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Monitoring of activity dynamics of an anaerobic digester bacterial community using 16S rRNA polymerase chain reaction–single-strand conformation polymorphism analysis

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Summary

The influence of parameter changes on the bacterial community of a laboratory-scale anaerobic digester fed with glucose was investigated using a culture-independent approach based on single-strand conformation polymorphism (SSCP) analysis of total 16S rDNA and 16S rRNA amplification products. With the digester operating at steady state, the 16S rDNA SSCP patterns of the bacterial community showed eight peaks, whereas the 16S rRNA patterns showed six peaks with a very prominent one corresponding to a *Spirochaetes*-related bacterium. An acidic shock at pH 6 caused an increase in the 16S rRNA level of two *Clostridium*-related bacteria. After a 1 week starvation period, the major bacteria present reverted to a basal 16S rRNA level proportional to their 16S rDNA level. Starvation revealed the presence of a previously undetected peak whose corresponding sequence was deeply branched into the low G+C Gram-positive bacteria phylum. Twenty-four hours after a spiked addition to the starved digester community of starch, glucose, lactate or sulphate, an upsurge in several new 16S rRNA-derived peaks was observed. Thus, the perturbation approach combined with 16S rRNA analysis revealed bacteria that had not been detected through 16S rDNA analysis.

Introduction

Anaerobic treatment offers great potential as a biotechnological process for degrading polluting organic wastes. Populations involved in anaerobic digestion ecosystems can generally be assigned to four trophic groups (hydrolytic, acidogenic and acetogenic bacteria and methanogens), whose concerted activity is required for the complete degradation of carbohydrates to CH_4 and CO_2 (Archer and Kirsop, 1990; Fernández *et al*., 1999). Knowledge of the most effective metabolizing microorganisms involved in each step of the anaerobic digestion pathway, and how they react on perturbations, is a prerequisite for an optimal running of the process over time.

Molecular inventories of anaerobic digesters that revealed a high microbial diversity were carried out recently (Godon *et al*., 1997; Delbès *et al*., 1998; Sekiguchi *et al*., 1998). However, these results based on 16S rDNA molecule analysis did not provide any data on the role and activity of these uncultivated microorganisms. Analysis based on 16S rRNA can partly overcome this limitation, in so far as metabolically active bacteria contain more rRNA than resting or starved cells (Kemp *et al*., 1993; Maaløe and Kjeldgaard, 1966). Hybridization probes targeting 16S rRNA molecules have been used to correlate the activity of specific microorganisms with defined environmental conditions (Kramer and Singleton, 1993; Poulsen *et al*., 1993). Fingerprinting techniques, such as temperature or denaturing gradient gel electrophoresis (T/DGGE) (Muyzer *et al*., 1993), terminal restriction fragment length polymorphism (T-RFLP) (Liu *et al*., 1997) and single-strand conformation polymorphism (SSCP) (Lee *et al*., 1996; Clapp, 1999; Zumstein *et al*., 2000), which give a rapid overview of complex microbial communities without prior identification of sequences, have been developed. Approaches combining these techniques and reverse transcription–polymerase chain reaction (RT–PCR) of 16S rRNA have been used to estimate the diversity of active bacteria in various environments (Teske *et al*., 1996; Felske *et al*., 1998; Zoetendal *et al*., 1998). In addition, an approach based on cDNA synthesis and cloning has been used recently to identify the metabolically active members of a soil bacterial community (Nogales *et al*., 1999). In this study, we used an approach combining RT–PCR of 16S rRNA and SSCP analysis. The aim of this study was to investigate the diversity of the presumptive metabolically

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most active populations of the bacterial community of a laboratory-scale digester fed with glucose, and to monitor the activity dynamics of these populations over defined perturbations. The 16S rRNA dynamics revealed the presence of species that had not been detected through 16S rDNA analysis, as well as their metabolic responses *in situ* to specified environmental changes.

Results

Strategy used to analyse the anaerobic digester community and reproducibility of the experiments

The experiments that were conducted in this study are outlined in Fig. 1. All experiments were performed in duplicate tubes of cells that yielded reproducible SSCP patterns. Figure 2 shows, as an example, the RNA-derived SSCP patterns obtained from the two tubes of cells amended with glucose. The total 16S rRNA fragments of the bacterial community gave a pattern of peaks through SSCP analysis, whereas a pool of fragments containing a unique 16S rRNA sequence gave one peak. In order to identify the bacteria corresponding to the different peaks within the complex SSCP patterns, clone libraries were prepared either from PCR products of the total 16S rDNA or from RT–PCR products of the V3 region of the 16S rRNA (see *Experimental procedures*). The SSCP peak of the 16S rDNA of each clone was compared with the community 16S rDNA and 16S rRNA SSCP patterns.

