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Quantification of Methanogenic Groups in Anaerobic Biological Reactors by Oligonucleotide Probe Hybridization

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The microbial community structure of anaerobic biological reactors was evaluated by using oligonucleotide probes complementary to conserved tracts of the 16S rRNAs of phylogenetically defined groups of methanogens. Phylogenetically defined groups of methanogens were quantified and visualized, respectively, by hybridization of ^{32}P- and fluorescent-dye-labeled probes to the 16S rRNAs from samples taken from laboratory acetate-fed chemostats, laboratory municipal solid waste digestors, and full-scale sewage sludge digestors. *Methanosarcina* species, members of the order *Methanobacteriales*, and *Methanosaeta* species were the most abundant methanogens present in the chemostats, the solid-waste digestors, and the sewage sludge digestors, respectively.

Anaerobic processes have been used for more than a century for the treatment of concentrated municipal and industrial wastewater, for the stabilization of wastewater sludge, and for the treatment of solid waste (24, 26, 29, 31). Applications of anaerobic processes are now being extended to the treatment of dilute industrial wastewaters (25), and even anaerobic sewage treatment is considered achievable under certain conditions (17).

Even though great advances in our understanding of the role of methanogens in anaerobic processes have been made since the discovery of the *Archaea* (formerly *Archaebacteria*) and the placement of the methanogens in this domain (50, 51), much needs to be learned about microbial interactions in anaerobic systems. From a practical standpoint, given the importance of methanogens in anaerobic treatment processes, an understanding of their ecology is essential to make effective control of the startup and operation of anaerobic bioreactors possible.

We are at present using molecular techniques to study the ecology of anaerobic reactor systems containing methanogenic consortia. These techniques take advantage of the phylogenetic framework provided by comparative rRNA sequencing (30). Within this framework, DNA probes have been designed for studies of microbial population structure in relationship to system performance (methane production rates, substrate utilization rates, and carbon flow analyses). Quantification of different populations is based on hybridization of fluorescent-dye- or ^{32}P-labeled oligonucleotide probes to rRNA (most commonly the 16S rRNA) (42). Fluorescent-dye-labeled probes have been used for whole-cell hybridizations to identify and quantify single cells (2, 9). The use of ^{32}P-labeled probes requires extraction of the RNAs from environmental samples prior to hybridization and provides a more general measure of population abundance (43). We earlier described a set of probes for major groups of sulfate-reducing bacteria (SRB) (10) and describe the characterization of group-specific methanogen probes in the accompanying paper (35).

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The following characteristics of rRNA-based techniques make them particularly well suited to studying methanogenic consortia. (i) Methanogens can be identified and quantified at different levels of specificity (phylogenetic depth). The classification of methanogens was based on comparative rRNA sequencing, and the oligonucleotide probes that we designed for their study cover several taxonomic levels, from the *Archaea* domain, to order, family, and genus level (35). Species-specific probes can be designed for more specific applications. (ii) Uncultured (undescribed) methanogens can be identified and quantified by molecular techniques that are presently standard and include selective amplification of the target group 16S rRNA or rDNA from mixed-population nucleic acids using the PCR, cloning, and comparative sequencing of the cloned DNA. By relating the newly obtained sequence(s) with a data base of aligned 16S rRNA sequences, it is now possible to design additional probes specific for the uncultured organisms (e.g., see references 3, 8, 11, and 19). (iii) The use of whole-cell hybridizations provides a basis to estimate the in situ growth rates of methanogens in natural populations, since the cellular ribosome content and, consequently, the rRNA concentration vary with the growth rate (34). (iv) Methanogenic consortia can be studied intact by whole-cell hybridization, allowing individual community members to be identified and enumerated in their natural spatial positions. The characterization of spatial relationships should be particularly useful in the study of the colonization of surfaces, the study of attached-film reactors, and the formation of granular microbial consortia in, for example, upflow anaerobic sludge blanket (UASB) reactors.

The goal of this study was to demonstrate the potential usefulness of rRNA-based techniques to study complex anaerobic microbial communities. The use of taxon-specific methanogen probes to quantify and visualize phylogenetically defined groups of methanogens in laboratory chemostats and solid-waste digestors and in full-scale sewage sludge digestors is described in this paper. To a limited extent, chemical data were related to probe results; however, a rigorous description of the link between microbial structure and function did not fall within the scope of the present study. We anticipate that the most complete understanding of these and similar systems

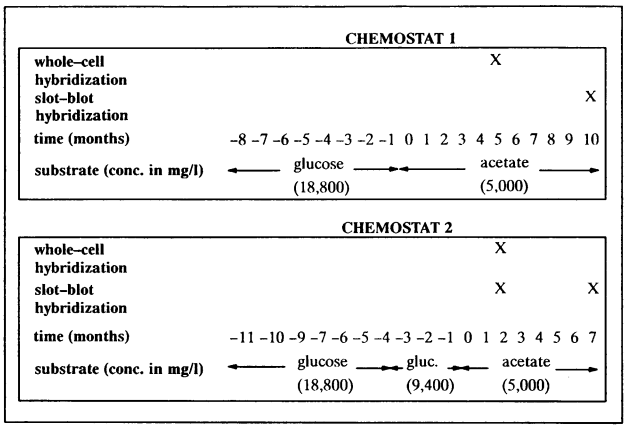

FIG. 1. Time schedule for changes in medium composition and hybridizations with samples taken from chemostats 1 and 2. Conc., concentration.

will come from the use of molecular techniques to complement more traditional culture-based and microscopic identification techniques and chemical analyses.

