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Quantitative Immunologic Analysis of the Methanogenic Flora of Digestors Reveals a Considerable Diversity

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To determine which methanogens occur in digestors, we performed a quantitative immunologic analysis of a variety of samples. A comprehensive panel of calibrated polyclonal antibody probes of predefined specificity spectra was used. This allowed precise identification of bacteria by antigenic fingerprinting. A considerable diversity of methanogens was uncovered, much larger than previously reported, encompassing at least 14 strains of 11 species. Strategies were developed to measure the load of any given methanogen in a sample and to compare samples quantitatively. Two methanogens were found to predominate which were antigenically closely related with either *Methanobacterium formicicum* MF or *Methanobrevibacter arboriphilus* AZ. Fundamental data, probes, and methods are now available to monitor methanogenic subpopulations during digestor operation and thus learn about their respective roles and predictive significance.

The need for recycling wastes is imperative in view of the disposal and public health problems created by the large amounts of municipal and industrial refuse (6, 9, 10, 13, 17; M. S. Switzenbaum, ASM News **49**:532–536, 1983; S. H. Zinder, ASM News **50**:294–298, 1984). Methanogens (1, 20) play a key role in recycling organic wastes along with other bacteria (5, 7, 16, 18, 19; Switzenbaum, ASM News; Zinder, ASM News). These microbes can be used to advantage for processing organic materials in digestors, but they are not well understood. Important questions concerning diversity and ecophysiology of methanogens remain unanswered. Crucial issues must be clarified before optimization of digestors becomes a reality. It is of interest to establish the variation, if any, of the methanogenic flora in relation to type of waste and digestor and to time during digestor operation (10, 12, 13). The most meaningful data will, however, only be obtained by means that identify methanogens at the species and subspecies levels, for example, at the immunotypic level. Quantification of immunotypic subpopulations should be done to increase the usefulness of the data for process control.

Immunology provides strategies and tactics useful for rapid and accurate identification and quantification of methanogens (2, 10, 14). Methods and probes have been developed to study methanogens in axenic cultures (11, 14) and in complex ecosystems (8, 12, 15, 19). This report describes the use of immunotechnology for quantitative analysis of the methanogenic flora of digestors. Major topics examined are determination of (i) the composition of the flora, (ii) the loads of any given methanogen in different samples, and (iii) the loads of various methanogens in a single digestor.

MATERIALS AND METHODS

Panel of reference methanogens. The following strains in our antigen repository (2, 13) were used as references: *Methanobrevibacter smithii* PS and ALI; *Methanobacterium formicicum* MF; *Methanosarcina barkeri* MS 227, W, and R1M3; *Methanobacterium bryantii* MoH and MoHG; *Methanospirillum hungatei* (*Methanospirillum hungatii*) JF1; *Methanobrevibacter ruminantium* M1; *Methanobrevibacter arboriphilus* (*Methanobrevibacter arboriphilicus*) DH1, AZ, and DC; *Methanobacterium thermoautotrophicum* GC1 and ΔH; *Methanococcus vannielii* SB; *Methanococcus voltae* PS; *Methanogenium marisnigri* JR1; *Methanogenium cariaci* JR1; *Methanosarcina mazei S6*; *Methanosarcina thermophila* TM1; *Methanomicrobium mobile* BP; *Methanothermus fervidus* V24S; *Methanolobus tindarius* Tindari; *Methanococcus maripaludis* JJ; *Methanosphaera stadtmaniae* MCB3; *Methanoplanus limicola* M3; *Methanococcus thermolithotrophicus* SN1; *Methanotrix soehngenii* Opfikon; and *Methanothrix* sp. strain CALS-1.

Antibody probes. Polyclonal antibody probes were used which were derived from antisera made against the 31 reference methanogens listed above. Each antiserum was titrated against the homologous (immunizing) methanogen, and the S probe (i.e., the highest dilution of the plateau of the antiserum titration curve) was used for indirect immunofluorescence (IIF) (4, 13). The D (digestor) probe was obtained by diluting the S probe twofold; the D/4 probe was obtained by diluting the D probe by a factor of 4. Both the D and D/4 probes were used for quantitative slide immunoenzymatic assay (SIA) (3, 4) and were adopted after a series of preliminary experiments. In these, series of twofold dilutions of the antisera were tested with digestor samples to determine the kinetics of the enzymatic reaction. The purpose was to determine which dilutions would give A_{450} readings within the range 0 to 0.8 and would yield normal hyperbolic curves. The D probe satisfied the above criteria.