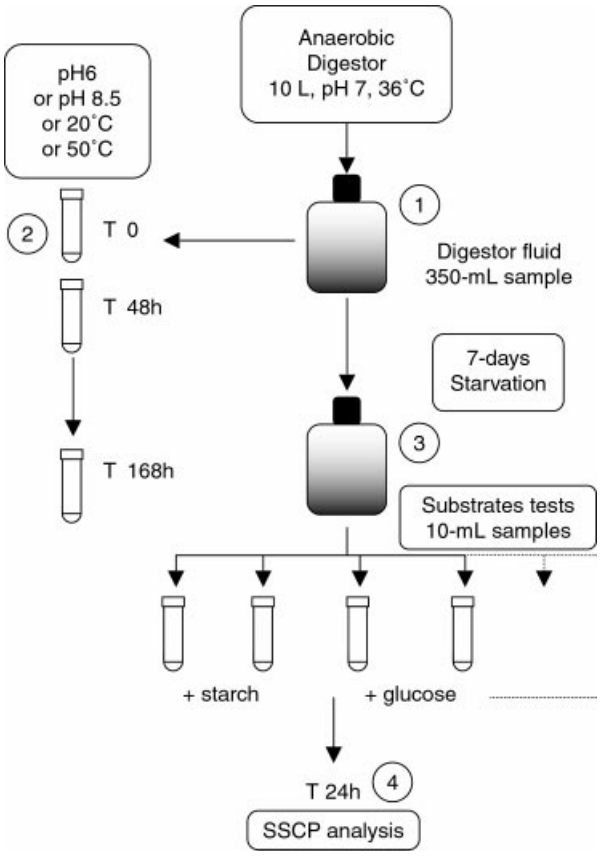


Fig. 1. Outline of the perturbations and substrate addition tests performed in duplicate with samples of the anaerobic digester ecosystem.

16S rDNA and 16S rRNA of the bacterial community in the anaerobic digester

16S rDNA and 16S rRNA were amplified from a sample collected from the digester and compared by SSCP electrophoresis (Fig. 3). The 16S rDNA pattern (Fig. 3A) showed eight prominent peaks, which should represent the most abundant sequences in the digester community. The 16S rDNA and 16S rRNA patterns were confirmed to be aligned, with all the peaks observed on the 16S rRNA pattern being present on the 16S rDNA pattern as well. However, the 16S rRNA pattern revealed only six peaks, with one clearly prominent (Fig. 3B). The pattern data are outlined and normalized in Fig. 4.

A total of 106 clones from the AA, AB and AC 16S rDNA libraries were analysed by SSCP. The estimated percentage coverage, defined as $[1-(n/N)] \times 100$, where n is the number of unique clones detected in a subsample of size N (Good, 1953), was 84%. Furthermore, 89% of the total clones were distributed among the eight defined peaks of the 16S rDNA SSCP pattern (Fig. 3A) and allowed the assignment of these peaks. Analysis of the libraries prepared from the RT–PCR SSCP products also confirmed this assignment. The phylogenetic affiliation of the 16S rDNA sequences from assigned peaks and from the remaining 11% of clones that did not fall into any peak is detailed in Table 1. The sequence AA14, corresponding to the major peak of the 16S rRNA pattern (Fig. 3B), belonged to the *Spirochaetes* group (Table 1). The

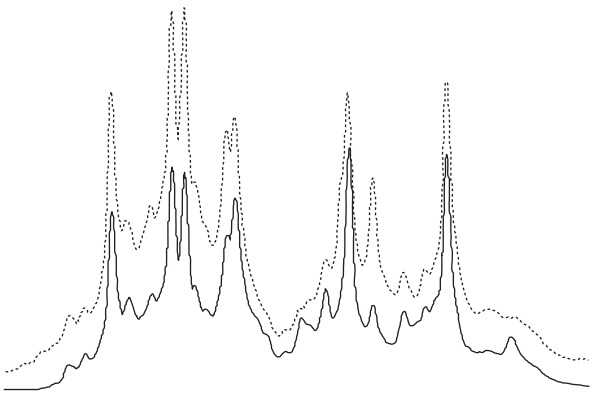
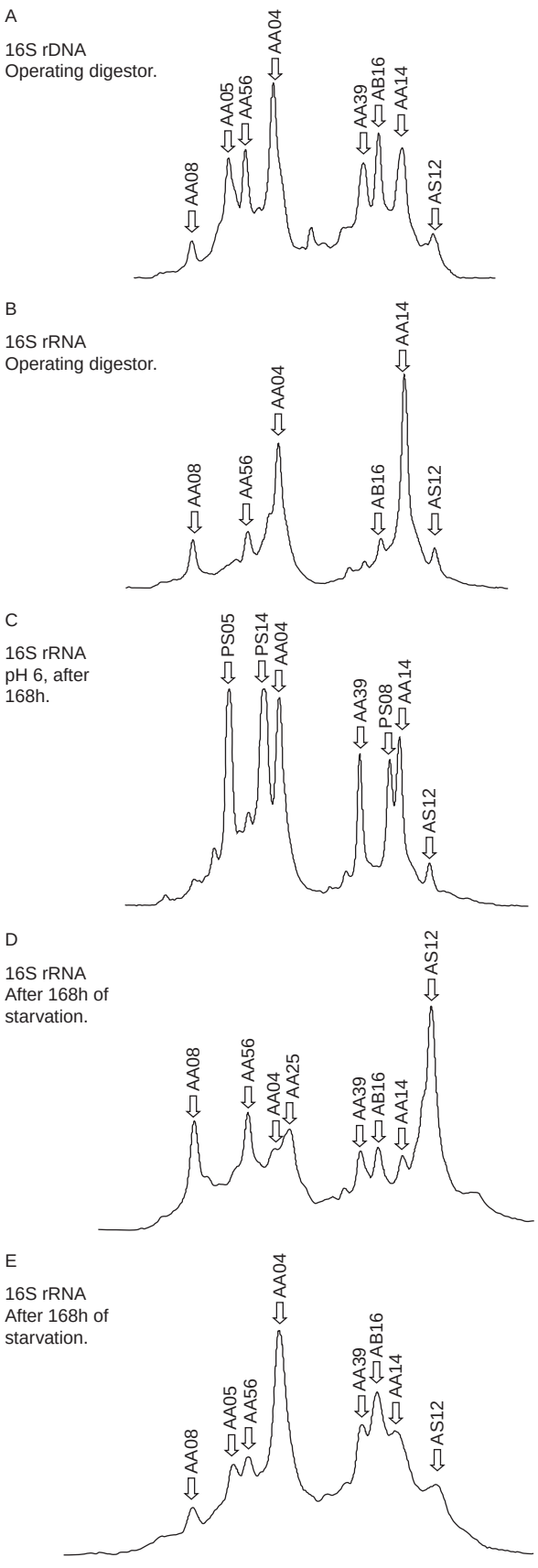


