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Novel predominant archaeal and bacterial groups revealed by molecular analysis of an anaerobic sludge digester

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Summary

A culture-independent molecular phylogenetic approach was used to study prokaryotic diversity in an anaerobic sludge digester. Two 16S rRNA gene libraries were constructed using total genomic DNA, and amplified by polymerase chain reaction (PCR) using primers specific for archaeal or bacterial domains. Phylogenetic analysis of 246 and 579 almost full-length 16S rRNA genes for *Archaea* and *Bacteria*, respectively, was performed using the ARB software package. Phylogenetic groups affiliated with the *Archaea* belong to *Euryarchaeota* and *Crenarchaeota*. Interestingly, we detected a novel monophyletic group of 164 clones representing 66.6% of the archaeal library. Culture enrichment and probe hybridization show that this group grows better under formate or H_2–CO_2. Within the bacterial library 95.6% of the operational taxonomic units (OTUs) represent novel putative phylotypes never described before, and affiliated with eight divisions. The *Bacteroidetes* phylum is the most abundant and diversified phylogenetic group representing 38.8% of the OTUs, followed by the Gram-positives (27.7%) and the *Proteobacteria* (21.3%). Sequences affiliated with phylogenetic divisions represented by few cultivated representatives such as the *Chloroflexi*, *Synergistes*, *Thermotogales* or candidate divisions such as OP9 and OP8 are represented by <5% of the total OTUs. A comprehensive set of 15 16S and 23S rRNA-targeted oligonucleotide hybridization probes was used to quantify these major groups by dot blot hybridization within 12 digester samples. In contrast to the clone library, *Firmicutes* and *Actinobacteria* together accounted for 21.8 ± 14.9% representing the most abundant phyla. They were surprisingly followed by the *Chloroflexi* representing 20.2 ± 4.6% of the total 16S rRNA. The *Proteobacteria* and the *Bacteroidetes* group accounted for 14.4 ± 4.9% and 14.5 ± 4.3%, respectively, WWE1, a novel lineage, accounted for 11.9 ± 3.1% while *Planctomycetes* and *Synergistes* represented <2% each. Using the novel set of probes we extended the coverage of bacterial populations from 52% to 85.3% of the total rRNA within the digester samples.

Introduction

Anaerobic digestion is a process of microbial decomposition in which, under anaerobic conditions, a community of microorganisms convert organic matter into methane, carbon dioxide, inorganic nutrients and humus. During this microbial process known as biomethanogenesis, microorganisms, including protozoa, fungi and bacteria, decompose organic matter using carbon dioxide and the methyl group of acetate as electron acceptors in the absence of dioxygen or other compounds. This microbial activity is responsible for carbon recycling in anaerobic environments, including wetlands, rice fields, intestines of animals, aquatic sediments and manure. The overall process involves a consortium of different species of microorganisms, which decompose organic matter in a series of steps that ultimately produce methane and carbon dioxide as terminal products (Chynoweth *et al*., 1991). Methane is formed from two primary substrates, acetate and hydrogen/carbon dioxide (or formate). In the absence of methanogens to utilize these substrates, both hydrogen and acetic acid build up back-up for the reactions. Organic acids accumulate causing a decrease in pH, which ultimately inhibits and stops the fermentation. The overall role of biomethanogenesis in the biosphere is to complete the degradation process by removal of inhibitory fermentation products.

In the last decade, several microorganisms have been isolated from granular sludge and characterized (Archer

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and Kirsop, 1990; Wu *et al*., 1992; Harmsen *et al*., 1996). However, cultivation-dependent methods have limitations in elucidating diversity of the complex microbial ecosystems, and thus many of the component species may remain to be identified (Goebel and Stackebrandt, 1994; Suau *et al*., 1999; Juretschko *et al*., 2002). Knowledge of the microbial diversity of wastewater microbial communities has been greatly extended recently by molecular studies based on the analysis of 16S rRNA gene sequences. Numerous novel 16S rRNA gene sequences were retrieved from both municipal or industrial wastewater treatment plants (WWTP). They indicate that the vast majority of species found in these communities have not been cultivated yet (Godon *et al*., 1997; Snaidr *et al*., 1997; Crocetti *et al*., 2000; Dabert *et al*., 2001; Daims *et al*., 2001; Juretschko *et al*., 2002). While microbial diversity can be readily studied by polymerase chain reaction (PCR)-based 16S rRNA gene cloning, community structure cannot be deduced from cloning studies because of potential biases introduced during DNA retrieval and amplification (Reysenbach *et al*., 1992; Farrelly *et al*., 1995; Suzuki and Giovannoni, 1996). For reliable characterization of community structure, qualitative or quantitative methods such as fluorescent *in situ* hybridization (FISH) or dot blot hybridization (DBH) are more suitable (Amann *et al*., 1995). To date, a number of studies have been performed using one of these two methods to quantify different groups in WWTP. Most of these studies, however, focused on specific microbial groups within the bacterial or archaeal domains (Raskin *et al*., 1994; De los Reyes *et al*., 1997, 1998; Snaidr *et al*., 1997; Juretschko *et al*., 2002).

In the present work we describe archaeal and bacterial molecular diversity of an anaerobic sludge digester, revealing the occurrence of novel hitherto non-described phylogenetic groups and phylotypes. Second, we investigate the contribution of the bacterial and archaeal populations to the total microbial community through the development of three novel probes targeting major bacterial and archaeal populations and application of a set of 12 16S–23S rRNA probes using DBH to quantify the targeted populations within the digester.

Results

Overall molecular diversity of the digester microflora

The present molecular analysis shows that the anaerobic digester ecosystem is composed of at least 226 different organisms belonging to the *Archaea* and *Bacteria* domains. Only a small fraction of 16S rRNA gene sequences represented by 16 operational taxonomic units (OTUs) (7% of the total OTUs) was affiliated with already existing sequences in public databases with ≥97% sequence similarity. The remaining 16S rRNA gene sequences were remotely related to 16S rRNA gene sequences of known bacterial or archaeal species, and represent novel phylotypes not described in previous analyses.

