *Deltaproteobacteria* (Delta), *Bacteroidetes* (Bact), *Firmicutes* (Firm), *Actinobacteria* and *Deinococcus-Thermus* clone (DT)]. FISH data correspond to the mean of samples from years 2007 and 2008. The arrows with question marks indicate that an unknown proportion of phylogenetic groups cannot be retrieved by the set of primers used in both DGGE and clone libraries.

1

Table 1. Phylogenetic affiliation of sequences obtained from DGGE bands, with closest uncultured and cultured matches, and relative intensity of the bands

Band	Closest match	% similarity (nº bases)[a]	Taxonomic group	Accession nº (Gen Bank)	Cultured closest match (% similarity)	Relative intensity (%)
DER_1	Uncultured Bacteroidetes/Chlorobi group clone 3B02-03	92.1 (498)	Bacteroidetes	DQ431894	*Marinicola seohaensis* (84.9)	5.6
DER_2	Uncultured Bacteroidetes clone ML617.5J-33	93.3 (502)	Bacteroidetes	AF507866	*Owenweeksia hongkongensis* (86.5)	24.9
DER_3	Uncultured Bacteroidetes clone 02D2Z22	92.8 (482)	Bacteroidetes	DQ330313	*Owenweeksia hongkongensis* (88.8)	4.0
DER_4	*Vitellibacter* sp.	77.7 (383)	Bacteroidetes	EU642844	The same	0.6
DER_5	Clone nsmp VI41	97.2 (529)	γ-proteobacteria	AB212895	*Luteibactor rhizovicina* (85.8)	5.6
DER_6	Clone nsmp VI41	82.0 (437)	γ-proteobacteria	AB212895	*Aquimonas* sp. (75.7)	2.1
DER_7	Clone Strom2G11	87.2 (449)	α-proteobacteria	EU918039	*Parvibaculum* sp.	7.9
DER_8	*Nitratireductor* sp.	94.4 (487)	α-proteobacteria	EU564843	The same	7.9
DER_9	Clone 101-91	99.8 (539)	δ-proteobacteria	EF157196	*Desulfonatronum cooperativum* (84.1)	2.0
DER_10	Clone nsmo VI20	99.6 (541)	γ-proteobacteria	AB212894	*Frateuria aurantia* (94.1)	2.0
DER_11	Clone OTU_23	85.1 (430)	Deinococcus-Thermus	EU083501	*Truepera radiovictrix* (80.0)	1.5
DER_12	Clone OTU_23	96.1 (493)	Deinococcus-Thermus	EU083501	*Truepera radiovictrix* (89.3)	1.4
DER_13	Clone Hua6-s78	83.7 (385)	Euryarchaea	EU481593	*Methanobacterium aarhusense* (78.8)	11.8
DER_14	Clone ss037b	93.8 (379)	Euryarchaea	AJ969783	*Aciduliprofundum boonei* (84.2)	7.8
DER_15	Clone ss037b	94.4 (476)	Euryarchaea	AJ969783	*Methanobrevibacter* sp. (79.3)	1.3
DER_16	Clone ss037b	95.3 (487)	Euryarchaea	AJ969783	*Aciduliprofundum boonei* (79.1)	2.8
DER_17	Clone ss037b	96.3 (494)	Euryarchaea	AJ969783	*Methanobrevibacter* sp. (79.7)	26.7

[a] Number of bases used to calculate the levels of sequence similarity

2

3

1

Table 2. Phylogenetic affiliation of clones from the bacterial clone library to the closest match and to the closest cultured strain in GenBank