Fig. 2. Reproducibility of the effect of glucose addition to the cells, as analysed by the SSCP approach. Two tubes of cells were amended with glucose and processed in the same way (incubation, nucleic acids extraction, RT–PCR, SSCP electrophoresis). Tubes 1 and 2 are indicated by black and grey lines respectively.



sequence of the second major peak of the 16S rRNA pattern (AA04) showed more than 97% similarity to the *Clostridium indolis* 16S rDNA sequence. The sequences corresponding to the peaks AA05 (low G+C Gram-positive bacteria), AA39 (*Cytophagales*), AA08 (green non-sulphur bacteria) and AA56 (*Synergistes*) clustered with sequences retrieved from another anaerobic digester fed with distillery slops and studied in our laboratory by Godon *et al*. (1997). The sequence AB16 was not closely related (less than 90% 16S rDNA sequence similarity) to any 16S rDNA sequence in the databases and was deeply branched into the *Cytophagales* group.

Dynamics of the bacterial populations upon perturbations

Four stress conditions (pH 6, pH 8.5, incubation at 20°C and 50°C) were applied to subsamples in Hungate tubes (Fig. 1). Only the patterns obtained after the shock at pH 6 were markedly different from the control patterns. Forty-eight hours after the acidic shock, an upsurge in intensity of a new 16S rRNA peak (PS05) was detected (data not shown). A second 16S rRNA peak (PS14) also appeared within 1 week ($T = 168$ h) to be as high in intensity as the first one (Figs 3 and 4). An upsurge in the peak PS14 was also detectable as 16S rDNA signal after 1 week, whereas 16S rDNA signal for peak PS05 did not change (data not shown). Both peaks corresponded to sequences closely related to *Clostridia* species (Table 1).

Effect of a 1 week starvation period on the bacterial community

A starvation stage was applied to the microbial community as a conditioning of the cells before the addition of different substrates. The test was conducted with samples of digester fluid collected anaerobically and incubated at 36°C for 1 week (Fig. 1). The cells produced decreasing daily amounts of biogas, ceasing by the sixth day. The 16S rDNA patterns showed the same peaks before and after starvation, whereas the 16S rRNA pattern showed eight peaks and more closely resembled the 16S rDNA pattern than the 16S rRNA pattern before starvation (Figs 3 and 4). However, a new peak, AS12, was prominent on the 16S rRNA pattern after starvation. The sequence AS12 was recovered from the three 16S rRNA libraries AS, PS and MS, but did not cluster with any of the

Fig. 3. Dynamics of SSCP patterns of 16S rDNA and 16S rRNA V3 region amplification products.
A. 16S rDNA pattern of the operating digester.
B. 16S rRNA pattern of the operating digester.
C. 16S rRNA pattern 1 week after a shock at pH 6.
D. 16S rRNA pattern after 1 week of starvation.
E. 16S rDNA pattern after 1 week's starvation. Arrows indicate the representative clone sequence assigned to each peak.

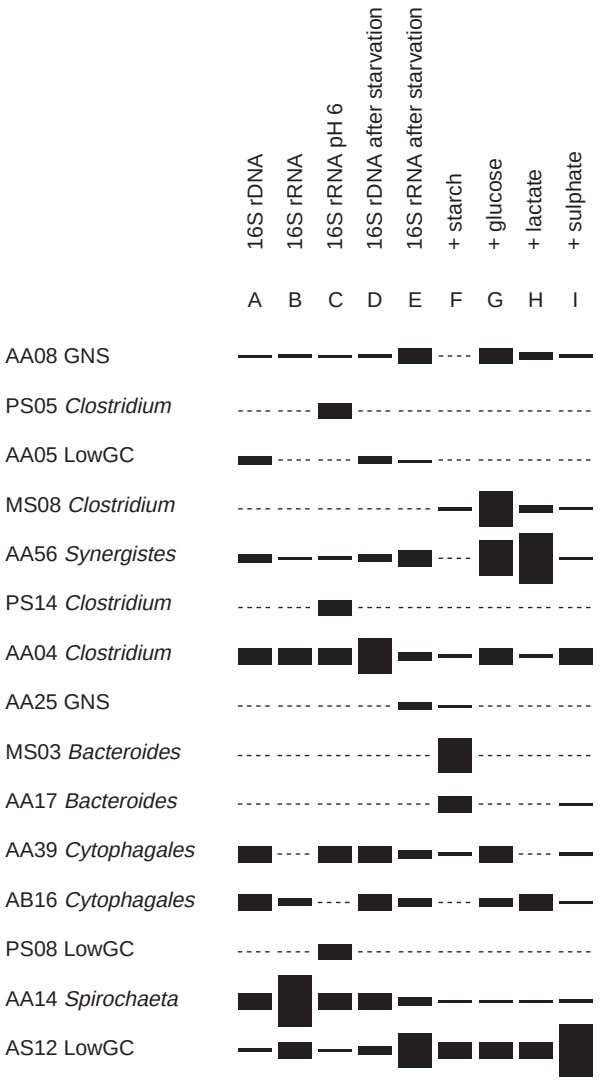


Fig. 4. Outline of the SSCP patterns showing the relative intensities in 16S rDNA or 16S rRNA signal of each peak relative to the different parameter conditions (letters A to I refer to the patterns shown in Figs. 3 and 5). Peak layout is the same as in the patterns. The thickness of each band is proportional to the relative area of the corresponding peak, which was estimated according to the ratio between the height of the peak and the sum of the heights of all peaks within the pattern. GNS, green non-sulphur bacteria group; lowGC, low G+C Gram-positive bacteria group.