Microorganisms belonging to the three domains *Bacteria*, *Eukarya* and *Archaea* were quantified using domain-specific probes (Table 1). Bacterial rRNA quantified using EUB338, EUBI and EUBII probes, represents 77.2 ± 12.4% of the total rRNA hybridized to the universal probe. Archaeal and eukaryal rRNA quantified using the Arch915 and EUK502 probes represent 7.1 ± 4.4% and ≤1% respectively (data not shown).

Archaeal diversity analysis

The *Archaea* domain encompasses at least 20 OTUs among which 65% represent novel phylotypes. Phylogenetic analysis indicates that *Archaea*-affiliated sequences belong to the *Crenarchaeota* represented by only 39 sequences (6 OTUs) and the *Euryarchaeota* represented by 207 sequences (14 OTUs), which accounted for 80% of the *Archaea* clone library (Table 2). Among the *Euryarchaeota* we detected 16S rRNA gene sequences affiliated with the *Methanomicrobiales* (7 OTUs, 39 sequences), *Methanosarcinales* (2 OTUs, 4 sequences) and a novel archaeal lineage we called Arc I, represented by only 5 OTUs but 164 sequences making up to 70% of the retrieved archaeal rRNA gene sequences of the *Archaea* clone library. This group of sequences was closely related to sequences retrieved from an oil-contaminated aquifer in the United States (AF050615) (Dojka *et al*., 1998), or from an oil-contaminated groundwater in Japan (AB077224) (Watanabe *et al*., 2002). Sequence similarity with these closely related sequences ranges between 93.5% and 97.8% (data not shown). In the phylogenetic analysis, we used a comprehensive number of cultivated and non-cultivated archaeal 16S rRNA gene sequences and the three treeing methods: Maximum parsimony (MP), maximum likelihood (ML) and Neighbour-joining (NJ) as indicated in *Experimental procedures*. Results indicate that the novel archeal cluster is clearly monophyletic and branches distinctly from the *Methanobacteriales*, *Methanomicrobiales* or *Methanosarcinales* (Fig. 1A). We show that the affiliated sequences from the reports by Dojka and colleagues (1998) and Watanabe and colleagues (2002), along with our 16S rRNA gene sequences cluster in a monophyletic lineage with a bootstrap value of 100%. Thus this group of sequences appears clearly distinguishable and forms a completely independent lineage (Fig. 1A). The bootstrap support for the association of Arc I with the *Methanobacteriales* and *Methanosarcinales* is low (75%). The branching order of this cluster of 16S rRNA gene sequences changes if a

Table 1. Oligonucleotide probes and primers used in this study.

Probe	Specificity	Sequence (5′-3′)	Target RNA (nucleotide range)	Position[a]	Dot blot T_d (°C)	Reference
UNI1390	universal	GACGGGCGGTGTGTACAA	16S, 18S	1390–1407	44	Zheng *et al.* (1996)
ARCH915	*Archaea*	GTGCTCCCCCGCCAATTCCT	16S	0915–0935	56	Amann *et al.* (1995)
ARCH21F	*Archaea*	TTCCGGTTGATCCYGCCGGA	16S	0007–0026	ND	DeLong (1992)
EUK502	*Eukarya*	ACCAGACTTGCCCTC	18S	0502–0517	52	Lim *et al.* (1993)
EUB338	*Bacteria*	GCTGCCTCCCGTAGGAGT	16S	0338–0355	54	Amann *et al.* (1995)
EUB338II	*Planctomycetales*	GCAGCCACCCGTAGGTGT	16S	0338–0355	54	Daims *et al.* (2001)
EUB338III	*Verrucomicrobiales*	GTCGCCACCCGTAGGTGT	16S	0338–0355	54	Daims *et al.* (2001)
Bacteria	*Bacteria*	CAGGCCTAACACATGCAAGTC	16S	0043–0063	ND	Marchesi *et al.* (1998)
ALPH19	α- and δ-*Proteobacteria* some Spirochaetes	CGTTCGYTCTGAGCCAG	16S	0019–0035	55	Manz *et al.* (1992)
GP1199	Most Gram-positives	AAGGGGCATGATG	16S	1199–1211	34	MacGregor *et al.* (2001)
GAM42a	γ-*Proteobacteria*	GCCTTCCCACATCGTTT	23S	1027–1043	60	Manz *et al.* (1992)
BET42a	β-*Proteobacteria*	GCCTTCCCACTTCGTTT	23S	1027–1043	58	Manz *et al.* (1992)
PLA46R	*Planctomycetales*	GACTTGCATGCCTAATCC	16S	0046–0063	55[b]	Neef *et al.* (1998)
CF319a	*Cytophaga-Flavobacterium*	TGGTCCGTGTCTCAGTAC	16S	0319–0336	56	Manz *et al.* (1996)
Bacto1080	*Bacteroides* cluster	GCACUUAAGCCGACACCU	18S	1080–1097	46	Doré *et al.* (1998)
Arc864	Novel lineage Arc I	CCCTACAGCACAGGGCCA	16S	0846–0864	ND	This study
Syn773	Synergistes	CTAGCTTTCGCACATGAG	16S	0756–0773	48	This study
WWE1	Candidate phylum WWE1	CTTCCTCTGCGTTGTTAC	16S	1164–1181	50	Chouari *et al.* (2005)
GNSB1126	*Chlofoflexi*	AACACACAGCGAGGG	16S	1112–1126	44	This study