MATERIALS AND METHODS

Anaerobic biological reactors. (i) Laboratory chemostats. For a period of 8 months, two anaerobic chemostats were operated at 35°C with glucose (influent concentration, 18,800 mg/liter) as the only added carbon source and electron donor. The inoculum for these reactors came from a mixture of glucose- and acetate-fed enrichments maintained in the laboratory of G. F. Parkin at the University of Iowa. After 8 months of operation, glucose was withheld from chemostat 1, while acetate (influent concentration, approximately 5,000 mg/liter) was added to the feed solution as the only added carbon source and electron donor. Chemostat 2 continued to operate with glucose as the only added carbon source and electron donor; however, the concentration was decreased to 9,400 mg/liter. Three months later, glucose was also withheld from chemostat 2, and acetate (influent concentration, approximately 5,000 mg/liter) was added to the feed solution. Figure 1 summarizes the time schedule for the media changes of chemostats 1 and 2.

The chemostats consisted of 2-liter vessels to which anaerobic medium was fed for 30 s every 30 min and from which effluent was withdrawn at the same rate. The retention time of the reactors was approximately 50 days. Mixing was supplied by recirculating the produced gas with diaphragm pumps. The composition of the glucose-acetate growth medium was as follows (milligrams per liter): $C_6H_{12}O_6$ (18,800, 9,400, or 0), CH_3COOH (0 or 1,743), $CH_3COONa \cdot 3H_2O$ (0 or 7,337), NH_4Cl (1,780 or 890), K_2HPO_4 (290 or 145), $MgCl_2 \cdot 6H_2O$ (30), $CaCl_2 \cdot 2H_2O$ (20), $FeCl_2 \cdot 4H_2O$ (2.5), $MnCl_2 \cdot 4H_2O$ (10), KI (2.5), cysteine (10), $NiCl_2 \cdot 6H_2O$ (1.0), $CoCl_2 \cdot 6H_2O$ (1.0), H_3BO_3 (0.5), and $NaHCO_3$ (6,000 or 2,000). Dissolved oxygen was removed from autoclaved media by bubbling the hot media extensively with oxygen-free nitrogen. Subsequently, the media were kept under an oxygen-free atmosphere by maintaining a constant pressure of approximately 4 lb/in^2 of 100% nitrogen. Sulfide was added, after autoclaving, to reduce the media (final concentration of $Na_2S \cdot 9H_2O$ was 250 mg/liter).

Samples (10 ml) for analysis by hybridization were taken from the chemostats after the switch from glucose to acetate media. Chemostat 1 was sampled at 5 and 10 months and chemostat 2 was sampled at 2 and 7 months after the changeover. Figure 1 summarizes the time schedule for sampling the two chemostats. Chemical analyses were performed as described elsewhere (28).

(ii) Laboratory solid-waste digestors. Two anaerobic, benchtop, continuously stirred fermentors with a 3-liter working volume were operated in the laboratory of R. I. Mackie (Department of Animal Science, University of Illinois at Urbana-Champaign). The digestors were fed shredded municipal solid waste in a buffer solution. The municipal solid waste was screened to remove any hard plastic, glass, metal objects, and yard waste. One digestor was operated at mesophilic conditions (40°C), and the other digestor was kept at thermophilic conditions (60°C). The digestors were seeded with approximately 500 g of cattle feces and 500 g of material from a waste lagoon at the University of Illinois swine research farm. After several months of operation at a retention time of 20 days, 10-ml samples were taken from both digestors. The samples were sonicated for 15 min as described for the sewage sludge digestors (see below).

Detailed information about the municipal solid waste digestion system (buffer solution, inoculum, reactor operating conditions, biogas volume produced, biogas composition, and chemical analyses) has been previously published (21).

(iii) Full-scale sewage sludge digestors. The three primary digestors at the Urbana and Champaign Sanitary District (Urbana, Ill.) were fed approximately 60% primary sludge and 40% secondary sludge. The sludge leaving these three digestors was further digested in a secondary digestor. The temperature in all of the digestors ranged from 31 to 37°C.

Samples (10 ml) taken from the three primary digestors and the secondary digestor were transferred to 15-ml Corex tubes. The four Corex tubes were placed in a beaker filled with ice water, which was subsequently placed in a 1200 Bransonic Ultrasonic Cleaner (Branson Cleaning Equipment Company, Shelton, Conn.), filled with ice water to approximately 2 cm from the top, and sonicated for 30 min. The sonicated material was continuously inspected microscopically to obtain a complete disaggregation of the flocs without apparent disruption of the cells. Sludge and biogas analyses were performed at the Urbana and Champaign Sanitary District according to the method described by Greenberg et al. (14).