Representative clone	Closest match (Acc. number)	% similarity	Cultured closest match (Acc. number)	% similarity	Nº clones (%)
α-Proteobacteria					
BACDER07_1C3	Clone DR938CH110701SACH95 (DQ230971)	94.0	*Nitratireductor* sp. (EU564843)	93.4	1 (0.4)
BACDER07_1C12	Clone SC71 (EU735614)	97.8	*Brucella* sp. (DQ167235)	90.2	1 (0.4)
BACDER07_1F8	Clone 81 T12d-oil (FM242433)	95.7	*Subaequorebacter tamlense* (AM293856)	90.1	1 (0.4)
BACDER07_1H9	*Nitratireductor* sp. (EU564843)	94.2	The same	94.2	4 (1.4)
BACDER07_1D12	Clone B1-43 (AM229476)	98.2	*Sneathiella chinensis* (DQ219355)	95.6	1 (0.4)
BACDER07_2B7	Clone Cl75cm.2.18 (EF208711)	98.6	*Methylocystis* sp. (AJ868421)	94.7	2 (0.7)
BACDER07_2F8	Clone Strom2G11 (EU918039)	96.3	*Maricaulis* sp. (AJ301666)	90	2 (0.7)
BACDER07_2G10	Clone 256ds10 (AY212705)	98.0	*Sphingomonas* sp. (D16149)	97.1	1 (0.4)
δ-Proteobacteria					
BACDER07_1D11	Clone 101-91 (EF157196)	98.9	*Desulfonatronum cooperativum* (AY725424)	80.7	7 (2.5)
γ-Proteobacteria					
BACDER07_1B8	Clone nsmpVI41 (AB212895)	98.9	*Kangiella koreensis* (AY520560)	85.4	16 (5.8)
BACDER07_1D2	Clone nsmpVI20 (AB212894)	99.6	*Rhodanobacter lindaniclasticus* (DQ507211)	91.6	21 (7.6)
BACDER07_3B12	Clone nsmpVI20 (AB212894)	98.1	*Frateuria aurantia* (AB091195)	90.2	1 (0.4)
Deinococcus-Thermus					
BACDER07_1B5	Clone GZKB22 (AJ853517)	95.8	*Truepera radiovictrix* (DQ022077)	89.3	**212 (76.3)**
BACDER07_1G6	Clone GZKB22 (AJ853517)	94.5	*Truepera radiovictrix* (DQ022077)	89.8	2 (0.7)
BACDER07_1F5	Clone 6 (EU017377)	94.5	*Truepera radiovictrix* (DQ022077)	89.5	1 (0.4)
CFB Group					
BACDER07_1F4	Clone 6 (DQ015772)	96.3	*Lewinella marina* (AB301495)	84.7	1 (0.4)
BACDER07_2D6	Clone Er-LLAYS-51 (EU542514)	97.5	*Owenweeksia hongkongensis* (AB125062)	87.3	1 (0.4)
BACDER07_2H3	Clone HF500_26D14 (EU361310)	95.6	*Sphingobacterium* sp. (AM411964)	90.0	1 (0.4)
Firmicutes					
BACDER07_2F2	Clone p816_b_3.45 (AB305600)	81.7	*Bacillus* sp. (EF422410)	79.0	1 (0.4)
Unclassified bacteria					
BACDER07_3D4	*Denitromonas indolicum* (AY972852)	95.2	The same	95.2	1 (0.4)

2

3

Table 3. Phylogenetic affiliation of clones from the archaeal clone library to the closest match and to the close cultured strain in GenBank