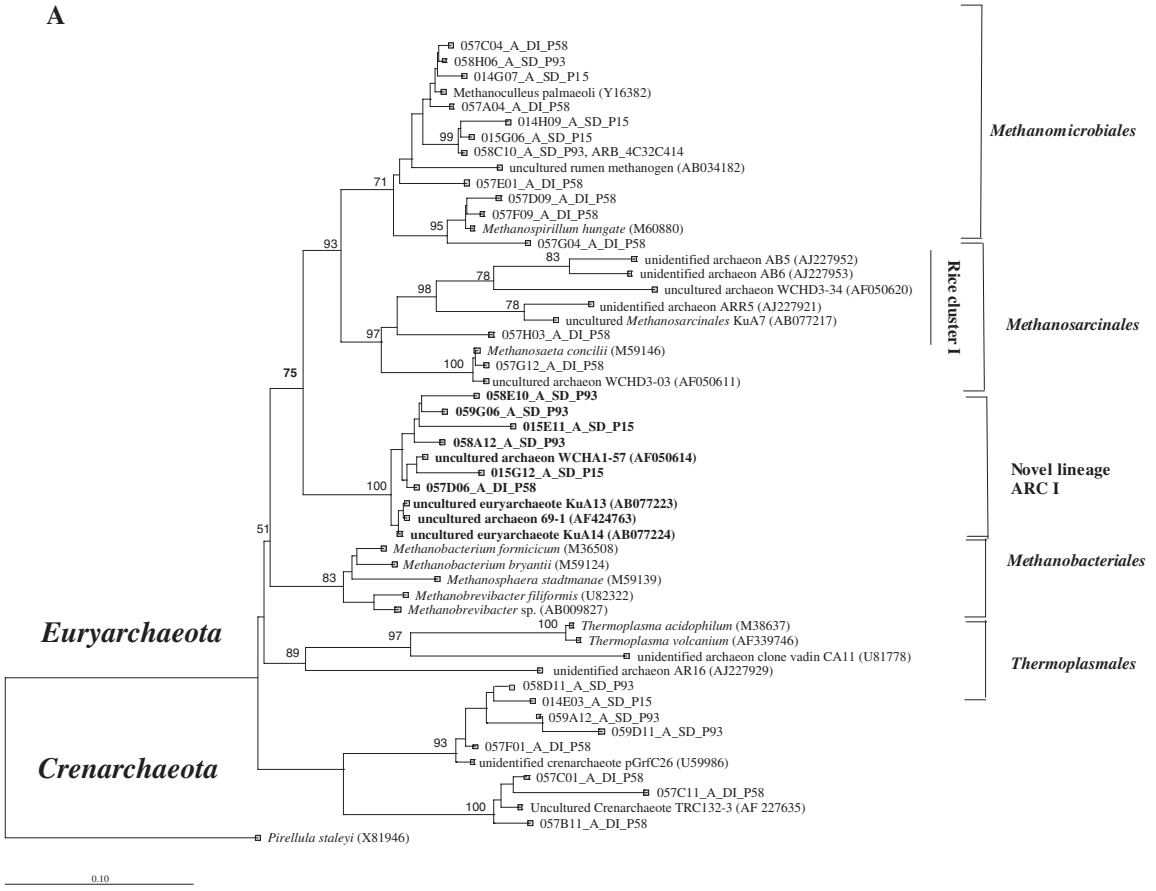
a. Position in the 16S or 23S rRNA of *E. coli* (Brosius *et al.*, 1978).
b. Determined in our study.
ND, not determined.

Table 2. Cultivated and non-cultivated phylotypes within the *Archaea* domain.

	Euryarchaeota				
	Methanosarcinales	*Methanomicrobiales*	Novel lineage (Arc I)	*Crenarchaeotes*	Total
No. of OTUs	2	7	5	6	20
% of OTUs	10	35	25	30	100
No. of clones	4	39	164	39	246
% of clones	1	15.8	66.6	15.8	100
OTUs ≥97% cultivated	0	3	0	0	3
% of OTUs	0	15	0	0	15
OTUs ≥97% not yet cultivated	1	0	1	2	4
% OTUs	5	0	5	10	20
Novel OTUs	1	4	4	4	13
% of novel OTUs	5	20	20	20	65

Coverage estimates: 92% calculation using the formula $[1 - (n/N)] \times 100$ (Good, 1953).

Fig. 1. Evolutionary distance dendrogram constructed using the NJ method and showing the affiliation of the environmental 16S rRNA gene sequences recovered from the anaerobic digester. Trees were calculated using the ARB software package, as described in *Experimental procedures*.
A. A neighbour-joining tree of the archaeal 16S rRNA gene sequences showing the position of the novel phylogenetic group among the *Euryarchaeota*. The number at branch nodes indicates bootstrap values (per 100 trials). The scale bar corresponds to a 10% estimated difference in nucleotide sequence positions. *Pirellula staleyi* (X81946) was used as an outgroup.
B. A neighbour-joining tree of the *Bacteria* 16S rRNA gene sequences showing the affiliation of the different OTUs retrieved in this study. The number of OTUs is indicated on the branches, the total number of clones is indicated in brackets. The scale bar corresponds to a 10% estimated difference in nucleotide sequence positions. *Methanobacterium formicicum* (M36508) was used as an outgroup.