Oligonucleotide probe synthesis and labeling. The oligonucleotide probes, their target groups, and properties relevant to this study are listed in Table 1. All of the oligonucleotide probes are complementary to regions of the small subunit rRNA molecules. The probes were synthesized on a DNA synthesizer (Applied Biosystems, Foster City, Calif.) at the University of Illinois Biotechnology Center Genetic Engineering Facility. Oligonucleotides used for conjugation with fluorescent dye were synthesized with an aminohexylphosphate linker at the 5′ end (Aminolink 2; Applied Biosystems). Aminolink oligonucleotides were coupled with tetramethylrhodamine isothiocyanate or fluorescein isothiocyanate and purified as previously described (2). Oligonucleotides used for radioactive labeling were purified by high-performance liquid chromatography and 5′-end labeled with ^{32}P by using polynucleotide kinase and [γ-^{32}P]ATP (35).

Cell fixation and whole-cell hybridization. Approximately 1 ml of the cell suspensions obtained after sonication of the samples from the sludge digestors and of the cell suspensions sampled from the chemostats was transferred to a 1.7-ml Eppendorf tube and centrifuged at 14,000 rpm in an Eppendorf microcentrifuge for 3 to 5 min. A 750-μl volume of the supernatant was removed, and the cell pellets were resuspended in 750 μl of fixative, which was prepared immediately

TABLE 1. Oligonucleotide probes and relevant characteristics of each target group

Probe	Target group	Relevant characteristic(s)	Reference
MC1109	*Methanococcales*	Most use H_2-CO_2 and formate	35
MB310	*Methanobacteriales*	Most use H_2-CO_2; some use H_2-CO_2 and formate	35
MG1200	*Methanogenium* relatives	Most use H_2-CO_2 and formate	35
MSMX860	*Methanosarcinaceae*	Most use acetate	35
MS1414	*Methanosarcina* + relatives	All use methanol and methylamines; some use acetate and H_2-CO_2	35
MS821	*Methanosarcina*	Use acetate and other substrates (H_2-CO_2, methanol, and methylamines); generally have high minimum threshold, K_s, and μ_{max} values for acetate	35
MX825	*Methanosaeta*	Use only acetate; generally have low minimum threshold, K_s, and μ_{max} values	35
ARC915	*Archaea*		43
EUB338	*Bacteria*		1
EUK516	*Eucarya*		1
UNIV1392	Virtually all known organisms		29

before use (see below). The resuspended cells were vortexed for 1 min and incubated for 16 h at 4°C. The fixed cells were pelleted by centrifugation at 6,000 rpm (Eppendorf microcentrifuge) for 5 min, the fixative was removed, and the cells were resuspended by vortexing in a solution containing 900 ml of 1× phosphate-buffered saline (PBS) buffer (130 mM NaCl, 10 mM sodium phosphate [pH 7.2]) and 100 μl of 0.1% Nonidet P-40 (Sigma) and were repelleted by centrifugation. This washing step was repeated with 500 μl of 0.1% Nonidet P-40. Finally, the cell pellets were resuspended in 100 to 200 μl of storage buffer (20 mM Tris-HCl [pH 7.2], 0.1% Nonidet P-40), and an equal amount of 100% ethanol was added. The fixed cells were stored at 4°C. The fixative was prepared by addition of 1 drop of 10 M NaOH, 2 g of paraformaldehyde, and 16.5 ml of 3× PBS buffer to 33 ml of double-distilled (dd) H_2O at 60°C. After the paraformaldehyde was dissolved, the solution was cooled on ice, the pH was adjusted to 7.2, and the solution was filtered through a 0.45-μm-pore-size filter.

Fixed-cell samples (1 to 3 μl) were applied to wells on gelatin-coated slides (2), allowed to air dry, and dehydrated by serial immersion of the slides in 50, 80, and 100% ethanol (3 min each). The slides were air dried, and 9 μl of hybridization solution was mixed with 0.5 to 1 μl of rhodamine- or fluorescein-labeled oligonucleotide probe (25 to 50 ng) in the wells. The hybridization solution consisted of 0.9 M NaCl, 0.1% sodium dodecyl sulfate, 20 mM Tris-HCl (pH 7.2), and 40% formamide. The ARC344 and ARC915 probes were used together (dual probing) to enhance signal intensity and alleviate problems of low rRNA content or reduced target availability (1). The slides were incubated at 37°C for 4 h in a moisture chamber (2), rinsed three times with 20 μl of hybridization solution, washed twice for 15 min at 37°C in a moisture chamber using 20 μl of hybridization buffer, and rinsed with ddH_2O at room temperature. The slides were air dried, and samples were mounted in ddH_2O or Citifluor (Citifluor, Ltd., London, United Kingdom) and visualized as previously described (2). Photomicrographs were made with Kodak Ektachrome 400 ASA film. Exposure times were 0.5 s for phase-contrast micrographs and 30 to 45 s for epifluorescence micrographs.

