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Examination of Thermophilic Methane-Producing Digesters by Analysis of Bacterial Lipids†

J. MICHAEL HENSON,[1,2]‡* PAUL H. SMITH,[3] AND DAVID C. WHITE[2]

Agricultural Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, Monticello, Florida 32344[1]; Department of Biological Sciences, The Florida State University, Tallahassee, Florida 32306[2]; and Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611[3]

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Thermophilic methane-producing digesters were examined by the analysis of lipids to determine the microbial biomass, community structure, and nutritional status of the microbes within the digesters. The digesters received a daily feedstock of cattle feed and Bermuda grass, with some digesters receiving additional supplements of propionate, butyrate, or nitrate. Microbial biomass, measured as total extractable lipid phosphate, was decreased in slurries from digesters receiving continuous addition of the fermentation intermediates propionate or butyrate as compared with slurries from control digesters receiving the feedstock alone. In slurries from digesters that received continuous addition of nitrate, the microbial biomass was higher than in the slurries from control digesters. The control digesters had ca. 2.5×10^{11} bacteria per g (dry weight) as determined from total extractable lipid phosphate. Shifts in microbial community structure were observed by analysis of ester-linked phospholipid fatty acids. Statistical analysis of the patterns of phospholipid fatty acids indicated that the digesters receiving different supplements could be distinguished from the control digester and from each other. Poly-β-hydroxybutyric acid, an indicator of metabolic stress, was detected in slurries from all the digesters. Slurries from the nitrate-amended digester had the highest concentration of poly-β-hydroxybutyric acid, whereas slurries from the propionate-amended digester had the lowest concentration. These chemical analyses offer a quantitative means to correlate shifts in microbial biomass, community structure, and nutritional status in complex fermentation systems to the production of a specific end product.

Anaerobic environments are composed of communities characterized by diverse populations of microorganisms. The rumen, for example, has over 200 species of bacteria and at least 13 genera of protozoa (26). Descriptions of the rumen and its microbial ecology are provided by Hungate (16, 17) and Bryant (5, 6). The methane-producing digester is another anaerobic environment with diverse populations of microorganisms and is presently being extensively studied (7, 9, 18, 19, 27, 28, 33). Anaerobic digesters are generally used to stabilize municipal and animal wastes with the concomitant production of methane.

In anaerobic digesters, organic matter is degraded to methane and carbon dioxide by the integrated actions of three metabolically distinct groups (8, 23). These groups are composed of a large number of different species of bacteria, many of which have not been isolated (19). Kirsh (20) and Mah and Sussman (22) recovered about 10% of the nonmethanogenic bacteria present in digesters. Varel (28) was able to culture 8.5 to 14.1% of the direct count of bacteria from a thermophilic digester. Methanogenic bacteria were not cultured. Sixty-four strains were characterized in more detail and were found to be gram-negative fermentative rods (28). Iannotti et al. (19) achieved the highest percentage of colony counts when they were able to record colony counts that were 60% of the direct counts. One-hundred and thirty nonmethanogenic bacterial strains were characterized (19) and were found to be mostly gram-positive anaerobes.

The above studies indicate the difficulties associated with culturing bacteria from environments where bacteria are found in consortia, where metabolic interactions occur, and where bacteria are attached to surfaces. White (29) discussed these problems as they relate to microbial ecology and described new methods developed to overcome them. These analytical methods are based on the examination of lipids to obtain information about the microbial biomass, community structure, and nutritional status of complex microbial ecosystems without the selective removal or growth of bacteria from an ecosystem (29). Total lipid phosphate is an indicator of microbial biomass (32) and correlates well with extractable ATP (31). Analysis of ester-linked phospholipid fatty acids (PLFA) allows the characterization of the microbial community structure (3). Some fatty acids are unique to specific bacteria or to groups of bacteria and function as signatures for these bacteria (29). Gillan and Hogg (13) used this concept to divide the bacterial community of mangrove sediments into several subgroups. Poly-β-hydroxybutyric acid (PHB) has been used as an indicator of the metabolic status of microbial communities (11, 12, 25).