A
057C04_A_DI_P58
058H06_A_SD_P93
014G07_A_SD_P15
Methanoculleus palmaeoli (Y16382)
057A04_A_DI_P58
014H09_A_SD_P15
015G06_A_SD_P15
058C10_A_SD_P93, ARB_4C32C414
uncultured rumen methanogen (AB034182)
057E01_A_DI_P58
057D09_A_DI_P58
057F09_A_DI_P58
Methanospirillum hungate (M60880)
057G04_A_DI_P58
unidentified archaeon AB5 (AJ227952)
unidentified archaeon AB6 (AJ227953)
uncultured archaeon WCHD3-34 (AF050620)
unidentified archaeon ARR5 (AJ227921)
uncultured Methanosarcinales KuA7 (AB077217)
057H03_A_DI_P58
Methanosaeta concilii (M59146)
057G12_A_DI_P58
uncultured archaeon WCHD3-03 (AF050611)
058E10_A_SD_P93
059G06_A_SD_P93
015E11_A_SD_P15
058A12_A_SD_P93
uncultured archaeon WCHA1-57 (AF050614)
015G12_A_SD_P15
057D06_A_DI_P58
uncultured euryarchaeote KuA13 (AB077223)
uncultured archaeon 69-1 (AF424763)
uncultured euryarchaeote KuA14 (AB077224)
Methanobacterium formicicum (M36508)
Methanobacterium bryantii (M59124)
Methanosphaera stadtmanae (M59139)
Methanobrevibacter filiformis (U82322)
Methanobrevibacter sp. (AB009827)
Thermoplasma acidophilum (M38637)
Thermoplasma volcanium (AF339746)
unidentified archaeon clone vadin CA11 (U81778)
unidentified archaeon AR16 (AJ227929)
058D11_A_SD_P93
014E03_A_SD_P15
059A12_A_SD_P93
059D11_A_SD_P93
057F01_A_DI_P58
unidentified crenarchaeote pGrfC26 (U59986)
057C01_A_DI_P58
057C11_A_DI_P58
Uncultured Crenarchaeote TRC132-3 (AF 227635)
057B11_A_DI_P58
Pirellula staleyi (X81946)
0.10
Euryarchaeota
Crenarchaeota
Methanomicrobiales
Rice cluster I
Methanosarcinales
Novel lineage ARC I
Methanobacteriales
Thermoplasmales
B
12 (19)
beta-proteobacteria
2 (2)
gamma-proteobacteria
10 (12)
alpha-proteobacteria
19 (89)
delta-proteobacteria
Proteobacteria
unidentified proteobacterium (U70713)
053A03_B_DI_P58
Novel lineage
15 (112)
group I
053G06_B_DI_P58
012G06_B_SD_P15
3 (5)
group II
4 (5)
group III
22 (44)
group IV
Bacteroides
28 (133)
group V
Cytophaga-Flavobacter
3 (3)
group VI
3 (11)
Rhodothermu-group
Bacteroidetes
7 (33)
Chloroflexi
3 (4)
Actinomycetes
54 (63)
Firmicutes
9 (18)
Synergistes
3 (4)
OP9
1 (3)
OP8
5 (16)
Thermotogales
Methanobacterium formidcum (M36508)
0.10

multiple data set is used in the analysis, or when ML, MP or NJ methods were used as treeing algorithms. This cluster may constitute a novel coherent clade within the phylogenetic radiation represented by members of the three orders *Methanobacteriales*, *Methanosarcinales* and *Methanomicrobiales* (Fig. 1A).

We developed a new 16S rRNA oligonucleotide probe S-*-ArcI-0864-a-A-18 targeting this group of sequences (Table 1) and attempted enrichment cultures of members of this group using modified Balch medium as described in *Experimental procedures*. *In situ* localization of members of this group using a CY3-labelled probe within culture enrichment samples shows that members of the novel predominant group grow using formate or H_2–CO_2 (Fig. 2). They may represent different species or genera showing some metabolic versatility. More experiments are being conducted in this context to isolate members of this group.

Bacterial diversity analysis

The *Bacteria* domain encompassed at least 206 phylotypes, of which 96% represent novel putative species dis-