Nucleic acid extraction, slot blot hybridization, and quantification. Approximately 5 to 10 ml of cell suspensions was centrifuged for 5 min at 8,000 × *g*. The supernatant was removed, and the cell pellets were used for nucleic acid extractions (phenol-chloroform-isoamyl alcohol extraction) as previously described (43). The phenol-chloroform-isoamyl alcohol (100:24:1 [vol/vol/vol]) extraction was repeated until no material was observed at the interface between aqueous and organic phases. The nucleic acids were precipitated overnight at −20°C using 0.6 to 0.7 volumes of isopropanol and ammonium acetate (final concentration, 2 μ) as the salt. After a 10-min centrifugation in a microcentrifuge at 14,000 rpm, the RNA pellet was washed with 1 ml of 70% (vol/vol) ethanol and resuspended in ddH_2O. The concentration of the recovered nucleic acid was measured spectrophotometrically, assuming that 1 mg of RNA per ml is equal to 20 optical density units at a wavelength of 260 nm. Nucleic acids from reference organisms were extracted as previously described (43), and the slot blot hybridizations and quantifications of bound probe were performed as described elsewhere (35). Magna Charge membranes (Micron Separation Inc., Westboro, Mass.) instead of MagnaGraph membranes were used. Alternatively, bound probe was quantified by exposing the membranes to Storage Phosphor screens (Molecular Dynamics, Sunnyvale, Calif.) and by scanning the exposed screen with a model 400 Series PhosphorImager (Molecular Dynamics) to obtain a digitized image of the radioactive membrane. This image was analyzed with the ImageQuant software package (Molecular Dynamics).

The abundances of specific groups of organisms (genera, families, orders, or domains) are expressed as percentages of the total 16S rRNA in the sample. The total 16S rRNA was quantified with a universal probe as previously described (43) in combination with RNA standards. To relate the universal and specific probe quantifications, the same reference series of RNAs extracted from pure cultures at different concentrations were applied to the membranes used in each hybridization. The least-squares method was used to calculate the slopes and intercepts of the reference RNA standard curves. RNAs from the following reference organisms were used: *Methanococcus voltae* PS (DSM 1537^T) (MC1109), *Methanobacterium bryantii* M.o.H.G. (DSM 862) (MB310), *Methanogenium cariaci* JR1 (DSM 1497^T) (MG1200), *Methanosarcina* sp. strain WH2 (MSMX860, MS1414, MS821, and ARC915), *Methanosaeta concilii* FE (DSM 3013) (MX825), *Desulfobacterium vacuolatum* (DSM 3385^T) (EUB338), and *Dictyostelium discoideum* (EUK516).

RESULTS AND DISCUSSION

Laboratory chemostats. Since glucose was the only electron donor and carbon source added to the laboratory anaerobic chemostats during the first period of their operation, four major metabolic groups of microorganisms were expected to be present: (i) hydrolytic-fermentative bacteria that convert

glucose and complex polymers (from decaying biomass) to acetate, hydrogen, carbon dioxide, and to a mixture of fatty acids, alcohols, succinate, and lactate; (ii) proton-reducing syntrophic bacteria that biotransform fatty acids, alcohols, succinate, and lactate to acetate, hydrogen, and carbon dioxide; (iii) hydrogenotrophic methanogens that convert hydrogen and carbon dioxide (as well as methanol, methylamines, and formate) to methane and water; and (iv) acetoclastic methanogens that utilize acetate to produce methane and carbon dioxide (55). The presence of two other metabolic groups cannot be ruled out. Acid-forming bacteria produce butyrate and propionate from, for example, acetate and ethanol (37), and homoacetogenic bacteria form acetate from carbon dioxide, while at least some homoacetogens carry out the reverse reaction in the presence of hydrogen-utilizing methanogens (20, 33, 57). Even after glucose was withheld and acetate was added to the chemostats, nonmethanogenic organisms were expected to be present, since the hydrolysis products from decaying biomass could provide additional substrates. However, a gradual depletion of these substrates and fermentation intermediates, along with a corresponding reduction in the abundance of populations which are dependent on those compounds, was expected.