sequences recovered from the 16S rDNA libraries. The closest relative sequence (BB60, 84% similarity to the V3 region of 16S rRNA; Table 1) was recovered from the digester studied by Godon *et al*. (1997) and was to be found deeply branched into the low G+C% Gram-positive bacteria group and related to the *Mycoplasma* subgroup.

Response of the bacterial community to the addition of different substrates

The tests were conducted on the previously starved cells incubated under anaerobic conditions. Eleven different substrates were added to each pair of tubes to a final concentration of 2 COD (chemical oxygen demand) g l^{-1}. The volume of biogas produced was measured after 24 h of incubation at 36°C, and the responses of the bacterial community were analysed by SSCP. Significant amounts of biogas (10–40% of the expected final total volume) were produced within each tube, except for the control tubes and the tubes that had received sulphate, indicating that anaerobic microbial degradation of the substrates down to methane was carried out by the cells. Although the 16S rDNA patterns did not change in any situation after 24 h of incubation (data not shown), responses could be detected through 16S rRNA pattern analysis. The clearest variations were obtained in the starch-, glucose-, lactate- or sulphate-amended tubes (Fig. 5), whereas patterns obtained after additions of ethanol, acetate, propionate, butyrate, pyruvate, succinate or formate were not different from the control pattern. The results of the 16S rRNA pattern analysis corresponding to the different substrates are summarized in Fig. 4. An upsurge in three peaks was observed 24 h after the addition of starch to the ecosystem. Two peaks corresponded to sequences related to *Bacteroides* species (MS03, which had not been detected previously, and AA17), and the last peak corresponded to a sequence related to *Clostridium* species (MS08). Lactate addition led to an upsurge in the peak AA56, which had also been detected in the operating digester and corresponded to sequences related to the *Synergistes* group. Finally, sulphate addition led to an upsurge in the peak AS12, which corresponded to sequences related to the low G+C Gram-positive bacteria group.

Discussion

In the present study, the presence and metabolic activity dynamics of the bacterial populations of a laboratory-scale anaerobic digester were investigated using SSCP analysis applied in parallel to 16S rDNA PCR products and 16S rRNA RT–PCR products. The SSCP analysis provided a rapid picture of numerically dominant 16S rDNA sequences of the operating digester bacterial community. The 16S rDNA pattern showed eight peaks, corresponding to 16S rDNA sequences falling into genera classically identified within anaerobic digesters (*Clostridium*, *Bacteroides*; Archer and Kirsop, 1990) and other difficult-to-cultivate groups detected through microscopy (*Spirochaetes*) or molecular inventory studies (*Spirochaetes, Synergistes* and GNS bacteria; Godon *et al*., 1997; Fernández *et al*., 1999). The SSCP approach reflected the diversity of prominent sequences among the sequences that were amplified. The comparison between SSCP patterns and clone libraries showed that the eight peaks of the 16S rDNA pattern represented 89% of the

Table 1. Phylogenetic affiliation and frequency of the 16S rDNA and 16S rRNA sequences.