Samples. A variety of thermophilic (e.g., digestors A, C, and D) and mesophilic digestor samples were chosen from our collection for this study. Portions of these samples were formalinized, and just before use the solid was separated from the fluid phase by centrifugation. The pellet was washed two times with phosphate-buffered saline (pH 7.2) and then resuspended in phosphate-buffered saline to obtain an A_{660} of 0.200 to 0.300. Of this suspension, 5 μl was deposited onto each circle of an SIA slide, allowed to dry by evaporation, and then heat fixed as described previously (4, 12). These cell concentration and volume per circle values were found to be suitable after testing higher and lower

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concentrations and other volumes per circle in addition to 5 μl.

Assays. SIA constellation (12) was used to study all samples. The sample and the reference methanogens corresponding to the probes used in the assay were tested in parallel. Gram staining, IIF, and SIA were done as described previously (3, 4, 12, 13). Sera from nonimmunized rabbits at the same dilutions as the probes were used as negative controls for IIF and SIA. In each sample, the morphotypes present were identified by phase-contrast microscopy in unstained preparations and by bright-field illumination microscopy in Gram-stained preparations. Findings were correlated with autofluorescence results from fresh samples. The reactivity of each morphotype with all S probes was then established by IIF. Thus, each immunoreactive morphotype was identified on the basis of morphology, reactivity with the most reactive probe(s), and reactivity with other probes when a morphotype reacted with more than one probe (i.e., every reactive morphotype was antigenically fingerprinted [11, 14]). There was always agreement between the morphology of a reactive morphotype and that of the corresponding reference methanogen. There was also agreement between the known specificity spectra of the probes and the range of morphotypes found to react with the probes. IIF-reactive morphotypes were evaluated as to reaction intensity with each probe. Two main categories of data were then collected. One included all morphotypes which reacted with an intensity of at least 2 with at least one probe and, when present in numbers of five or more, per SIA circle. The other category included morphotypes that reacted with an intensity of no more than 1 with any probe no matter their abundance or the number of probes which were reactive with them, and morphotypes which were present in numbers of four or fewer per SIA circle regardless of the intensity of their reaction and of the number of probes that reacted with them.

Quantitative measurements by SIA were done as described in Results, using the D and D/4 probes of each antiserum in the bank. The A_{450} readings obtained with the negative controls were subtracted from the readings obtained with the probes to calculate absolute values.

Quantitative comparisons between strain loads in any given sample and the loads of any given strain in all digestors were done by comparing SIA reactions over a minimum of 45 min. To simplify matters, final comparisons are presented with the peak value of each curve. If this peak A_{450} value was less than 0.100, the result was considered negative, i.e., it was assumed that the methanogen measured by the probe was not present in the sample or that it was present but in quantities below the limits set by the cutoff criterium of 0.100. This cutoff level was adopted because it corresponds to a reading of 0.150, which is threefold higher than the mean A_{450} reading obtained with all negative controls (this mean value was 0.0386 [n = 545]).

RESULTS

Methanogens identified by antigenic fingerprinting by using IIF. The immunologic identity of the reactive morphotypes found in all digestor samples examined is shown in Fig. 1. The morphotypes included are only the ones that reacted with an intensity of ≥2 with the most reactive probe and that numbered five or more per SIA circle. The number of bars, their height, and the list of reference methanogens in the abscissa form a unique pattern for each digestor sample.

A total of 52 findings were made in the 14 digestor samples, including 14 strains of 11 species (Fig. 2). The digestors contained variable numbers of strains from eight in digestor B to only one in digestor K. The most common ranges were three strains in four digestors and two strains in three digestors. The strain found most often was detected with the probe for *Methanobacterium formicicum* MF, immediately followed by a strain demonstrated by the probe for *Methanobrevibacter arboriphilus* AZ. These methanogens were found in 13 and 12 digestors, respectively. Strains CALS-1, JF1, DC, and ALI (or methanogens closely related with them) were the least frequent since each was found in