Representative clone	Closest match (Acc. number)	% similarity	Cultured closest match (Acc. number)	% similarity	N° clones (%)
Methanosarcinales					
ARCHDER07_1A12	*Methanococcoides* sp. (Y16946)	99.5	The same	99.5	5 (4.3)
ARCHDER07_1C3	Clone Z3-Arc-1 (EU999009)	98.6	*Methanolobus profundi* (AB370245)	97.8	2 (1.7)
ARCHDER07_1D4	Clone TFC20L31Ar (EU362350)	97.0	*Methanosaeta harundinacea* (AY970347)	96.2	2 (1.7)
Methanomicrobiales					
ARCHDER07_2C9	Clone WIP (EF420166)	98.5	*Methanoculleus marisnigri* (CP000562)	97.5	2 (1.7)
ARCHDER07_1B2	Clone GoM-GC234-015R (AY211693)	96.1	*Methanoculleus sp.* (AJ133793)	93.8	6 (5.1)
ARCHDER07_2D6	Clone PMMV-Arc14 (AJ937680)	92.2	*Methanoculleus sp.* (AJ133793*)*	89.8	1 (0.9)
Methanobacteriales					
ARCHDER07_1A2	Clone 4B09 (AY835426)	94.7	*Methanothermus fervidus* (M32222)	81.8	8 (6.8)
ARCHDER07_1B11	Clone ALAS95 (EU616776)	99.2	*Methanobacterium aarhusense* (DQ649334)	84.4	1 (0.9)
DER_1					
ARCHDER07_1D11	Clone ss037b (AJ969783)	97.1	*Methanomethylovorans sp.* (EU544305)	78.0	5 (4.3)
ARCHDER07_1G10	Clone HARR41 (AJ699117)	99.5	*Aciduliprofundum boonei* (DQ451875)	81.2	2 (1.7)
DER_2					
ARCHDER07_2C8	Clone GNA03E09 (EU731492)	94.3	*Methanobrevibacter gottschalkii* (U55239)	70.8	1 (0.9)
ARCHDER07_1C11	Clone MOB4-5 (DQ841225)	91.7	*Methanobrevibacter gottschalkii* (U55239)	70.1	1 (0.9)
ARCHDER07_1E6	Clone GNA03E09 (EU731492)	96.0	*Methanobacterium sp.* (DQ517520)	71.0	3 (2.6)
ARCHDER07_1A5	Clone GNA03E09 (EU731492)	98.2	*Methanococcus infernus* (AF025822)	72.8	**47 (40.2)**
ARCHDER07_1A10	Clone GNA03G10 (EU731491)	94.9	*Methanococcus vulcanus (AF051404)*	71.9	1 (0.9)
ARCHDER07_2D4	Clone CaR3b.h02 (EU244267)	90.1	*Methanobrevibacter sp.* (AJ550156)	70.8	2 (1.7)
ARCHDER07_2A4	Clone CaS1s.h02 (EF014578)	88.9	*Methanococcus infernus* (AF025822)	72.5	1 (0.9)
ARCHDER07_1A4	Clone A21 (EU328111)	88.6	*Methanococcus aeolicus* (CP000743)	72.8	12 (10.3)
ARCHDER07_1D1	Clone KAB187-14 (AB366595)	89.8	*Methanobacterium sp.* (EU366499)	73.8	1 (0.9)
DER_3					
ARCHDER07_1B4	Clone 1ACC-29 (AB175599)	97.2	*Methanothermococcus sp.* (AB175514)	78.0	9 (1.7)
ARCHDER07_1G6	Clone GNA03F04 (EU731409)	95.1	*Methanocaldococcus jannaschii* (L77117)	78.8	1 (0.9)
ARCHDER07_1D10	Clone GNA02E03 (EU731293)	97.4	*Methanococcus aeolicus* (CP000743)	78.0	2 (1.7)
ARCHDER07_2B5	Clone GNA01D07 (EU731138)	91.6	*Methanothermococcus sp.* (AB260046)	77.3	1 (0.9)
ARCHDER07_1A1	Clone ML23_ANME 9 (AY245465)	83.2	*Methanocaldoccus indiensis* (AF547621)	76.1	1 (0.9)