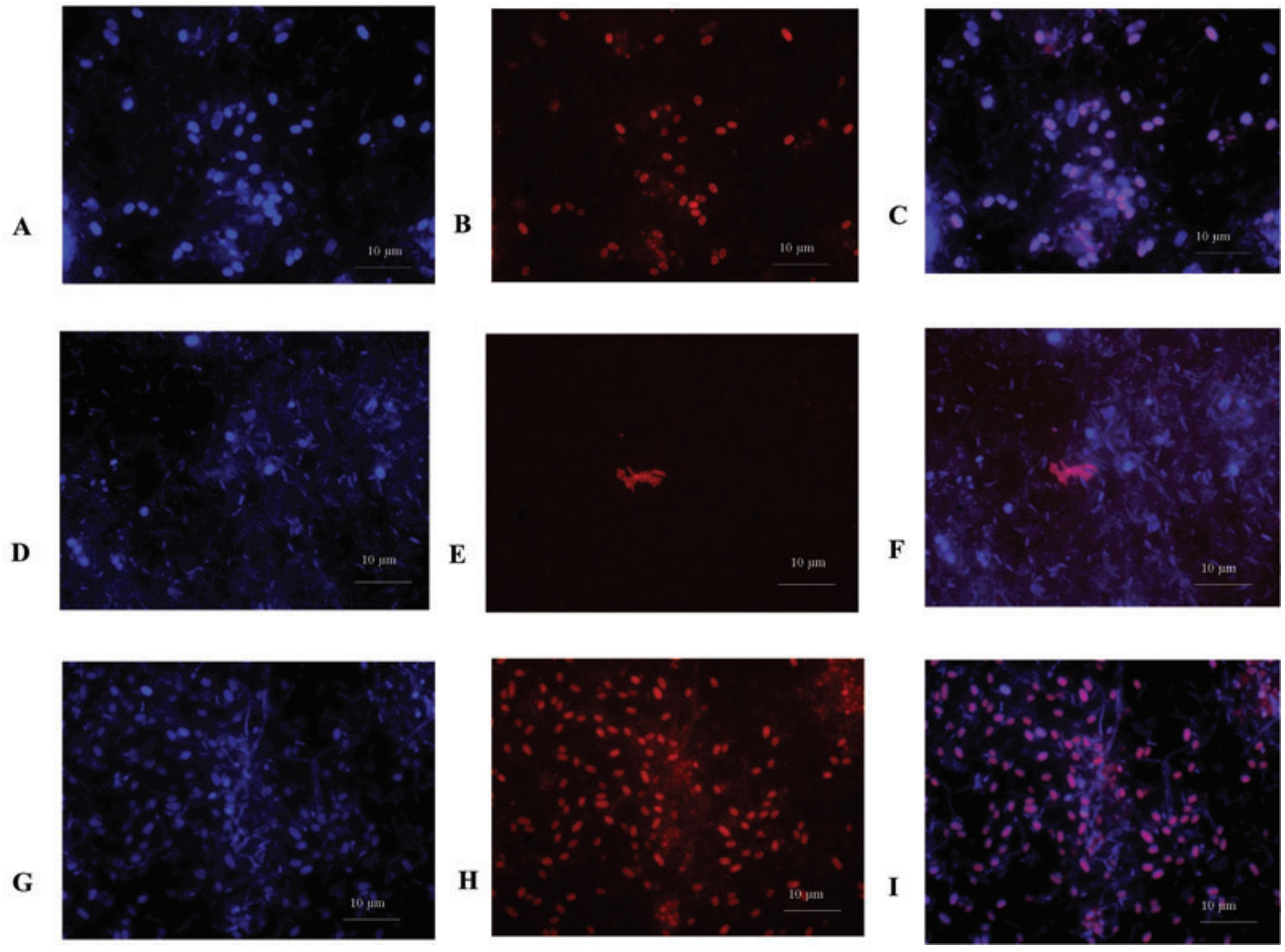


Fig. 2. *In situ* identification of the novel archaeal group in enrichment cultures of a digester sample using Balch medium and formate, methanol and H_2–CO_2, respectively, as energy and carbon sources. Microorganisms were identified using a CY3-labelled probe, S-*-ArcI-0864-a-A-18. Images were obtained using an axioplane epifluorescence microscope.
A. DAPI staining of microorganisms in enrichment culture under N_2–CO_2 (80:20, v/v) and formate.
B. Hybridization using probe S-*-ArcI-0864-a-A-CY3.
C. Superposition of pictures A and B.
D. DAPI staining of microorganisms in enrichment culture under N_2–CO_2 (80:20, v/v) and methanol.
E. Hybridization using probe S-*-ArcI-0864-a-A-CY3.
F. Superposition of pictures D and E.
G. DAPI staining of microorganisms in enrichment culture under H_2–CO_2 (80:20, v/v).
H. Hybridization using probe S-*-ArcI-0864-a-A-CY3.
I. Superposition of pictures G and H.

tributed among at least eight phylogenetic divisions (Table 3). Operational taxonomic unit distribution within the bacterial library was the following: *Bacteroidetes* (38.8%), *Firmicutes* and *Actinobacteria* (27.7%), and the *Proteobacteria* (21.3%). The remaining phylogenetic groups such as the *Chloroflexi*, *Synergistes*, *Thermotogales*, OP8 and OP9 (Dojka *et al*., 1998) made up a total of 12.3% of the OTUs within the clone library. Only five OTUs were identified as cultured species, and four OTUs are affiliated with not yet cultivated microorganisms in public databases with ≥97% similarity. The remaining 197 OTUs (95.6%) show <97% sequence similarity with the closely related bacterial 16S rRNA gene sequences (Table 3). In many cases they form novel phylogenetic groups clearly separated from the other known bacterial clusters (Fig. 1B).

We detected one deeply branching OTU affiliated with the *Proteobacteria*. This OTU is closely related to a sequence, OM27 (U70713), retrieved from a marine coastal picoplankton (Rappé *et al*., 1997). Both sequences are related to the *Proteobacteria* but do not affiliate with any of its major subclasses. Twelve more closely related OTUs were retrieved from the analysis of an aerobic and anoxic basin of the same WWTP (data not shown). They may constitute a novel subclass within the *Proteobacteria* (R. Chouari, D. Le Paslier, P. Daegelen, P. Ginestet, J. Weissenbach and A. Sghir, in preparation).

Bacterial diversity at the phylogenetic group level (Table 3 and Fig. 1B)

The *Bacteroidetes* phylum represents the predominant group of 16S rRNA gene sequences of our clone library and accounted for 38.8% of the total OTUs in the library. All of the *Bacteroidetes* OTUs represents novel OTUs and constitute at least six novel monophyletic clusters independently emerging within the known cultivated representatives of this group (Fig. 1B). They are mainly related to 16S rRNA gene sequences of cultured *Bacteroidetes*, or to uncultivated microorganisms such as WCHB1-29 (AF050544) retrieved from the methanogenic zone of a polluted aquifer undergoing intrinsic bioremediation (Dojka *et al*., 1998), an anaerobic digester treating waste wine (U81676) (Godon *et al*., 1997), or associated with benzoate degradation in methanogenic consortia (AF323778). Using DBH technique, application *of Cytophaga-Flavobacter* probe (CF319a) and *Bacteroides-Porphyromonas-Prevotella* (Bacto-1080) shows that both probes accounted for 5.3 ± 2.4% and 9.2 ± 4.3% of the total rRNA, respectively, as measured by the *Bacteria* domain probe (Table 4).

The Gram-positive bacteria (*Firmicutes* and *Actinobacteria*) represent the second major phylogenetic group, with 27.7% of the OTUs. The *Actinobacteria* are represented by only four sequences defining three OTUs represented only by sequences affiliated with cultivated microorganisms with <97% sequence similarity. *Firmicutes* are much more dominant (54 OTUs, 63 sequences) and are represented by sequences affiliated with either cultivated or uncultivated microorganisms with sequence similarity ranging between 81.5% and 98%. Quantification by DBH using probe GP1199, which targets most of the Gram-positives (MacGregor *et al*., 2001), shows that this group exceeded the *Bacteroidetes*, with a rRNA index of 21.8 ± 4.3% (Table 4).

The *Proteobacteria* with 44 OTUs (21.3% of the total OTUs) represents the third major predominant phylogenetic group. Within this phylum, the delta subclass is an important group (9.2%), encompassing sequences affiliated with WCHB1-12 (AF050534) and WCHB1-27 (AF050538) retrieved from the methanogenic zone of a polluted aquifer undergoing intrinsic bioremediation (Dojka *et al*., 1998). Only 4.8% and 5.8% of the OTUs represented the alpha and beta subclasses, respectively, while only two OTUs represented the gamma subclass (Table 3). DBH shows that the *Proteobacteria* represent 14.5% of the rRNA index, making this phylum an important component in the degradation of organic matter in the digester (Table 4).

The Chloroflexi: *predominant members of the digester microbial community*

Phylogenetic analysis of our clone sequences shows that only seven OTUs (3.4%) represent this phylum. Most of the sequences are affiliated with a 16S rRNA gene sequence (Z94009) retrieved from activated sludge (Snaidr *et al*., 1997) and forming a monophyletic group (Fig. 1B). To our great surprise, application of a novel designed probe targeting this bacterial division by quantitative DBH shows a rRNA index of 20.2 ± 4.6% (Table 4), which makes this phylum comparable in terms of abundance to the Gram-positive bacteria.