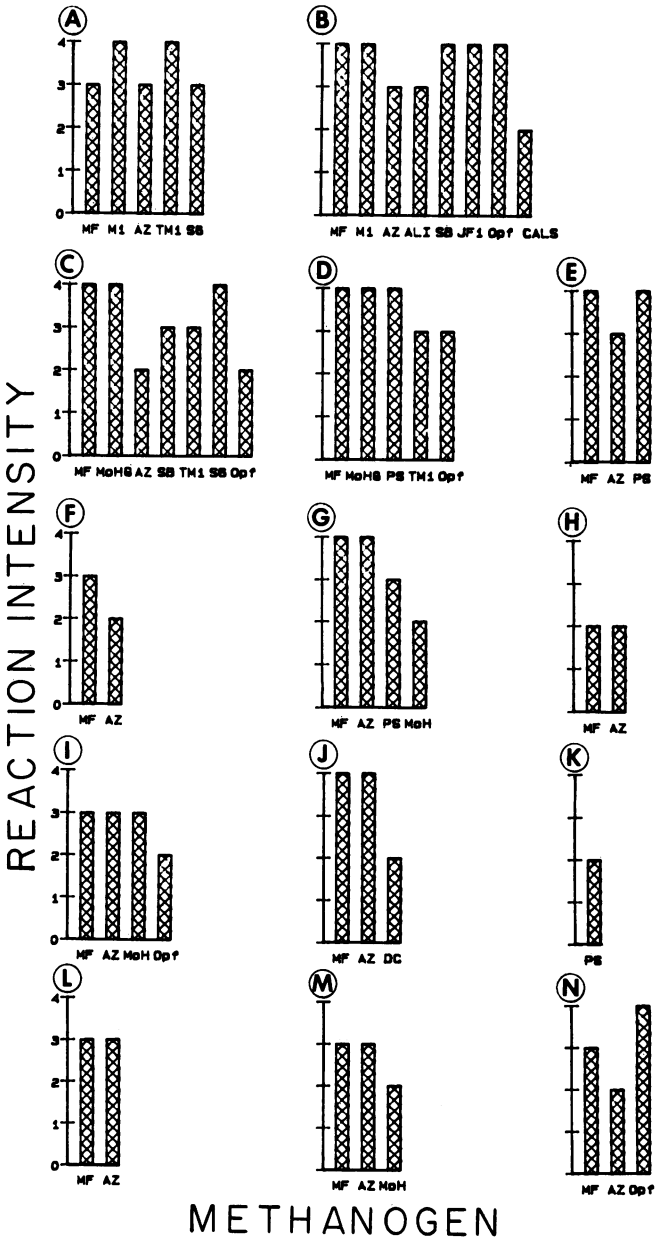


FIG. 1. Methanogenic flora of digestors (A through N). Each bar represents the IIF reaction intensity of the reactive strains with the S probe for the reference methanogens shown in the abscissa. See list of reference methanogens in Materials and Methods. Opf, Opfikon.

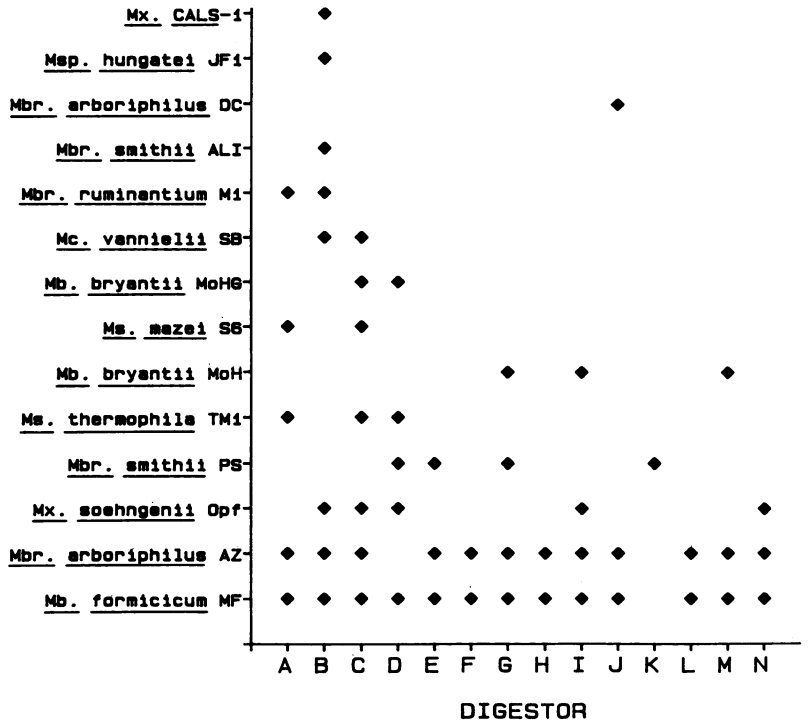


FIG. 2. Methanogens identified in the digestors shown in the abscissa, using IIF and the S probes for the reference organisms listed in the ordinate. Opf, Opfikon.

only one digestor. Examples of some of the morphotypes found are shown in Fig. 3.

Measurement of strain load per digestor sample by SIA. Every sample was assayed with the D and D/4 probes derived from the 31 antisera in the bank. Each SIA run included the D and D/4 probes from at least two antisera and the corresponding negative controls. The negative control readings were subtracted from those obtained with the corresponding probes to calculate the absolute A_{450} values. Typical SIA reaction curves are illustrated in Fig. 4. These types of curve were obtained with all D probes, except those for strains TM1 in digestor A and AZ in digestor N. In these cases, supraoptimal curves were obtained as illustrated in Fig. 5 for D probe anti-TM1. The D/4 probe, however, did produce a typical curve. Data in Fig. 5 also show a typical antibody titration effect apparent only when the two curves are compared in their optimal regions, i.e., up to 15 min into the enzymatic reaction. In this portion of the curves, readings obtained with the D/4 probe are approximately fourfold lower than those obtained with the D probe, as one would expect from the fact that the D/4 probe is fourfold less concentrated than the D probe.