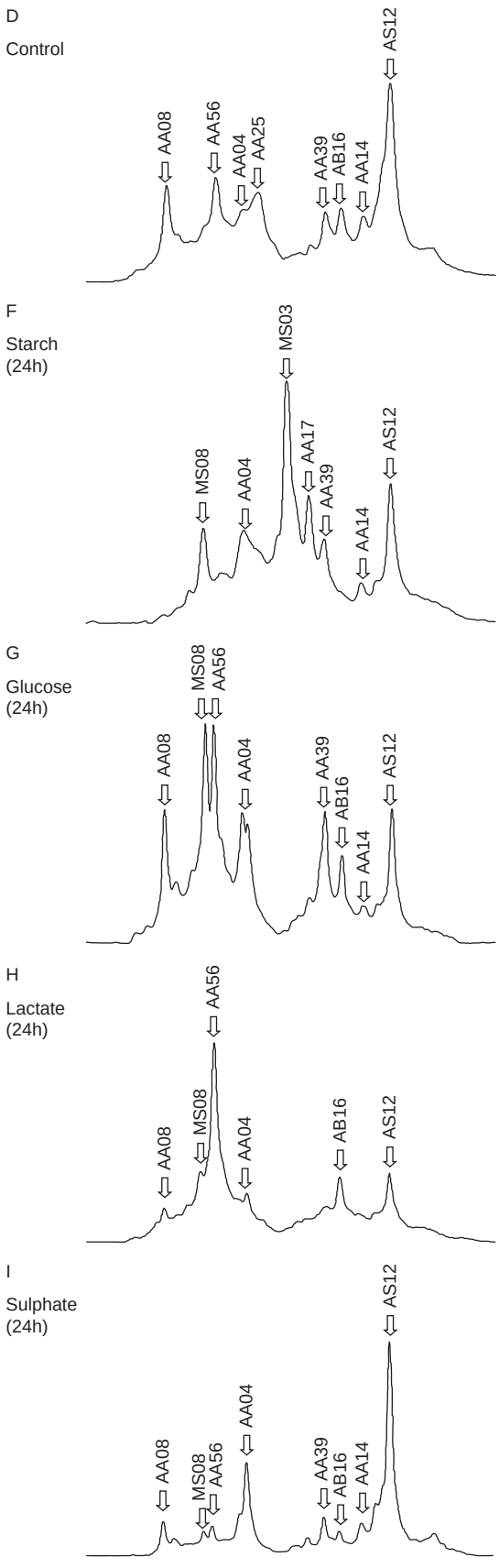
Phylum or group	Clone name[a]	Frequency[b]	Closest neighbour	Accession numbers	Similarity of 16S rDNA (%)	Similarity of V3region (%)
Low G+C Gram-positive bacteria	AA01	10	Clone SJA143	AJ009494	89.1	88.8
	AA05	8	Clone BB35	U81761	95.4	95.6
	[AA33]	1	Clone BSV 27	AJ229189.1	94.4	97.8
	[AA64]	1	*Syntrophomonas* sp.	AF022249	86.6	81.1
	AS12	*	Clone BB60	U81670	NA	84.0
	[PS08]	*	*Enterococcus dispar*	Y18358	NA	98.5
Clostridia	AA04	38	*Clostridium indolis*	AF028351	97.3	99.4
	MS08	*	Clone adhufec 3525	AF153853	NA	97.8
	PS05	*	*Clostridium paraputrificum*	AB032556	NA	98.9
	PS14	*	*Clostridium butyricum*	M59085	NA	100.0
Cytophagales	AA39	10	Clone BB12	U43697	99.2	ND
	AB16	5	Clone ii-12	Z95724	75.5	78.6
	[AA26]	1	Clone SB-5	AF029041	85.6	92.5
	[AC08]	1	*Sphingobacter comitans*	X91814	71.8	78.5
Bacteroides	AA17	1	Clone BB23	AF129860.1	90.8	95.6
	MS03	*	*Bacteroides stercoris*	X83953	NA	87.4
Green non-sulphur bacteria	AA08	5	Clone HB65	U81755	97.6	99.5
	AA25	2	Clone SJA-116	AJ009487	89.8	92.3
	[AB13]	1	Clone SJA-170	AJ009500	92.1	94.5
	[AC05]	1	Clone SJA-68	AJ009475	94.4	ND
Spirochaeta	AA14	8	*Treponema* sp.	Y08894	87.9	89.3
	[AC31]	1	Clone SJA-182	AJ009506	99.6	99.0
Synergistes	AA56	12	Clone BB48	AF129869.1	95.2	97.3
High G+C Gram-positive bacteria	[AS14]	*	*Kineosporia aurantiaca*	X87110	NA	84.9
Total		106				

a. Clone names in brackets do not correspond to a SSCP peak of the total 16S rDNA or 16S rRNA patterns.
b. Number of clones among the total 106 clones of the AA, AB and AC 16S rDNA libraries. Clones with * were only detected in 16S rRNA libraries.
NA, unavailable data, for clones obtained only from 16S rRNA V3 region libraries.
ND, not determined.

total clones but only 68% of the diversity observed among the libraries (Table 1). It is likely that numerous other sequences were present at a lower ratio than the detection limit of the method. The additional peaks that were detected within the 16S rRNA patterns after substrate additions supported this hypothesis.

As rRNA per cell ratio is roughly proportional to growth rate, and ribosome synthesis is immediately affected by changing environmental conditions (Maaløe and Kjeldgaard, 1966; Wagner, 1994), the fingerprinting of 16S rRNA essentially reflects the most metabolically active bacteria of the community. *Spirochaetes* have been detected previously at low frequency in anaerobic digesters (Godon *et al*., 1997), and little is known about their ecological role in these ecosystems. Our finding of a *Spirochaete* (AA14) among the major bacteria of the anaerobic digester ecosystem was consistent with the results of Fernández *et al*. (1999) obtained with the bacterial community of a similar reactor fed with glucose. Furthermore, the analysis of the 16S rRNA pattern of the bacterial community within our digester ascertained the metabolic significance in the ecosystem of the AA14 *Spirochaete* bacteria. The prominence of the AA14 peak within the 16S rRNA pattern suggested that these *Spirochaete*-related bacteria were metabolically highly active. According to pure culture studies, non-pathogenic *Spirochaetes* are expected to retrieve energy from the fermentation of either carbohydrates or amino acids (Stanier *et al*., 1987; Fernández *et al*., 1999). As both digesters were fed with glucose as the sole carbon source, it may be assumed that the AA14 *Spirochaete* used glucose and might have been the main glucose consumer in the operating digester at the time of sampling. The AA04 *Clostridium indolis*-related bacteria was the second most active bacteria, according to the 16S rRNA pattern. This result was coherent, as *Clostridia* species are known to take part in a broad spectrum of fermentation reactions (Archer and Kirsop, 1990; Melvin and Hobson, 1994).