Less known lineages such as the *Synergistes*, *Thermotogales* and candidate divisions OP9 and OP8, were also represented in our clone library, but in low percentages. However, using purportedly universal primers, we were not able to retrieve any representative of some bacterial division rRNA gene sequences such as *Spirochaetes* or *Planctomycetales*. Quantitative analysis using DBH and *Planctomycetales*-specific (Pla46R) and *Synergistes*-specific probes (Syn773R) shows that these groups accounted for <2% respectively (Tables 1 and 4).

Discussion

In the present study we characterized both archaeal and bacterial populations within an anaerobic sludge digester. Regarding the *Archaea* population we show that among

Table 3. Distribution of bacterial OTUs within the main phylogenetic groups within the digester.

	Proteobacteria							Gram-positives						
	alpha	beta	gamma	delta	unid Prot.	*Chloroflexi*	Bacteroidetes	LGC	HGC	Candidate *Thermotogales*	division OP9	Candidate *Synergistes*	division OP8	Total
Total number of clone sequences	12	19	2	89	1	33	315	63	4	16	4	18	3	579
% of clones	2	3.2	0.4	15.4	0.2	5.7	54.4	10.9	0.7	2.8	0.7	3.1	0.5	100
Total OTUs	10	12	2	19	1	7	80	54	3	5	3	9	1	206
% of OTUs	4.8	5.8	1	9.2	0.5	3.4	38.8	26.2	1.5	2.4	1.5	4.4	0.5	100
OTUs ≥97% cultivated[a]	1	0	2	0	0	0	0	2	0	0	0	0	0	5
OTUs ≥97% not yet cultivated[b]	0	2	0	1	0	1	0	0	0	0	0	0	0	4
Novel OTUs[c] (<97%)	9	10	0	18	1	6	80	52	3	5	3	9	1	197 (95.6%)
Intradivergence (% phylogenetic depth)	23.1	19.5	18.6	27.3	–	23.7	33.4	29.5	18.1	24.1	–	20.7	–	–
Coverage estimate[d]		64.4%												

a and b. ≥97% similarity sequence to the most related sequence having a cultivable or with no cultivable representative.
c. <97% similarity sequence to any known sequence.
d. $[1 - (n/N)] \times 100$ (Good, 1953).
LGC and HGC, Low G+C and high G+C Gram-positive bacteria.

Table 4. Ribosomal RNA indexes of 12 sludge samples from April 2001 through March 2002 measured by quantitative dot blot hybridization using the *Bacteria* domain-probe as reference and probes specific for: the alpha (ALPH19), beta (BET42a) and gamma (GAM42a) subclasses of *Proteobacteria*.

Sample	Date of sampling	alpha and delta	beta	gamma	Gram-positives	*Cytophaga-Flavobacter*	Bacto1080	GNSB1126	*Planctomycetales*	Syn773	Total
P 63	April	4.6	6.6	3.7	20.4	2.8	5.5	22.3	1.2	1.9	69 ± 20.8
P 68	May	7.1	1.3	1.6	5	1.5	1.5	ND	1.9	0.4	ND
P 72	June	6.8	4.2	6.5	17.1	6.4	3.6	22.7	1.9	0.9	70.1 ± 20.9
P 80	July	6.3	1.9	8.5	23.3	12.9	6.6	ND	3.3	1.3	ND
P 86	August	5.3	6.9	6.7	11.1	5	4	24.8	2.2	1.5	67.5 ± 20.1
P 93	September	6.5	5.5	3.4	16.5	7.9	5.5	26.5	1.1	0.9	73.8 ± 22.2
P 98	October	5.1	1.1	1.9	14.6	11.5	3.8	18.6	0.7	1.2	58.5 ± 17.6
P 101	November	8.8	3.6	2.6	14.6	10.7	3	21	1.9	1	67.2 ± 20
P 106	December	5.5	4.2	1.5	ND	12.9	4.4	21.4	0.9	0.7	114.5 ± 37.3
P 109	January	8.3	0	4.2	32.2	12.2	8	10.5	0.7	1.3	77.4 ± 23.7
P 112	February	6.6	2.2	3.9	28.1	14.8	9.7	18.1	1	1	85.4 ± 25.5
P 115	March	9.2	5.6	4.4	15.2	11.7	7.5	16.5	1.2	1.8	73.1 ± 21.2
Mean ± SD		6.7 ± 1.5	3.6 ± 2.3	4.1 ± 2.2	21.8 ± 14.9	9.2 ± 4.3	5.3 ± 2.4	20.2 ± 4.6	1.5 ± 0.8	1.2 ± 0.4	73.45 ± 20.9

Gram-positives (GP1199), *Bacteroidetes* (CF319a and Bacto1080), *Chloroflexi* (GNSB1112), *Planctomycetales* (PLA46R) and *Synergistes* (Syn756).
ND, not determined.

the *Euryarchaeota*, *Methanomicrobiales* were largely represented along with *Methanosarcinales*. We did not detect any *Methanobacteriales* 16S rRNA gene sequence representative. This finding is in agreement with previous studies. In previous work Raskin and colleagues (1994) characterized methanogens in acetate-fed chemostats and digesters fed sewage sludge or municipal solid waste (MSW) using 16S rRNA targeted hybridization probes. *Methanobacteriales* were predominant in the mesophilic and thermophilic MSW digesters but not in the sewage sludge digesters (SSD). *Methanosarcinales* were abundant in the mesophilic MSW and SSD but not in the thermophilic MSW digesters. In another molecular inventory Godon and colleagues (1997) found that *Methanosaeta* were prevalent in a digester treating wine distillation wastes. Importantly, we found an important group of 16S rRNA gene sequences closely related to sequences retrieved from several different environments. These environments included an oil-contaminated aquifer undergoing an intrinsic bioremediation in the Wartsmuth Air Base in Michigan in the United States (AF050615) (Dojka *et al*., 1998), a municipal wastewater sludge (AF424763) and an oil-contaminated groundwater accumulated at the bottom of an underground crude oil storage cavity in Japan (AB077223) (Watanabe *et al*., 2002). Both authors showed that these sequences are closely related to *Methanobacteriales*. Watanabe and colleagues (2002) found that this group of sequences clustered in a monophyletic group (candidate division V) branching deeply within the *Euryarchaeota* and affiliated with low bootstrap support (76%) to the *Methanobacteriales*. Our phylogenetic analysis using a comprehensive set of 16S rRNA gene sequences show that this lineage (Arc I) is truly monophyletic, distinct from the other main orders of the *Euryarchaeota*, and representatives of this group of putative microorganisms are widely distributed in methanogenic environments. They may be of importance in the degradation of the organic matter within these ecosystems.