Figure 2 illustrates the results obtained with oligonucleotide probes in the analysis of the temporal changes occurring in the microbial community compositions in these chemostats. During the 5-month period between the two samplings of chemostat 2, a decrease in the relative concentration of bacterial 16S rRNA (EUB338) from 20.3% ± 4.0% to 3.4% ± 0.2% occurred (Fig. 2a). Conversely, the relative concentration of archaeal 16S rRNA (ARC915) increased from 76.3% ± 15.8% to 85.8% ± 5.8%. Figure 2b shows that this increase resulted from an increase in the relative concentrations of members of the family *Methanosarcinaceae* (MSMX860) and of the order *Methanobacteriales* (MB310), while the relative concentrations of *Methanogenium* relatives (MG1200) decreased. The use of a fluorescein-labeled MX825 probe and a rhodamine-labeled MS821 probe in whole-cell hybridizations of samples taken from chemostat 2 (2 months after switchover to acetate) showed that *Methanosarcina* species (MS821) were the major acetoclastic methanogens present in this chemostat, while *Methanosaeta* species (MX825) were virtually absent. A survey of kinetic parameters reported in the literature reveals that, in general, *Methanosarcina* species have higher maximum growth rate (μ_{max}) values than *Methanosaeta* species and that *Methanosaeta* species have lower *K* values (substrate concentration giving one-half the maximum growth rate) and minimum thresholds for acetate than *Methanosarcina* species (12, 15, 22, 27, 38, 40, 45, 47, 56, 57). The relatively high retention time of the chemostats (50 days) suggests that *Methansaeta* species should have been prevalent and that acetate concentrations should have been low. However, acetate levels were found to be relatively high (between 100 and 200 mg/liter), and *Methanosarcina* species apparently dominated in the chemostats, suggesting that other factors were important in the outcome of the competition (e.g., differences in ATP yield and maintenance requirements are factors determining the result of the competition for common substrates among different microorganisms in the rumen [36]). The increase in the relative concentrations of members of the order *Methanobacteriales* and the decrease in the relative concentrations of *Methanogenium* relatives are even more difficult to explain, since the competition for common substrates among different hydrogenotrophic methanogens has been studied less extensively than the competition for acetate among acetoclastic methanogens.

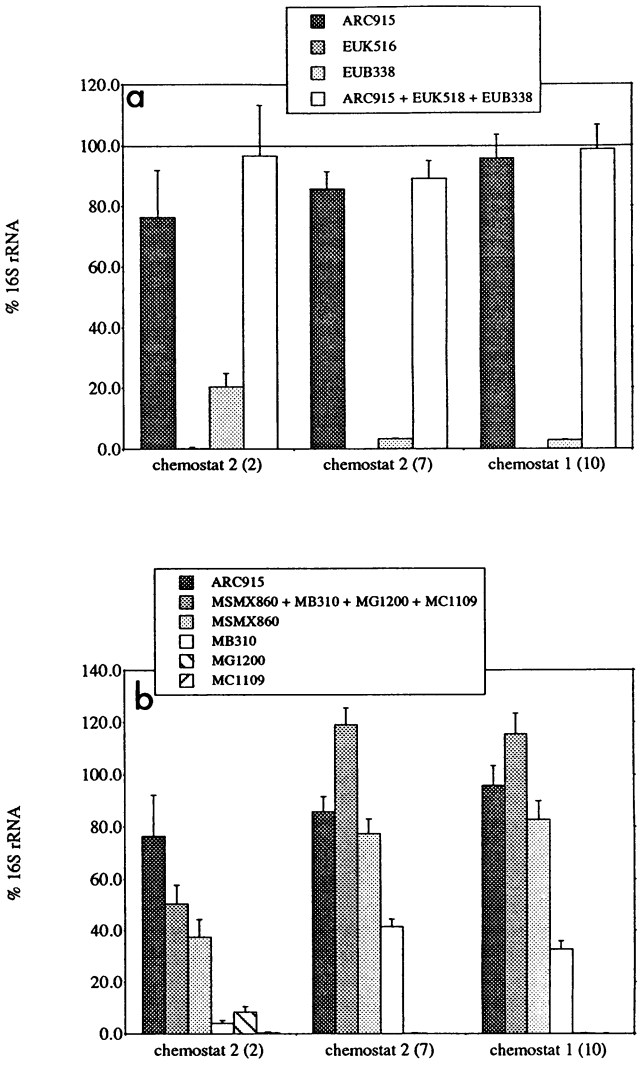

FIG. 2. Microbial community composition in chemostat 2 (2 and 7 months after the switch from glucose to acetate medium) and chemostat 1 (10 months after the changeover). (a) Percentages of 16S rRNAs of *Archaea* (ARC915), *Eucarya* (EUK516), and *Bacteria* (EUB338) and the sum of these three percentages; (b) percentages of 16S rRNAs of *Archaea* (ARC915) and of four specific methanogenic probes, *Methanosarcinaceae* (MSMX860), *Methanobacteriales* (MB310), *Methanogenium* relatives (MG1200), and *Methanococcales* (MC1109) and the sum of the percentages of the last four. Data are the averages of values from triplicate slot blot hybridizations (three neighboring slots on one membrane). The error bars indicate the standard deviations.

For chemostat 1, which was sampled 10 months after the switchover to acetate, the relative bacterial and archaeal 16S rRNA concentrations were even lower and higher, respectively, than those in the sample taken from chemostat 2 7 months after the switchover to acetate (Fig. 2a). Relative bacterial and archaeal 16S rRNA concentrations were 3.0% ± 0.1% and 95.8% ± 7.7%, respectively. Figure 2b shows that *Methanosarcinaceae* (MSMX860) and *Methanobacteriales* (MB310) were the major methanogens present in chemostat 1.

The principle of probe nesting (35) is demonstrated by the combined use of several specific probes and a general probe.