The SSCP approach permitted the observation, after perturbations imposed on the ecosystem, of dynamic changes in relative 16S rRNA levels of individual bacterial populations within the community. The increased resemblance of the 16S rRNA pattern to the 16S rDNA pattern after the starvation period suggested that the major bacteria present, and especially the highly active *Spirochaete*- and *Clostridium*-related bacteria, reverted to a basal 16S rRNA level proportional to their population level. The 16S rRNA pattern after starvation was useful in confirming the presence of the *Synergistes*, GNS and



other deep-branched sequences that had been detected on the 16S rDNA pattern. It also revealed the presence of the previously undetected AS12 peak. The results of the substrate addition tests allowed us to hypothesize about the physiological features of some of these bacteria. The increase in activity of the previously undetected MS03 *Bacteroides* bacteria after starch addition suggested that these bacteria used starch and was consistent with the well-established hydrolytic capacities of species of this group (Thiele, 1991). The AA56 sequence was related to the *Synergistes* group, which have only been detected by molecular inventories in anaerobic digestion ecosystems (Godon *et al*., 1997; Sekiguchi *et al*., 1998) and whose functional role in anaerobic digesters is unknown. The marked increase in activity of the bacteria corresponding to the AA56 sequence after lactate addition suggested that these *Synergistes*-related bacteria may take part in lactate degradation within the ecosystem studied in this work. Finally, the increase in AS12 activity, or its limited loss in activity compared with other populations, during either starvation or sulphate addition remains unclear. This microorganism fell into the low G+C% Gram-positive bacteria group, close to *Mycoplasma* whose parasitic capacities are well documented. The lack of detection of any response to several substrates or perturbation tests could be partly explained by the technical limitations of the SSCP approach, which permitted the detection of shifts involving dominant species. Shifts involving a large number of subdominant species would not have been detected. However, several parameters could have affected the bacterial activity and interfered with the expected response: (i) the time necessary for metabolic change; (ii) the management of the ribosome pool: some species may have retained excess ribosomes during starvation, as was shown for *Escherichia coli* cells (Koch, 1971) or *Vibrio* sp. (Flardh *et al*., 1992); (iii) the substrate concentrations applied.

Our approach through specific stimulation of the ecosystem was valuable in that it revealed the post-perturbation upsurge in bacteria that had not been detected at first sight, such as the MS03 *Bacteroides* bacteria or the PS05 and PS14 *Clostridium* bacteria. Hence, certain bacteria can grow and remain in the digester at subdominant levels and yet be capable of immediate and significant responses to environmental

Fig. 5. Dynamics of SSCP patterns of 16S rRNA V3 region amplification products 24 h after spiked additions of substrates.
D. Control pattern: 16S rRNA pattern after 1 week's starvation.
F. Starch-amended sample pattern.
G. Glucose-amended sample pattern.
H. Lactate-amended sample pattern.
I. Sulphate-amended sample pattern.
Arrows indicate the representative clone sequence assigned to each peak.

changes. Such subdominant bacteria take part in the reactive capacity of the digester ecosystem and may help it to face perturbations and foster functional stability.

The SSCP analysis of total 16S rRNA amplification products has been shown to be useful in investigating the rapid dynamics of activity of a complex microbial community in response to specified stimuli. SSCP was an alternative powerful technique to either DGGE or T-RFLP. Although other investigations will be required to cross-check data before drawing definitive conclusions about the functional role of the bacteria identified in this work, our results have demonstrated the promising nature of this strategy for gaining information about the physiological features of microorganisms *in situ*.

Experimental procedures

Reactor design

The experimental system consisted of a 10 l working volume all-glass reactor and time-controlled pumps for substrate feed and sludge removal. The contents of the anaerobic reactor were kept completely mixed and maintained at 36°C ± 1°C and a pH of 7.2. The seeding sludge consisted of a (v/v) mix of three sludges collected from an anaerobic lagoon and two industrial-scale anaerobic digesters, all fed with distillery slops. The chemical composition of the synthetic substrate, in which glucose was used as the sole carbon and energy source, was as follows (concentration in g l^{-1}): $C_6H_{12}O_6$ (40.0); $CaCl_2.2H_2O$ (0.045); NH_4Cl (1.15); $MgSO_4.7H_2O$ (0.5); $NiCl_2.6H_2O$ (0.0027); KH_2PO_4 (0.75) and mineral solution (3 ml l^{-1}). The stock mineral solution contained the following compounds (concentration in mg l^{-1}): $FeCl_3.6H_2O$ (1500.0); H_3Bo_3 (50.0); $CuSo_4.5H_2O$ (10.0); NaI (10.0); $MnCl_2.4H_2O$ (40.0); $Na_2MoO_4.2H_2O$ (20.0); $ZnSO_4.7H_2O$ (40.0). The substrate solution was sterilized by autoclaving and continuously fed to the reactor. The organic load was increased from 0.5 to 2 COD (chemical oxygen demand) g l^{-1} day^{-1} (≈0.47–1.87 g l^{-1} day^{-1} glucose) during start up and was then maintained within the range 0.2–0.5 COD g l^{-1} day^{-1} for steady-state operation.

Total gas production was measured on-line with a gas meter based on liquid displacement. Gas composition (methane and carbon dioxide) and volatile fatty acid (VFA) concentrations were determined by gas chromatography (Garcia-Calderon *et al.*, 1998) on a daily to weekly basis depending on reactor performance. Concentration of suspended solids (SS) within the digester stabilized at around 3 g l^{-1}. The anaerobic digester had been running for 10 months when the investigations were carried out.

Batch test procedure (Fig. 1)

All tests were conducted in duplicate either in 16 ml Hungate glass tubes or in 500 ml glass bottles using Hungate techniques to ensure anaerobic conditions.

Batch tests for studying the response of the cells to different temperature and pH conditions were conducted using digester fluid distributed among tubes as 10 ml subsamples. A first series of tubes was incubated horizontally on rotary shakers at 140 r.p.m., at 36 ± 1°C (control), 20 ± 1°C or 50 ± 1°C. In the second series of tubes, the pH was initially adjusted to either pH 6 or pH 8.5 by adding to the tubes 340 μl of 2 N HCl or 150 μl of 0.1 N NaOH respectively. This second series of tubes was incubated at 36 ± 1°C. Doses of the synthetic substrate were added daily to each tube over 1 week. Duplicate tubes for each disturbing condition were analysed by SSCP after 48 h of incubation and after 1 week.