Fig. 1

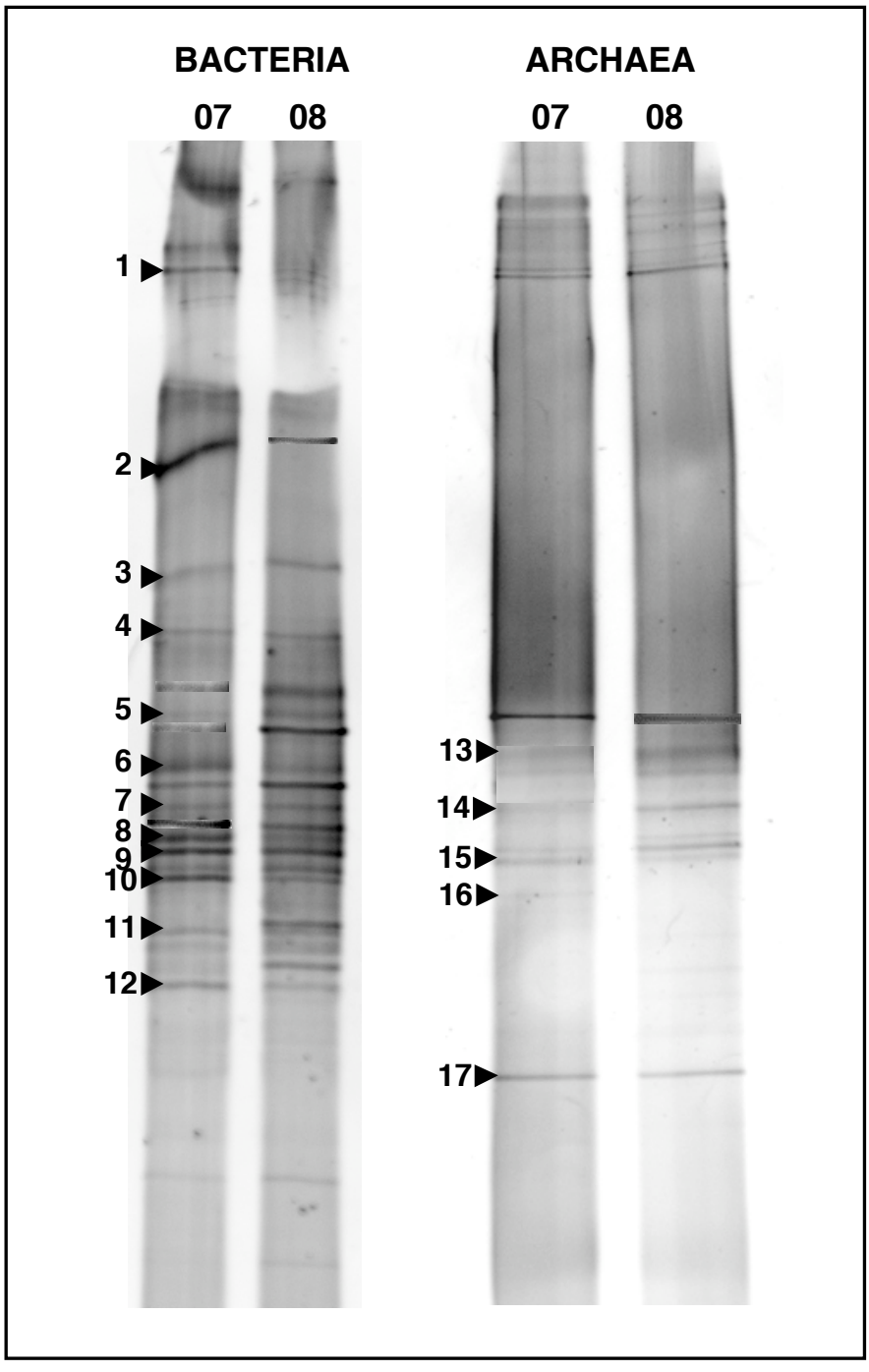