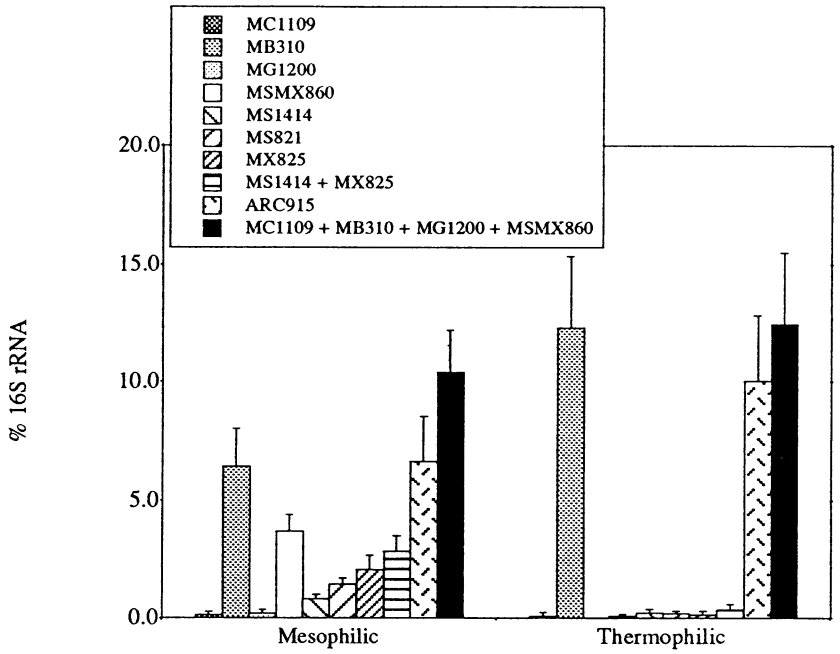

FIG. 3. Methanogenic population structure in mesophilic and thermophilic solid-waste digestors; percentages of 16S rRNAs of *Methanococcales* (MC1109), *Methanobacteriales* (MB310), *Methanogenium* relatives (MG1200), *Methanosarcinaceae* (MSMX860), *Methanosarcina* species and close relatives (MS1414), *Methanosarcina* species (MS821), *Methanosaeta* species (MX825), and *Archaea* (ARC915) and sums of percentages of MS1414 and MX825 and of MC1109, MB310, MG1200, and MSMX860. Data are the averages of values from triplicate slot blot hybridizations (three neighboring slots on one membrane). The error bars indicate the standard deviations.

For example, the percentage of ARC915 (*Archaea*) plus the percentage of EUK516 (*Eucarya*) plus the percentage of EUB338 (*Bacteria*) should equal 100, since all known life forms belong to one of these three domains (51). Similarly, the percentage of MSMX860 (*Methanosarcinaceae*) plus the percentage of MB310 (*Methanobacteriales*) plus the percentage of MG1200 (*Methanogenium* relatives) plus the percentage of MC1109 (*Methanococcales*) should equal the percentage of ARC915 (*Archaea*). Since environmental conditions did not favor the growth of nonmethanogenic *Archaea* (extreme halophiles, thermoacidophiles, the *Archaeoglobales*, the *Thermococcales*, and the thermophiles placed in the *Crenarchaeota* kingdom [51]), no *Archaea* other than methanogens were expected to be present in these chemostats. Figure 2a shows that the more general probe nesting requirement was well met, since the sum of the results obtained with the three domain-specific probes was close to 100%. The second requirement was reasonably well met. The sum of the results obtained by using the four methanogenic probes was relatively close to the result obtained with the *Archaea*-specific probe (Fig. 2b).

Whole-cell hybridizations with a rhodamine-labeled MS821 probe for samples taken from chemostat 2 (2 months after the switchover to acetate) and chemostat 1 (5 months after the switchover to acetate) showed that in chemostat 1, *Methanosarcina* species were present in multicellular clusters or cell packets, whereas their predominant appearance in chemostat 2 was unicellular (results not shown). *Methanosarcina* species are the only members of the domain *Archaea* for which uni- and multicellular forms have been observed (23, 54). For example, *Methanosarcina mazei*—one of the better-studied *Methanosarcina* species—normally grows as large aggregates of cells (multicellular clusters or cell packets); however, under certain conditions, *M. mazei* grows as individual cells or as laminae, i.e., multicellular flat shapes which can be observed macroscopically and which have minimal thicknesses compared with their lengths and widths (23, 54). The form of *M. mazei* appears to be controlled by environmental conditions, such as cation concentrations, substrate type and concentration, and inoculum size (23). Our observations of uni- and multicellular forms of *Methanosarcina* species are interesting in the context of these studies, but detailed analyses of the environmental conditions in the chemostats did not fall within the scope of this study.

Laboratory solid-waste digestors. An anaerobic digestor is a microbial community in which the same four major metabolic groups of microorganisms described earlier need to interact effectively for organic matter to be converted efficiently into methane. The effective conversion of organic matter to methane requires the microbial community structure to have unique characteristics, which may depend on the operating conditions (e.g., substrate, temperature, retention time, and other operating parameters). For example, since syntrophic and methanogenic bacteria are very slow growers, an upset of the community structure may require a long recovery period. Similarly, the startup (time period necessary to reach steady state) of an anaerobic digestor may require several weeks to months, depending on the operating conditions and the size and type of the inoculum used (6). The potential of 16S rRNA-targeted probes to study the startup period of anaerobic digestors is illustrated in Fig. 3, which gives the methanogenic population structure in a mesophilic and thermophilic solid-waste digestor several months after startup.