Comparative measurements. Two categories of quantitative comparisons were done: the respective loads of each methanogen occurring in a digestor (intradigestor comparison) and the loads of each strain in the various digestors (interdigestor or intrastrain comparison). An example of intradigestor comparison is shown in Fig. 4 (digestor B). The MF load is considerably greater than the loads of M1, AZ, and ALI, which are about equal.

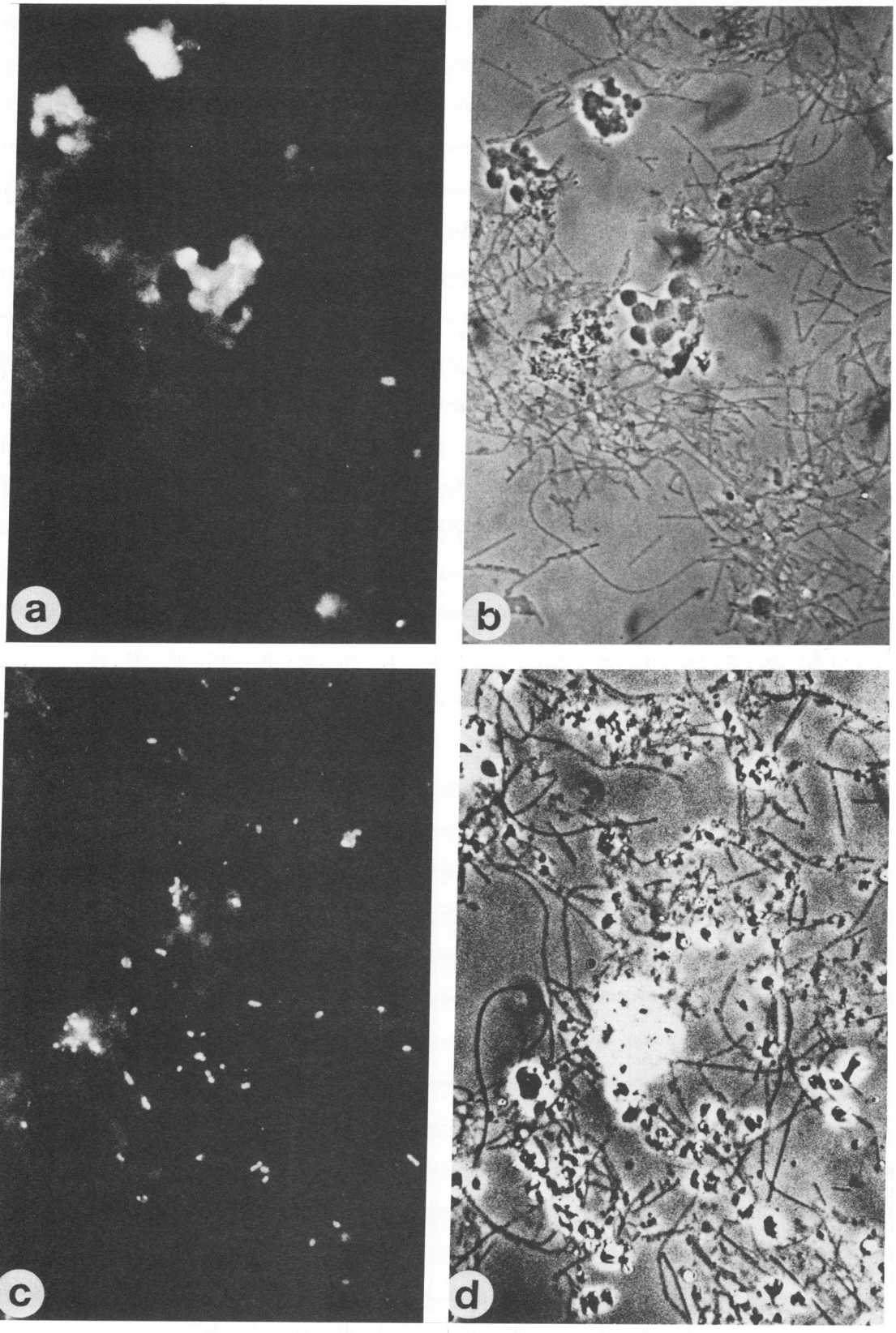
An example of interdigestor comparison is shown in Fig. 6. The load of strain MF was considerably greater in digestors M and N than in the rest; it was comparatively much lower in digestors K, C, D, and H; and it was intermediate in digestors A, B, E, F, G, J, I, and L.