Regarding bacterial diversity analysis, we show that mainly the *Bacteroidetes*, *Firmicutes* and the *Proteobacteria* represent the predominant phylogenetic groups. The extent of this diversity is highest at different phylogenetic levels and many novel phylogenetic clusters and OTUs were identified (Fig. 1B). Molecular diversity and abundance of these bacterial phyla in the anaerobic digester may be explained by the input of complex organic substrates present in primary and secondary sludge and, the ability of this anaerobic microflora to degrade a wide range of macromolecules such as cellulose, pectin, chitin, proteins and various xenobiotic compounds.

We are reporting that using DBH technique the *Chloroflexi* phylum forms an important component of the anaerobic sludge microflora. It accounted for 20.2% of the total rRNA, which is comparable to the *Firmicutes*. However, this phylum is represented by only 5.7% of the 16S rRNA gene sequences of the clone library. The discrepancy between both analyses is most likely explained by the non-quantitative nature of PCR-clone libraries, which can give skewed representations of the relative abundance of organisms present in a sample (Hugenholtz and Goebel, 2001). This also means that this division, considered in many molecular inventories as a minor one, represents a predominant phylum in the anaerobic digester.

The *Chloroflexi* have long been recognized as an evolutionary and environmentally significant group of bacteria (Oyaizu *et al*., 1987; Hugenholtz *et al*., 1998; Sekiguchi *et al*., 2001, 2003). Members of this phylum include phototrophic and chemotrophic genera (Holt and Lewin, 1968; Oyaizu *et al*., 1987). Cultivation-independent 16S rRNA studies suggest that many species of the *Chloroflexi* thrive in certain natural habitats such as freshwater lakes and anaerobic dechlorinating enrichments (Von Wintzingerode *et al*., 1997; Chandler *et al*., 1998; Hugenholtz *et al*., 1998; Gich *et al*., 2001). Our finding is corroborated by other studies like those of Bjornsson and colleagues (2002). Using FISH these authors have recently stressed the importance of the *Chloroflexi* in wastewater treatment processes. In another study Sekiguchi and colleagues (1998) found that *Chloroflexi* clone sequences were predominant in clone libraries constructed using genomic DNA extracted from both mesophilic and thermophilic methanogenic sludge granules.

Minor archaeal and bacterial lineages may represent an important majority hiding a large metabolic diversity

Many phylogenetic groups such as *Crenarchaeota*, *Thermotogales*, *Planctomycetales*, *Synergistes*, *Chloroflexi*, OP8 and OP9 are recurrently retrieved from sludge samples in many molecular inventories in low percentages of clone libraries (Godon *et al*., 1997, Sekiguchi *et al*., 1998). In this study these groups accounted for 13% of our clone library. We found that *Chloroflexi* represents the second major and predominant phylogenetic group after the *Firmicutes* in the anaerobic sludge digester with more than 20% of the total rRNA. Hence this minority tends to be the 'large majority' in terms of abundance and perhaps also metabolic activity in the anaerobic digester. Moreover, studies using bacterial division level-specific primers such *as Planctomycete–Verrucomicrobia*-specific primers (PV assay) revealed a huge hidden diversity at the (sub)division level (Derakshani *et al*., 2001; Chouari *et al*., 2003). This diversity has never been described using the so-called bacterial universal primers, simply because most often these primers have many mismatches with their targets in the 16S rRNA gene sequences of some important bacterial divisions. Using this strategy we retrieved a novel high order bacterial phylogenetic lineage

(WWE1: wastewater of Evry) within the anaerobic sludge digester. Application of a probe targeting this candidate division using DBH on six different RNA samples extracted from anaerobic digester samples shows that WWE1 represents 11.9 ± 3.1% of the total bacterial rRNA (Chouari *et al*., 2005).

Altogether, by using a set of four additional probes targeting the *Chloroflexi*, *Synergistes*, *Planctomycetes* and WWE1, respectively, we were able to account for an additional 35% of the total bacterial rRNA, thus extending the coverage of the bacterial rRNA index from 51% to almost 86% of total bacterial rRNA.

We are currently analysing more digester samples from other WWTP for the presence and the predominance of these supposedly important groups of the *Archaea* and the *Bacteria*.

Conclusions and perspectives

Using both qualitative and quantitative molecular approaches we have described the microbial diversity and the occurrence of novel high order bacterial phylogenetic lineages among the *Archaea* and the *Bacteria* within the anaerobic sludge digester. These lineages may represent predominant members of a microbial community widely distributed in different anaerobic habitats. The discovery of novel phyla or subphylum phylogenetic lineages is a step towards exploring the relationship between bacterial diversity and biogeochemical function within the digester ecosystem. The design and application of novel 16S rRNA oligonucleotide probes enabled us to account for >85% of the bacterial domain. These probes might be useful in elucidating bacterial population dynamics in various conditions within the digester microflora. However, further elucidation of their role in such a complex ecosystem may require new culturing approaches or metagenomic studies that allow the linkage of functional and ribosomal RNA genes. These approaches are being developed in our laboratory.