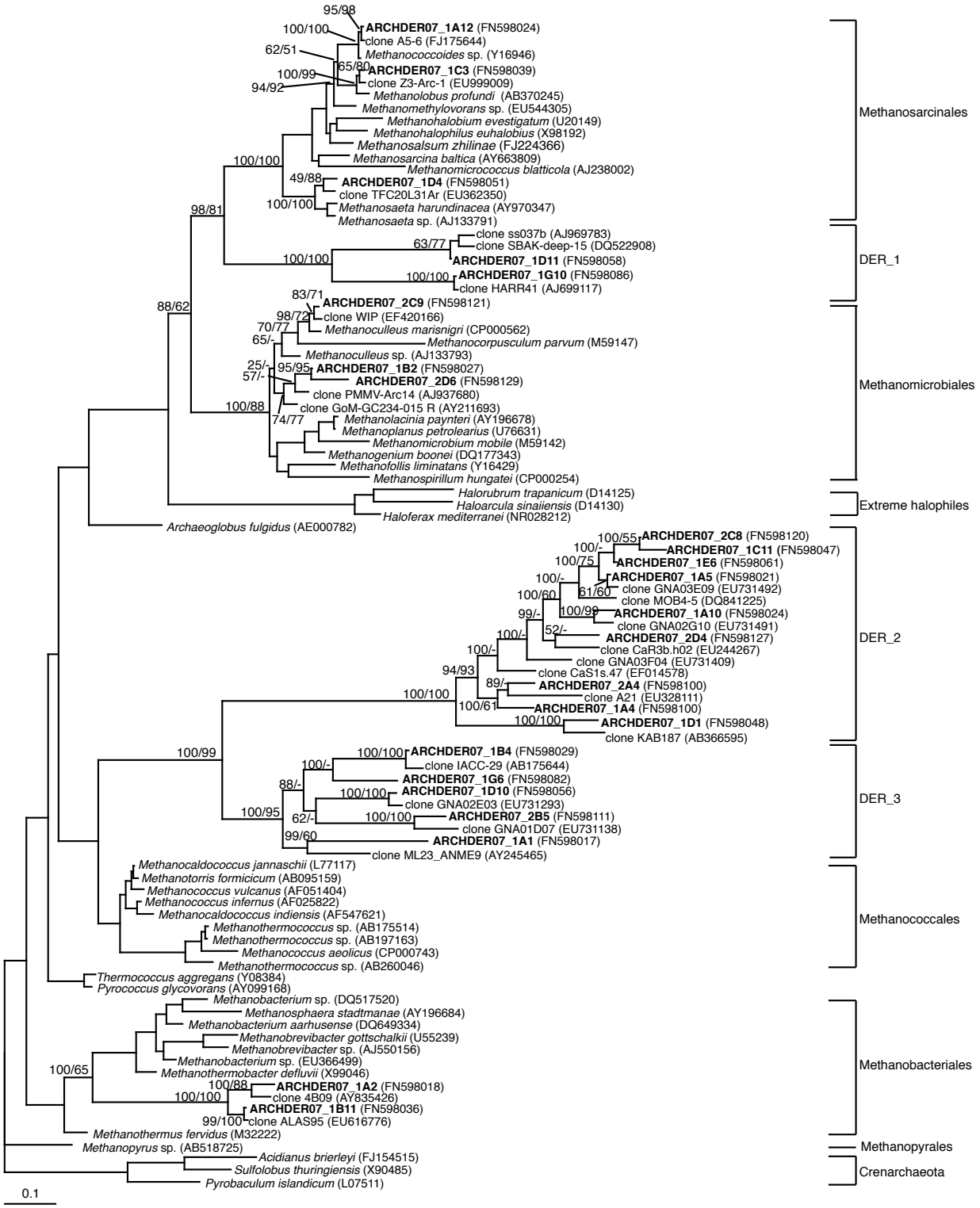