A simplified picture of all SIA measurements is shown in Fig. 7, in which only the peak A_{450} values in all curves were included provided they were ≥ 0.100. By applying this cutoff criterium, we found a total of 28 methanogens in 12 of the 14 digestor samples studied (Fig. 7 and 8). The most common strains measurable by SIA were shown with the probe for *Methanobacterium formicicum* MF and the probe for *Methanobrevibacter aboriphilus* AZ, which was also the case for IIF. Digestor I showed the widest range of different strains, with four of them. Digestor B showed only three strains, rather than the eight shown by IIF (Fig. 1). This indicated that five strains (i.e., those demonstrated by probes for M1, AZ, SB, Opf [Opfikon], and CALS-1) were present in digestor B in small numbers, and although the strains reacted relatively strongly (≥ 2) by IIF, they were not abundant enough to be measurable by SIA when the cutoff A_{450} value of 0.100 was used. However, if a cutoff value of 0.05 was used, seven strains could be demonstrated in digestor B (data not shown). In no case did SIA detect a strain that had not been demonstrated by IIF.

Figure 7 also shows that the number of bars, their height, and the list of reference methanogens in the abscissa form a unique pattern for each digestor sample.

DISCUSSION

Descriptions of the methanogenic flora of digestors have typically mentioned only two or three methanogens, such as rods resembling *Methanothrix* sp. and organisms similar to *Methanosarcina* species. This absence of strain variety and the vagueness of the terms reflect a lack of means for unequivocal identification. In this study, a comprehensive panel of antibody probes of predefined specificity spectra was used to identify precisely, at the immunotype level,


a
b
c
d

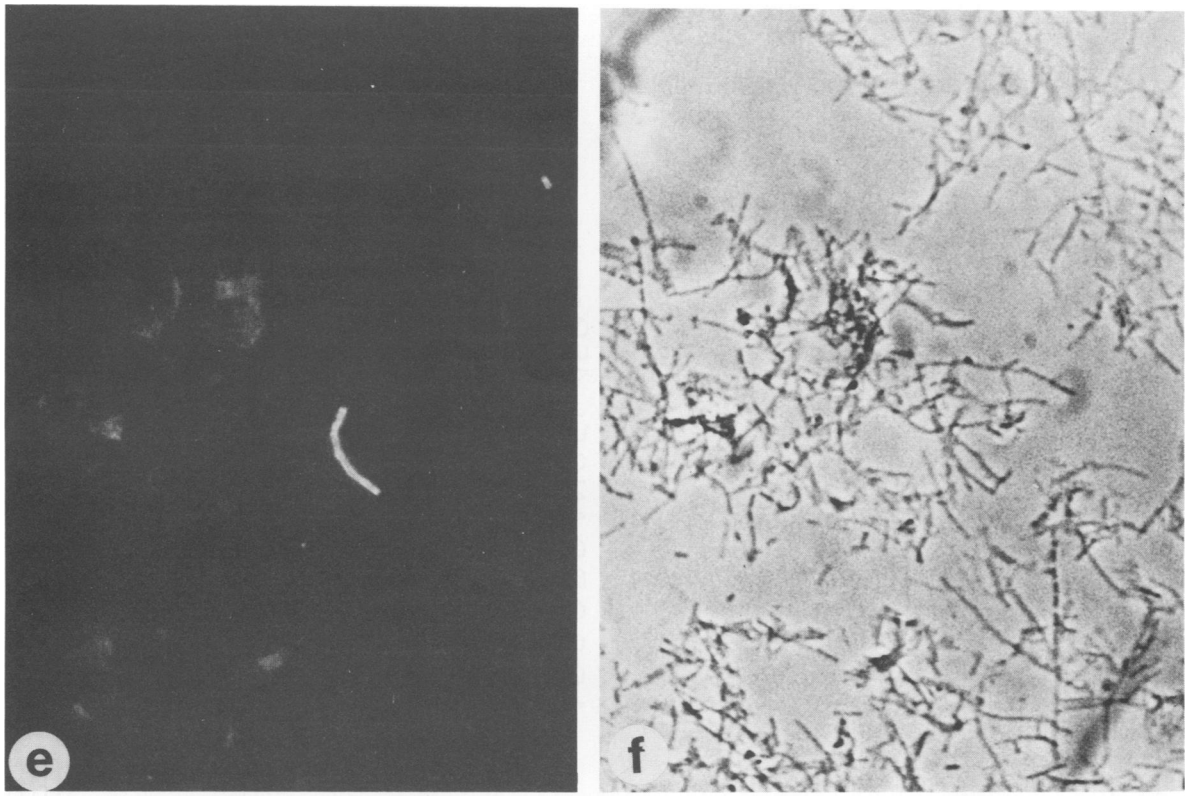


FIG. 3. Examples of methanogens revealed by IIF (epifluorescence optics) in digestor samples by using the S probes for *Methanosarcina mazei* S6 (a), *Methanobrevibacter smithii* PS (c), and *Methanobacterium formicicum* MF (e). These same microscopic fields are shown by phase-contrast optics in panels b, d, and f, respectively, to demonstrate the complexity of the microbial mixtures in which the methanogens were identified (magnification, × 1,000).