Batch tests to investigate the effect of starvation on the cells were conducted with samples of 350 ml of digester fluid. The bottles were incubated at 36 ± 1°C on a rotary shaker at 150 r.p.m. for 1 week. The amount of biogas produced by the cells was measured daily through the latex undertop with a syringe. Samples of cells (10 ml) were analysed by SSCP after 1 week of incubation.

Batch tests to study the response of the cells to the addition of different substrates were conducted with fluid collected from the 500 ml glass bottles after starvation and distributed as 10 ml subsamples. Solutions in water of the different substrates to be tested (starch, glucose, ethanol, lactate, acetate, propionate, butyrate, succinate, formate, pyruvate and sulphate) were prepared under an atmosphere of N_2 and sterilized by autoclaving, except for the starch solution, which was filtered. Solutions were added to the tubes as 500 μl doses such that the final concentration of the carbon sources in the tubes was 2 COD g l^{-1} and, for the sulphate test, the final concentration of $MgSO_4.7H_2O$ was 1.2 mg l^{-1}. Water without substrate was added to the control tubes. The tubes were then incubated horizontally at 36 ± 1°C in a rotary shaker at 140 r.p.m. After 24 h of incubation, the amount of biogas produced by the cells was measured, and the contents of each tube were treated for SSCP analysis.

Nucleic acids extraction

All glassware was sterilized by baking at 200°C overnight. Plastic ware was sterilized by autoclaving at 121°C for 20 min. GT solution contained 4 M guanidine thiocyanate and 0.1 M Tris-HCl (pH 7.5). SP buffer consisted of 0.1 M sodium phosphate, pH 6. AE buffer consisted of 50 mM sodium acetate and 10 mM EDTA, pH 5.5. Genomic DNA and rRNA were isolated simultaneously from liquid 10 ml samples, either retrieved directly from the reactor or originating from batch tests. The samples were collected in sterile centrifuge tubes and centrifuged for 5 min at 10 500 *g* in a precooled (4°C) centrifuge. GT solution (1 ml) and 150 μl of N-lauroyl-sarcosine 10% were added to the pellets, which were resuspended by vortexing for 1 min. The homogenized samples were either treated immediately or stored as 250 μl aliquots in 2 ml tubes at −80°C.

The tube containing 250 μl of the suspension was filled with 200 mg of zirconium beads, 100 μl of 20% (w/v) SDS solution, 400 μl of SP buffer, 400 μl of AE buffer and 400 μl of water-saturated phenol–chloroform–isoamyl alcohol (25:24:1), pH 5.5. The tube was vigorously shaken in a bead beater for 2 min and then heated in a water bath at 60°C for 2 min. These two steps, shaking and heating, were repeated a second time. Two washing steps were then

performed, the first one with phenol–chloroform–isoamyl alcohol (25:24:1) and the second one with chloroform–isoamyl alcohol (24:1). Nucleic acids were precipitated with one-tenth of the volume of 3 M sodium acetate and two volumes of ethanol. The tube was gently mixed and stored at −20°C overnight. After centrifugation at 17 500 *g* for 8 min, the resulting nucleic acid pellet was washed twice with 80% ethanol, dried in a vacuum concentrator for 10 min and resuspended in 100 μl of water. Subsamples (5 μl) of the nucleic acid suspension were analysed by electrophoresis on a 1.2% (w/v) agarose gel stained with ethidium bromide.

DNase and RNase treatments

Subsamples (5 μl) of the nucleic acid solution were treated with either RNase-free DNase or DNase-free RNase according to the manufacturer's instructions (Promega) in order to obtain purified RNA and DNA respectively. DNase was then inactivated by heating at 94°C for 5 min. The DNase and RNase treatments yielded purified RNA and DNA suitable for amplification by RT–PCR and PCR respectively.

PCR–SSCP and RT–PCR–SSCP of 16S rDNA and 16S rRNA

The variable V3 region (*E. coli* positions 331–533; Brosius *et al*., 1981) was amplified from DNA and RNA using identical buffer with *rTth*, which can carry out both reverse transcription and PCR amplification, as unique enzyme. The reactions were performed with the *rTth* DNA polymerase plus EZ buffer kit from Perkin-Elmer Cetus. RT–PCR reaction mixtures (50 μl) contained 1 × EZ buffer, 300 μM dNTPs, 0.6 μM primers W34 (5′-TETTTACCGCGGCTGCTGGCAC-3′, *E. coli* position R533; Brosius *et al*., 1981) and W49 (5′-ACGGTCCAGACTCCTACGGG-3′, *E. coli* position F_{331}), 5 U of *rTth* DNA polymerase and 2.5 mM $Mn(OAc)_2$. The primer W34 was labelled with the 5′-fluorescein phosphoramidite (TET; Applied Biosystems, Perkin-Elmer). After adding 4 μl of the extract digested with DNase, the samples were reverse transcribed and amplified in a Perkin-Elmer Cetus 9700 PCR thermocycler. After an initial denaturation step at 94°C for 15 s and an annealing step at 50°C for 1 min, the RT reaction was performed at 65°C for 20 min. After the RT reaction, a denaturation step was performed for 1 min at 94°C, followed by 25 cycles of a three-stage programme with 30 s at 94°C, 15 s at 50°C and 30 s at 65°C, and a final elongation for 10 min at 65°C.