Fig. 2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Methanomethylovorans sp. (EU544305)
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Methanohalophilus euhalobius (X98192)
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Methanosaeta harundinacea (AY970347)
Methanosaeta sp. (AJ133791)
clone ss037b (AJ969783)
clone SBAK-deep-15 (DQ522908)
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clone HARR41 (AJ699117)
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clone WIP (EF420166)
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ARCHDER07_2D6 (FN598129)
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clone GoM-GC234-015 R (AY211693)
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Methanomicrobium mobile (M59142)
Methanogenium boonei (DQ177343)
Methanofollis liminatans (Y16429)
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Halorubrum trapanicum (D14125)
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Methanothermococcus sp. (AB197163)
Methanococcus aeolicus (CP000743)
Methanothermococcus sp. (AB260046)
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Pyrococcus glycovorans (AY099168)
Methanobacterium sp. (DQ517520)
Methanosphaera stadtmanae (AY196684)
Methanobacterium aarhusense (DQ649334)
Methanobrevibacter gottschalkii (U55239)
Methanobrevibacter sp. (AJ550156)
Methanobacterium sp. (EU366499)
Methanothermobacter defluvii (X99046)
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ARCHDER07_1B11 (FN598036)
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Methanopyrus sp. (AB518725)
Acidianus brierleyi (FJ154515)
Sulfolobus thuringiensis (X90485)
Pyrobaculum islandicum (L07511)
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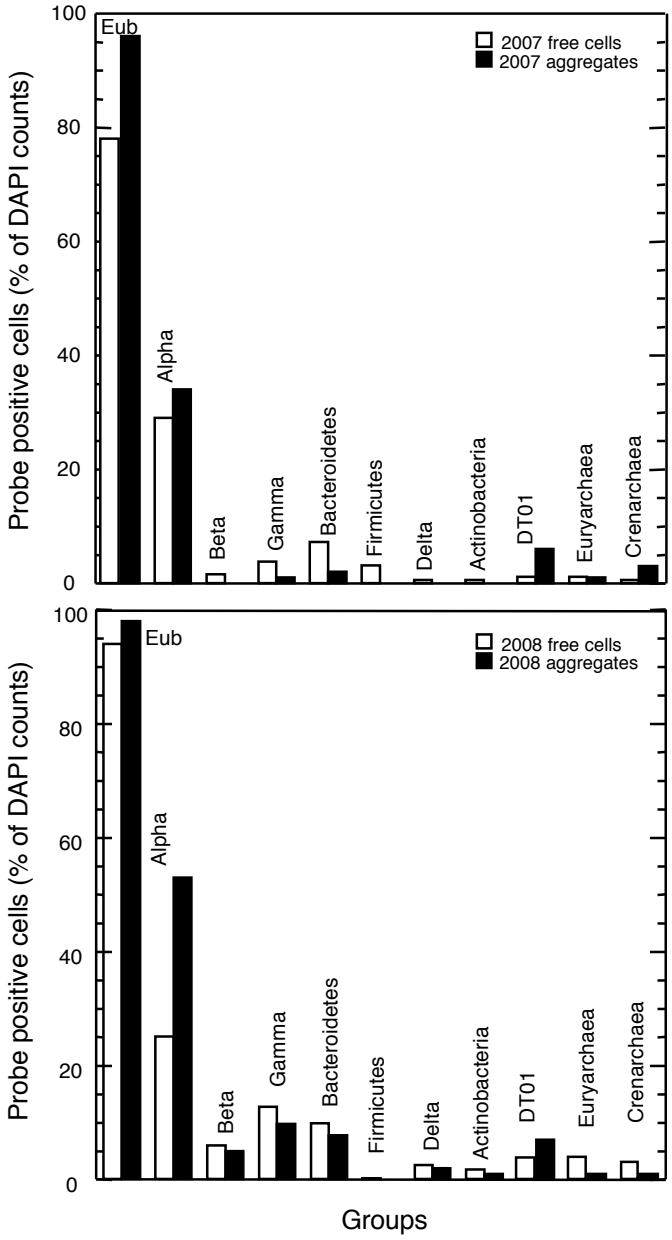
DER_1
Methanomicrobiales
Extreme halophiles
DER_2
DER_3
Methanococcales
Methanobacteriales
Methanopyrales
Crenarchaeota