methanogens in various digestor samples. The range of strains found was considerable larger than previously reported. Up to eight different methanogens were identified in a single digestor. Overall, 14 strains of 11 species were found. These are minimal estimations since strict criteria were applied. Methanogens occurring in relatively low numbers or weakly related with reference organisms were ignored.

Considering also weakly IIF reactive and rare strains (i.e., those giving a reading of <2 and those present in quantities lower than five per slide circle regardless of their reactivity), seven additional strains of five additional species were found, above and beyond those detected by applying more stringent criteria. These additional methanogens (not shown in Fig. 1) were demonstrated with the probes for *Methanobacterium thermoautotrophicum* ΔH and GC1, *Methanogenium cariaci* JR1, *Methanogenium marisnigri* JR1, and *Methanosarcina barkeri* R1M3, 227, and W. In addition another strain of *Methanobrevibacter arboriphilus* was also detected with the probe for DH1. This makes a grand total of 22 strains of 19 species.

IIF revealed the composition of the methanogenic flora and provided a pattern typical of each digestor sample (Fig. 1). This pattern tells which morphotypes are present in the sample and their degree of antigenic similarity with reference methanogens.

SIA reaction kinetics curves obtained with D and D/4 probes showed an ascending limb and then a plateau which remained for the entire observation period (45 to 60 min). In these instances, it was possible to observe a titration effect,

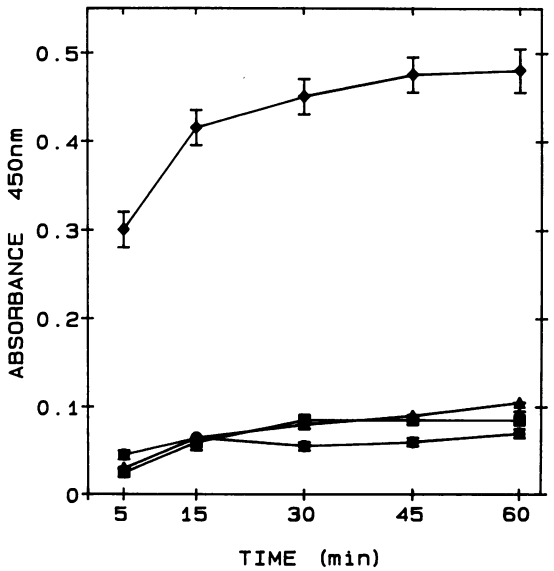


FIG. 4. Example of quantification of loads of various strains in a single digestor (B) by SIA. Sequential A_{450} readings after addition of substrate for peroxidase are shown to illustrate optimal kinetics curves. These were obtained with D probes for *Methanobacterium formicicum* MF (◆), *Methanobrevibacter smithii* ALI (▲), *Methanobrevibacter arboriphilus* AZ (■), and *Methanobacterium ruminantium* M1 (●) (arithmetic mean ± range).

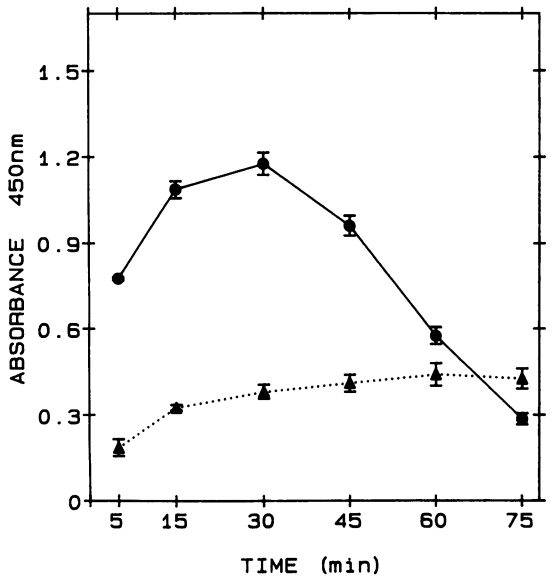


FIG. 5. Quantification by SIA of strain load in a digestor (A) using the D (●) and the D/4 (▲) probes for *Methanosarcina thermophila* TM1. Sequential A_{450} readings after addition of substrate for peroxidase are shown to illustrate a supraoptimal kinetics curve obtained with the D probe as compared with the optimal counterpart obtained with the D/4 probe (arithmetic mean ± range).