The PCR was performed from 4 μl of extract digested with RNase, with the same reaction mixture as RT–PCR, using only the three-stage programme.

Control experiments were performed by treating subsamples of nucleic acids with DNase plus RNase and checking that they were not amplified by RT–PCR. These control experiments proved that the products of RT–PCR were the result of RNA but not genomic DNA amplification.

SSCP electrophoresis

SSCP analysis relies on the fact that a single base modification can change the conformation of single-strand DNA molecules leading to a different electrophoretic mobility in a non-denaturing gel (Orita *et al*., 1989). Thus, DNA fragments of the same size but with a different base composition can be separated (Hayashi, 1991; Clapp, 1999). The use of the fluorescent dye-labelled PCR primer W34 permitted the laser detection of only one strand of each DNA fragment and provided optimal band separation on the automated DNA sequencer (Zumstein *et al*., 2000). The size standard using a different fluorophore (GeneScan-400 Rox; Applied Biosystems), which was added to the samples, permitted reliable comparison of patterns from each sample after computing correction (GENESCAN software; Applied Biosystems). The PCR or RT–PCR product (1 μl) was added to 18 μl of loading buffer TSR (template suppression reagent; Applied Biosystems) and 1 μl of the internal size standard GeneScan-400 Rox (Applied Biosystems). The sample was then denatured for 5 min at 94°C and placed directly on ice for 5 min. SSCP was performed using the ABI 310 Genetic Analyser (Applied Biosystems) equipped with a capillary tube (47 cm × 50 μm) filled with a polymer composed of 5.6% GeneScan polymer (Applied Biosystems), 10% glycerol and 1 × TBE. Electrophoresis was carried out at 12 kV and 32°C for 30 min per sample. Data processing was performed with the ABI Prism 310 collection software (Applied Biosystems). The second-order least square size calling method was used to analyse each sample and normalize mobilities from different runs (GENESCAN analysis 2.0.2 software; Applied Biosystems).

16S rDNA clone libraries and SSCP peaks assignments

Six clone libraries were prepared (AA, AB, AC, AS, MS and PS). The three libraries AA, AB and AC were prepared from three independent 16S rDNA amplification reactions of the same DNA extract, obtained from the sample taken after starvation of the cells. The three libraries, AS, MS and PS, were prepared from RNA extracts through a second amplification of the RT–PCR products of the V3 region of 16S rRNA. The AS library corresponded to the starved cells, the MS library to cells fed with starch and the PS library to cells shocked at pH 6. Amplifications were performed with the PfuTurbo DNA polymerase (Stratagene Cloning Systems), which gives blunt-end products, and primer pairs W2 (5′-GNTACCTTGTTAACGACTT-3′; *E. coli* position R1509) and W18 (5′-GGAGTTTGATCMTGGCTCAG-3′; *E. coli* position F9) or W34 and W49 (V3 region amplification). A three-stage programme with 25 amplification cycles was performed. The sizes of the amplification products were confirmed by electrophoresis on a 0.7% (w/v) agarose gel, and bands of the proper size range (≈1500 bases) were excised and eluted with the Qiaex II gel extraction kit (Qiagen). Plasmid ligation and *E. coli* transformation were carried out with the pCR 4Blunt-TOPO kit (Invitrogen). After the transformants had been grown for 24 h, single-clone colonies were taken up with sterile tips and transferred into 0.2 ml tubes containing 7.5 μl of water. The components necessary for PCR amplification with AmpliTaq DNA polymerase were added according to manufacturer's instructions (Perkin-Elmer Cetus), along with T7 and M13 primers. After an initial denaturation step at 94°C for 10 min to lyse the cells, plasmid inserts were amplified using 30 amplification cycles.

Clones providing an amplicon of the correct size (≈1.5 kb) were identified by agarose gel electrophoresis. Amplification products of the positively identified clones were amplified again (PCR–SSCP, see above) using the *rTth* DNA polymerase and the primers pair W34 andW49 for 30 amplification cycles. The PCR–SSCP product of each clone was analysed by SSCP electrophoresis, and the resulting patterns were compared with the digester total 16S rRNA and 16S rDNA patterns. After assignment, several clones belonging to independent libraries were sequenced for each peak.

Sequencing of PCR products from clone inserts

PCR products were sequenced as described previously (Godon *et al*., 1997) using the dideoxy chain-termination method (Sanger *et al*., 1977), with the PRISM ready reaction dideoxy termination cycle sequencing kit (Perkin-Elmer) and primers W12 (5′-TACGCATTTCACCKCTACA-3′; *E. coli* position R700), W07 (5′-CTCGTTGCGGGACTTAAC-3′; *E. coli* position R1100), T7 or M13, using the automated ABI model 373A DNA sequencer from Applied Biosystems (Perkin-Elmer).

Sequence analysis

The total 16S rDNA sequence for the clones obtained from libraries AA, AB and AC and an equal portion (≈200 bp in length) of 16S rRNA for the clones AS, MS and PS (*E. coli* positions 331–533) were used for sequence analysis. Each sequence was compared with sequences available in the databases GenBank and RDP (Ribosomal Database Project; Maidak *et al*., 1994). Comparisons of the sequences between themselves and with the sequences of the databases were performed using LASERGENE software (Dnastar). Phylogenetic trees were calculated by Jukes–Cantor (Jukes and Cantor, 1969) and neighbour-joining (Saitou and Nei, 1987) algorithms.

Nucleotide sequence accession numbers

The nucleotide sequence data reported in this work will appear in the GenBank nucleotide database under accession numbers AF275913to AF275937.

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