Experimental procedures

Study site, sampling, PCR amplification, cloning and sequencing of 16S rRNA gene sequences

Anaerobic sludge samples were obtained from an anaerobic mesophilic digester of Evry WWTP (250 000 population equivalents) located about 15 km south of Paris, France. The digester working parameters are: temperature 33°C, pH 7.2 and 37.5 days of retention time. Samples were centrifuged for 15 min at 20 000 *g*. Sludge pellets were washed three times with PBS and finally stored at −20°C. Genomic DNA extraction was performed as described in the study by Chouari and colleagues (2003). 16S rRNA genes were amplified from extracted DNA using *Archaea*-specific forward primer 21F (DeLong, 1992) or *Bacteria*-specific primer 63F (Marchesi *et al*., 1998) combined with the universal reverse primer 1390R respectively (Zheng *et al*., 1996). The thermal PCR profile, cloning, sequencing, chimera check and phylogenetic rRNA gene sequence analysis were performed as described in the study by [36]Chouari and colleagues (2003). Sequences from EMBL with the best BLAST score were imported into the ARB data set when necessary (http://www.arb-home.de). Chimeric sequences were searched using the procedure described by Juretschko and colleagues (2002) prior to phylogenetic analysis. All sequences with more than 1200 nucleotides were imported into the ARB database and automatically aligned with the existing 16S rRNA gene sequences. The resulting alignments were manually checked and corrected when necessary and 1122 unambiguously aligned nucleotide positions were used for *Archaea* phylogenetic analyses and 1179 for the *Bacteria* 16S rRNA gene sequences. Phylogenetic placement was performed in comparison with reference sequences representing the main lines of descent in the domain *Bacteria* using the ARB program and database package. Tree topology was evaluated by applying NJ analyses using Jukes and Cantor corrections and a 50% invariance criterion for inclusion of individual nucleotide sequence positions in the treeing analyses. We generated several trees that differed in the reference sequences, the set of alignment positions and the outgroup sequence used. Maximum parsimony and ML methods were also used. The statistical significance levels of interior nodes were determined by performing bootstrap analyses based on 100 re-samplings by the NJ and MP methods. A 97% similarity threshold was used for OTU assignment (Goebel and Stackebrandt, 1994).

Nucleotide sequence accession numbers

Sequences reported in this study have been submitted to EMBL, GenBank and DDBJ databases under accession numbers CR933117–CR933321.

RNA extraction, DBH and quantification

Total RNA was extracted from approximately 200 mg of wet samples by bead beating, phenol extraction and ethanol precipitation as described previously (Sghir *et al*., 2000). The quality of the RNA was checked by formaldehyde gel electrophoresis. Total rRNA amount was normalized with a universal probe after blotting 150 ng of RNA in triplicate onto nylon membranes (Nytran Super Charge Schleicher and Schuell) and hybridized with radioactively labelled oligonucleotide probes as described by Stahl and colleagues (1988) and Sghir and colleagues (2000). Membranes were washed at different temperatures depending on the probe dissociation temperature (T_w). The probes used and dissociation temperatures are given in Table 1. For probes BET42a, GAM42a, CF319a, washing buffer with a lower sodium dodecyl sulfate (SDS) concentration was used [1× SSC (150 mM NaCl, 15 mM sodium citrate; pH 7.0); 0.1% SDS]. However, for hybridization with probes Uni1390, EUB338, EUK502 and ARCH915, we used a washing buffer containing 1% SDS and 1× SSC. A mixture of probes (EUB338, II and III) was

used to quantify total bacterial rRNA expressed as a percentage of the signal intensity obtained using universal probe. Hybridization signal intensity was measured using a Beta Imager (Packard) and quantified as described previously (Sghir *et al*., 2000). Ribosomal RNA index of different phylogenetic groups within the *Bacteria* domain was calculated as a percentage of the total bacterial rRNA recovered. Reference rRNAs were collected from the following pure cultures: *Saccharomyces cerevisiae, Methanobrevibacter smithii* strain PS (DSM 861), *Pirellula staleyi* (DSM 6068), *Paracoccus denitrificans* (CIP 71.11), *Variovorax paradoxus* (CIP 103459T), *Microbacterium flavescens* (CIP 102401T) and *Flavobacterium johnsoniae* (CIP 100931T), as well as *Escherichia coli* (4 μg μl^{-1} Boehringer Mannheim, Germany).

Probe design and washing temperature determination (Table 1)

The probe search function of the ARB program software package was used to design novel probes (http://www.arb-home.de). They were checked using the probe function program of the RDP (http://rdp.cme.msu.edu/html/) (Cole *et al*., 2003). Oligonucleotide probes were purchased from Genosys (Sigma, France). For the probes targeting phylogenetic groups without cultured representatives, *in vitro* transcription of their respective plasmid rRNA gene inserts into RNA was used as positive and negative controls (Riboprobe® *in vitro* transcription system, Promega). The washing temperatures for the novel probes were determined, 40°C for the *Chloroflexi*, 44°C for *Synergistes*, using a washing buffer containing 3× SSC and 1% SDS and 55°C for *Planctomycetales* using a washing buffer containing 1× SSC and 1% SDS.

Culture enrichments under anaerobic conditions

Culture enrichments under anaerobic conditions were performed at 37°C in 50 ml bottles containing 25 ml of reduced anaerobic modified Balch medium (Balch *et al*., 1979) and 25 ml headspace of anaerobic gas [N_2–CO_2 (80:20, v/v), formate; N_2–CO_2 (80:20, v/v), methanol or H_2–CO_2 (80:20, v/v)] respectively. One millilitre of digester sample was inoculated and the enrichment was grown under these conditions for 4 weeks without shaking. Then, 500 μl of each enrichment was inoculated by syringe in 15 ml tubes containing 5 ml of modified Balch medium supplemented with antibiotics: cephalotin (4 μg ml^{-1}), D-cycloserine (4 μg ml^{-1}) and penicillin G (16 μg ml^{-1}). Culture transfer into new tubes was performed every month for 4 months. Putative members of the novel archaeal group were tracked by FISH technique, using the designed archaeal group-specific probe (Table 1, Fig. 2).

FISH experiments

Sludge samples were treated as described by Chouari and colleagues (2003). A stringent wash step was performed for 10 min at 48°C. We have tested the specificity of the new probe targeting the novel archaeal group S-*-Arcl-0864-a-A-18 using pure cultures of *M. smithii* as a negative control. Under these conditions no signal was obtained. However, a positive signal was obtained using the Arch915 FITC labelled probe specific for the *Archaea* domain for both *M. smithii* and our culture enrichment. Slides were visualized using a Zeiss axioplan epifluorescence microscope.

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