i.e., the readings given by the D probe were, as expected, approximately fourfold higher than those obtained with the D/4 probe. Occasionally, a supraoptimal kinetics curve was observed. These exceptions were produced by the probe for TM1 in digestor A and the probe for AZ in digestor N. In these cases, the peak values used for quantitative comparison were minimal estimates. For example, in Fig. 5, the D probe for TM1 produced a supraoptimal curve. By comparing this curve with the D/4 probe, it can be seen that the peak value of 1.175 at 30 min should have been 1.520, i.e., fourfold greater than the value given by the D/4 probe at 30 min, which was 0.380 (0.380 × 4 = 1.520). The same applies at 60 min, at which time the value for the D probe is only 0.575 when it should have been 1.760 (i.e., D/4 probe reading = 0.440 × 4 = 1.760).

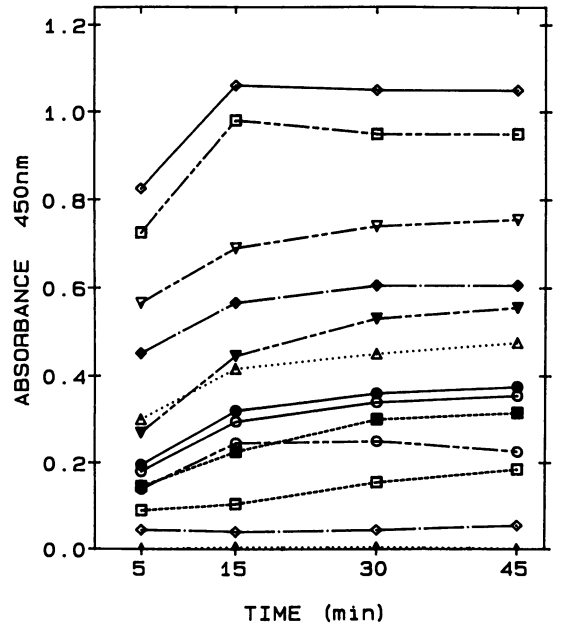


FIG. 6. Example of quantification of loads of a single strain in various digestors by SIA with the D probe for *Methanobacterium formicicum* MF. Symbols: ○, digestor A; △, digestor B; ⊡, digestor C; ◈, digestor D; ▽, digestor E; ⊙, digestor F; ●, digestor G; ▲, digestors H and K; ■, digestor I; ◆, digestor J; ▼, digestor L; □, digestor M; ◇, digestor N (arithmetic mean ± range).

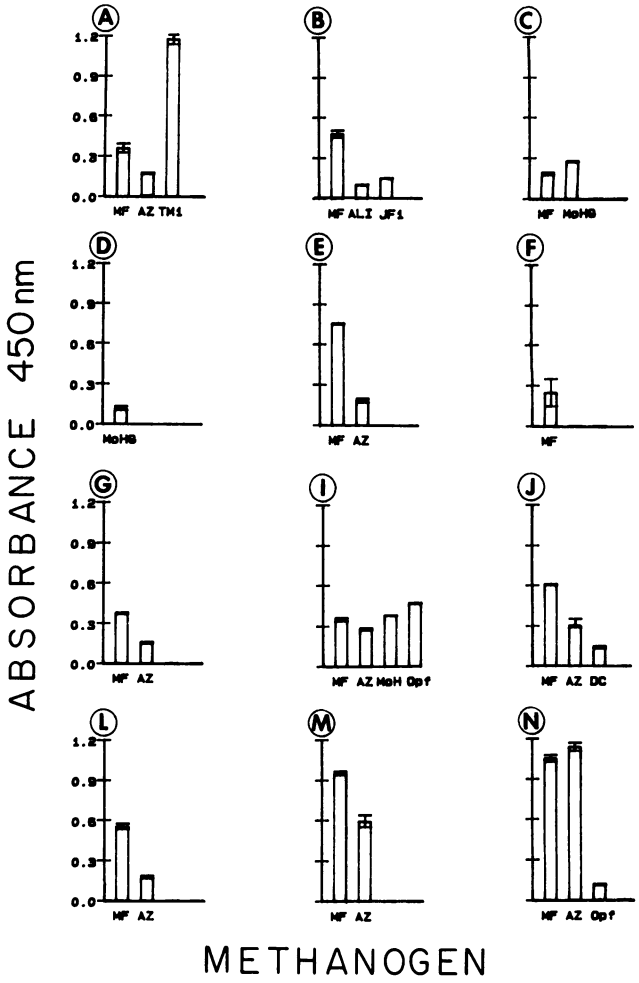


FIG. 7. Components of the methanogenic flora measured by SIA in 12 of the 14 digestors examined (digestors H and K contained methanogens at loads detectable only by IIF, see Fig. 1 and text). Each bar represents the load of the methanogen measured with the D probe for the reference organism shown under it and is the peak A_{450} reading of the SIA reaction curve. (See list of reference methanogens in Materials and Methods.) Opf, Opfikon. Arithmetic mean ± range.