Members of the order *Methanobacteriales* (MB310) constituted the major hydrogenotrophic methanogens present in both solid-waste digestors, whereas the other hydrogenotrophic methanogens—members of the order *Methanococcales* (MC1109) and *Methanogenium* relatives (MG1200)—were vir-

tually absent. The relatively high retention time of the digestors (20 days) suggests that *Methanosaeta* species should have been present in the reactors (see above). Although both *Methanosarcina* (MS821) and *Methanosaeta* (MX825) species were present in the reactors, their relative concentrations were low (especially in the thermophilic reactor). The total methanogenic population (represented by the percentage of ARC915 or by the percentage of MC1109 plus the percentage of MB310 plus the percentage of MG1200 plus the percentage of MSMX860) was slightly higher in the thermophilic digestor than in the mesophilic digestor, which is consistent with the slightly higher methane production observed in the thermophilic digestor (the percentage of methane in the biogas was 37.4% for the thermophilic digestor, versus 33.6% for the mesophilic digestor [21]). The percentages of methane in the biogas for both digestors were much lower than the corresponding values reported in the literature for the anaerobic digestion of the organic fraction of municipal solid waste (e.g., 53 to 73% [32]).

Preliminary probe results suggest that SRB were also present in significant amounts in both digestors (data not reported). SRB play a fundamental role in anaerobic ecosystems. In the presence of sulfate, they can compete with methanogens for available electrons and with proton-reducing syntrophs for fermentation products such as propionate, butyrate, lactate, and ethanol (48, 49). In the absence of sulfate, several SRB can act as syntrophic bacteria and favor methanogenesis by providing their substrates: hydrogen, formate, and acetate (4, 18, 44, 46, 52, 53). The observation that methanogens are generally poor competitors with SRB in the presence of sulfate has been observed in many environments (13, 16, 48). Although sulfate concentrations in the solid waste and sulfide production were not measured, the relatively low concentrations of methanogens and the low percentage of methane in the biogas are indications of the presence of sulfate in the solid waste. Typical sulfate concentrations in shredded domestic refuse are 0.44 mg/g (dry weight) of refuse (5), and a typical leachate from domestic refuse contained 1,380 mg of sulfate per liter (39). It is likely that SRB were competing more effectively for available electrons than methanogens, which resulted in low concentrations of methanogens and a low methane yield. A complete analysis, which integrates probe results, chemical analyses, and activity measurements, is necessary to address the suggested competition between SRB and methanogens in more detail.

The probe nesting requirement was reasonably well met for the two samples analyzed. The sum of all methanogen target groups (percentage of MC1109 [*Methanococcales*] plus the percentage of MB310 [*Methanobacteriales*] plus the percentage of MG1200 [*Methanogenium* relatives] plus the percentage of MSMX860 [*Methanosarcinaceae*]) nearly equals the percentage of ARC915 (*Archaea*). Similarly, the sum of MS1414 (*Methanosarcina* species and close relatives) and MX825 (*Methanosaeta* species) nearly equals the quantification by probe MSMX860 (*Methanosarcinaceae*).

Although the methanogenic makeup of the solid-waste digestors was determined at only one point in time, the potential for using 16S rRNA-directed probes in combination with chemical analyses to monitor the startup of anaerobic solid-waste digestors was illustrated. For example, operation problems associated with SRB and the types and/or sizes of inocula might be detected at an early stage of startup by using the methanogenic probes and probes for different phylogenetic groups of SRB (10). This could, in principle, provide a rational basis for modifications directed to improve digestor performance. For example, the addition of inhibitors for SRB (7) or the use of different types and/or sizes of inocula (6) could be investigated systematically.

Full-scale sewage sludge digestors. Although about two-thirds of the influent biological oxygen demand is processed through anaerobic sludge digestion in a conventional sewage treatment plant, this process usually receives less operational attention than the activated sludge process (41). For example, the routine analyses performed at the Urbana and Champaign Sanitary District include only the percentage of total solids, the percentage of volatile solids, alkalinity, pH, amount of total organic acids in the sludge, and composition of the biogas. There is a well-recognized need for diagnostic techniques to better evaluate the process operation of sludge digestors (41). Deviation from the steady-state microbial composition and activity could serve as an indication for process problems.