Fig. 3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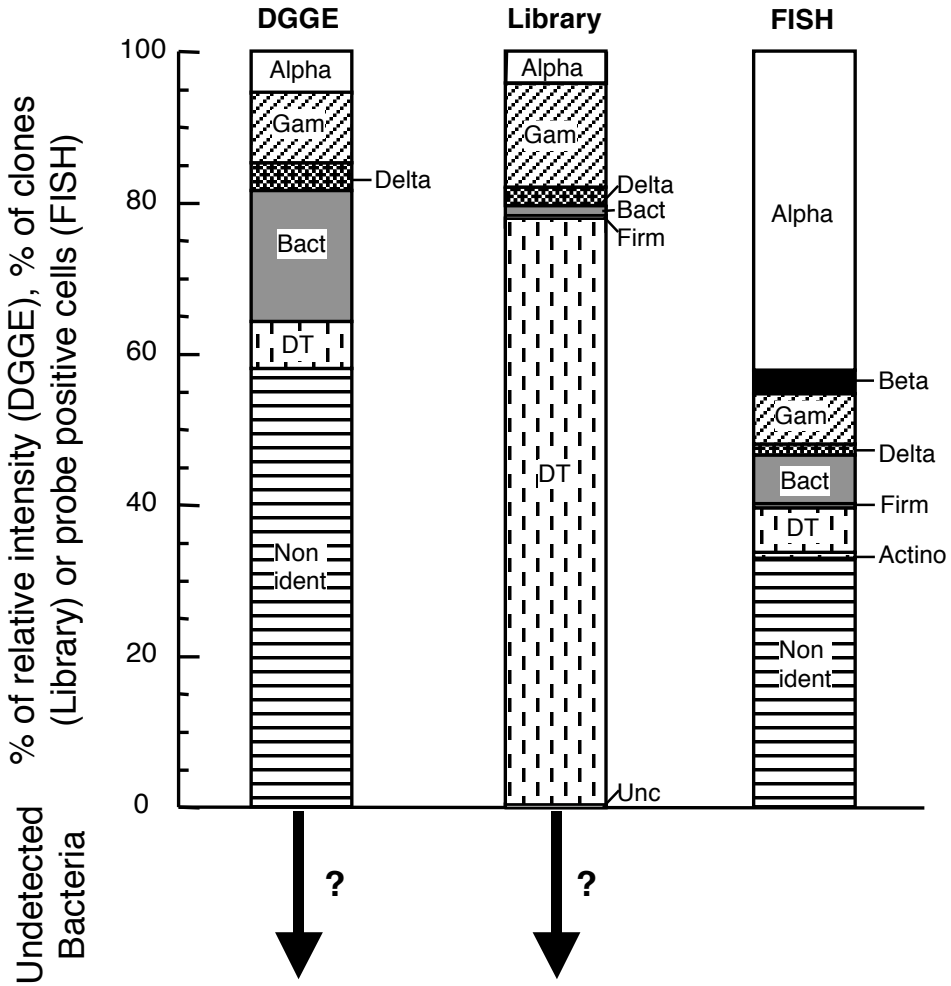
100
80
60
40
20
0
Eub
Alpha
Beta
Gamma
Bacteroidetes
Firmicutes
Delta
Actinobacteria
DT01
Euryarchaea
Crenarchaea
2007 free cells
2007 aggregates
2008 free cells
2008 aggregates
Groups

Fig. 4


DGGE
Library
FISH
% of relative intensity (DGGE), % of clones (Library) or probe positive cells (FISH)
100
80
60
40
20
0
Alpha
Gam
Delta
Bact
DT
Non ident
Unc
Beta
Firm
Actino
Undetected Bacteria
?
?