It is interesting that the strains showing the highest load in a single digestor, i.e., those demonstrated with probes for MF and AZ, were also those found most frequently in all the samples examined (Fig. 2 and 8).

SIA revealed a pattern distinctive of each digestor sample (Fig. 7) which, similar to the IIF pattern, shows the various strains occurring in the sample. However, SIA gives a quantitative measurement that is not provided by the IIF

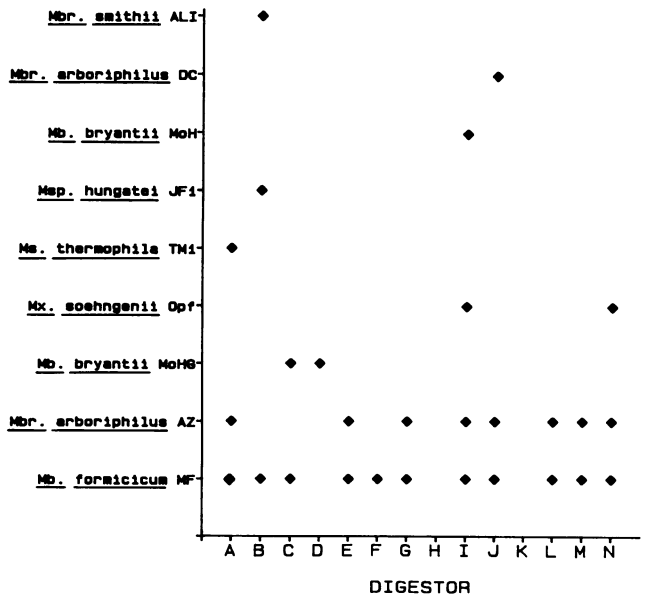


FIG. 8. Methanogens measured in the digestors shown in the abscissa by using SIA and the D probes for the reference organisms listed in the ordinate. No methanogens were detected in digestors H and K.

pattern. The latter furnishes, instead, information on the degree of similarity of each strain with reference organisms.

No matter which cutoff criterium was applied, IIF detected more strains than SIA. However, if an A_{450} of 0.05 was adopted as the cutoff for SIA, the total number of strains detected by this quantitative method increased considerably. In no case did SIA show a strain in a sample which had not been demonstrated by IIF. This is because reactive morphotypes can be identified by IIF even if they occur in minimal numbers (e.g., one or two per SIA circle). On the contrary, a greater number of methanogens of a given strain is required to show a positive result with SIA. While IIF provides a quantitative estimation of the intensity of the reaction of each individual morphotype, SIA measures the overall load of a reactive methanogen in a sample. Microbial counts under the microscope by IIF are useful but laborious. However, it may be necessary when the strain load is lower than that measurable by SIA. Fortunately, low methanogenic loads, i.e., when very few bacterial cells of a strain are present in a sample, can easily be quantified by counting the cells under the microscope by IIF. These counts are more difficult and inaccurate as the number of bacterial cells increases. When the load reaches levels at which direct microscopic counts become unreliable or unfeasible, it can be quantified by SIA. Thus, IIF and SIA complement each other well; they also complement the other elements of SIA constellation such as phase-contrast and bright-field illumination microscopy of unstained and Gram-stained preparations (12).

The SIA quantitative data referred to in this work are relative to each other. Absolute numbers of methanogens can be determined, if necessary, by comparing the SIA readings with standard curves constructed with known numbers of each pertinent reference organism (A. J. L. Macario and E. Conway de Macario, manuscript in preparation).

While the data in this report pertain only to bacterial cells, it should be emphasized that specific cell markers can also be measured in the fluid phase of digestors (12, 15). Thus, complementary profiles of bacterial cells and of their subcellular products can be determined to obtain a complete picture of the methanogenic flora. Elucidation of this flora under various sets of specified conditions should make it possible to advance the biologic design of digestors at least as far as other aspects have progressed (9, 17; Switzenbaum, ASM News).

Three main extrapolations are warranted from the observations in this paper. (i) Means and fundamental data are now available to begin monitoring the diversity and strain loads of methanogens during digestor operation to detect population shifts in relation to other parameters of practical interest. (ii) The probes that specifically identify methanogens in digestor mixtures have the potential of serving as highly refined tools for manipulating target populations (10). (iii) The same immunologic approach used for methanogens could be applied to the study and manipulation of other microbes also important in fermentation biotechnology (16).

ACKNOWLEDGMENTS

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