The use of the methanogen probes to characterize the steady-state microbial makeup of four digestors operated at the Urbana and Champaign Sanitary District is illustrated in Fig. 4. The four digestors demonstrated very similar methanogenic makeups. *Methanogenium* relatives (MG1200) and *Methanosarcinaceae* (MSMX860) were the most-abundant methanogens present in the digestors. Approximately 5 to 7% and 10 to 13% of the total 16S rRNA belonged to these two groups, respectively. Within the family *Methanosarcinaceae*, the *Methanosaeta* species (MX825) were dominant, while *Methanosarcina* species and close relatives (MS1414 and MS821) were present only at very low levels. The concentrations of organic acids in digestors D1, D2, and D3 varied from 66 to 77 mg/liter (as acetate), while the concentration of organic acid in digestor D4 was 213 mg/liter. Although the acetate concentrations were not determined, acetate levels had to be lower than or equal to the organic acid levels. The acetate and organic acid concentrations of sludge samples from 19 full-scale sewage sludge digestors (41) indicate that, on average, acetate concentrations were 73% ± 29% of the total organic acid concentrations. The relatively low levels of acetate give a competitive advantage to *Methanosaeta* species (see above), and, therefore, very low levels of acetoclastic methanogens other than *Methanosaeta* species were expected. Whole-cell hybridizations of sludge samples with a fluorescein-labeled MX825 probe and a rhodamine-labeled MS821 probe gave results which are consistent with slot blot hybridizations (although no precise quantification was attempted); very few *Methanosarcina* species were visible, while *Methanosaeta* species were abundant (results not shown).

The presence of H_2S in the digestor gas indicated that sulfate reduction was also taking place, but, since the H_2S concentration was relatively low (76 ppm), sulfate reduction did not appear to be very important. Generally, H_2S levels higher than this are observed in the digestor gas of sewage sludge digestors. Speece (41) reported an average H_2S content of 2,200 ± 1,800 ppm in the digestor gas of 30 full-scale sewage sludge digestors. Another indication for the limited extent of sulfate reduction in these digestors was the relatively high methane content of the digestor gas (61 to 63%). Thus, if SRB levels were significant, as our preliminary probe results suggest, although SRB were expected to compete with methanogens for available electrons, they were apparently simultaneously favoring methanogenesis by acting as syntrophs. However, a more complete analysis is necessary to address the suggested interaction between SRB and methanogens in more detail.

The probe-nesting requirements were reasonably met for all samples analyzed. Figure 4 shows that the sums of the values obtained with the four specific methanogenic probes are relatively close to the results obtained with the *Archaea*-

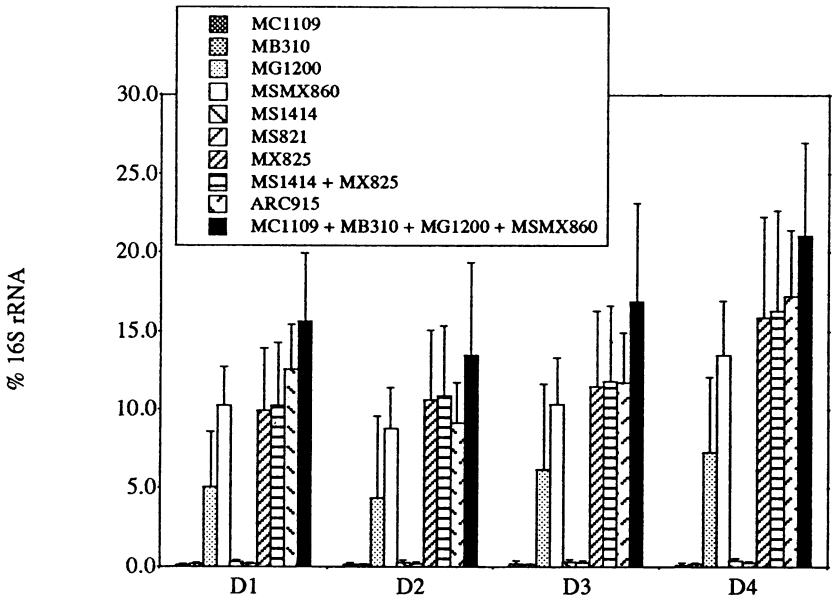

FIG. 4. Methanogenic population structure in four full-scale sewage sludge digestors; percentages of 16S rRNAs of *Methanococcales* (MC1109), *Methanobacteriales* (MB310), *Methanogenium* relatives (MG1200), *Methanosarcinaceae* (MSMX860), *Methanosarcina* species and close relatives (MS1414), *Methanosarcina* species (MS821), *Methanosaeta* species (MX825), and *Archaea* (ARC915) and sums of percentages of MS1414 and MX825 and of MC1109, MB310, MG1200, and MSMX860. Data are the averages of values from triplicate slot blot hybridizations (three neighboring slots on one membrane). The error bars indicate the standard deviations.

specific probe. The sums of the MS1414 and MX825 results nearly equal the MSMX860 values.

Conclusions. This study suggest that the use of 16S rRNA-targeted probes, in combination with chemical analyses, is well suited to the study of the microbial ecology in anaerobic treatment systems. An understanding of the fundamental role that methanogens and SRB seem to play in anaerobic treatment processes should aid in the evaluation and control of process operation. However, the economical and practical importance of anaerobic microbial communities is not limited to biological treatment systems. The presence of methanogens and SRB in the digestive tracts of humans and animals and the apparent importance of anaerobic microorganisms in corrosion make their ecology and techniques for studying these communities a subject of more-general interest. In addition, these probes should provide a perspective for the study of open environmental systems in which anaerobic microbial communities are important, such as marine and freshwater sediments.

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