In this study we report the results obtained when the techniques for lipid analysis were applied to thermophilic, anaerobic digesters. These digesters received daily input of feed, with some digesters receiving continuous supplementation of butyrate, propionate, or nitrate, which were added to determine their influences on the production of methane from biomass. The results indicate the usefulness of the application of these techniques to analyze the microbial ecology of anaerobic digestion (fermentation) systems.

MATERIALS AND METHODS

Chemicals. Chloroform and methanol were Baker Resi-analyzed grade and used as purchased (American Scientific

* Corresponding author.

† Florida Agricultural Experiment Station Journal Series no. 6712.

‡ Present address: Robert S. Kerr Environmental Research Laboratory, U.S. Environmental Protection Agency, Ada, OK 74820.

Products, McGaw Park, Ill.). Acetone was of pesticide grade (Fisher Scientific Co., Philadelphia, Pa.).

Digesters. The digesters were located and maintained at the Department of Microbiology and Cell Science, University of Florida, Gainesville. Digesters were constructed from 4-liter aspirator bottles. The digesters were fed once each day with a daily feed that consisted of Bermuda grass and cattle feed, in a 3:1 mix, to result in an organic loading rate of 3.6 g of volatile solids liter^{-1} day^{-1}. The volume of the digesters was 3.6 liters. The hydraulic retention time was 20 days. Digesters were stirred intermittently in 15-min cycles. A water bath was used to maintain the temperature at 55°C. Stock solutions of sodium *n*-butyrate or sodium propionate (Pfaltz and Bauer, Inc., Stamford, Conn.) or sodium nitrate (Fisher) were continuously infused into digesters which were also receiving the daily feed. The rates of addition of the supplements were as follows: sodium butyrate, 15 μmol ml^{-1} day^{-1}; sodium propionate, 10 μmol ml^{-1} day^{-1}; and sodium nitrate, 10 μmol ml^{-1} day^{-1}. Control digesters received only the daily feed. Effluent samples were removed just before feeding. The samples were frozen and transported to Florida State University where they were lyophilized. The lyophilized samples were stored at −70°C until analysis.

Analysis of lipids. Triplicate samples of 50 mg each were placed into 250-ml separatory funnels and were subjected to the modified (32) single phase chloroform-methanol extraction procedure of Bligh and Dyer (1). The lipids were recovered in the chloroform fraction, the chloroform was removed in vacuo, and the lipids were stored in Teflon-lined screw-cap test tubes at −20°C under dinitrogen. The lipid fraction was transferred in chloroform to a silicic acid column (Unisil, 100-200 mesh; Clarkson Chemical Co., Inc., Williamsport, Pa.) and separated into neutral lipids, glycolipids, and phospholipids by elution of 10 ml each of chloroform, acetone, and methanol, respectively. Phospholipids were subjected to mild alkaline methanolysis (33), and the fatty acid methyl esters (FAMEs) were separated from nonsaponifiable lipids by thin-layer chromatography. The thin-layer chromatography plates (Whatman K6 silica gel, 250 μm thick, 20 by 20 cm) were developed in nanograde hexane-ether 1:1 (vol/vol). A band 0.5 cm above and 1.5 cm below an authentic FAME standard was scrapped from the thin-layer chromatography plate, and the FAMEs were eluted with 5 ml of chloroform. The glycolipid fractions were analyzed for the presence of PHB (11).

Double-bond configuration. Double bond position and configuration were determined by analysis of Diels-Alder adducts formed by the reaction of FAMEs with 5,5-dimethoxy-1,2,3,4-tetrachlorocyclopentadiene (Aldrich Chemical Co., Milwaukee, Wis.) as described previously (24).

Gas-liquid chromatography. A Hewlett-Packard model 5880A gas chromatograph was used to measure FAMEs and Diels-Alder adducts. FAMEs were separated in a nonpolar 50-m by 0.2-mm cross-linked methyl silicone fused silica capillary column (Hewlett-Packard) and measured with a flame ionization detector. Hydrogen was the carrier gas. The injector and detector temperatures were 250 and 290°C, respectively. Samples were injected in the splitless mode with the vent opening after 30 s. The initial oven temperature was 80°C and was programmed to 140°C at 2°C min^{-1} and then to 260°C at 4°C min^{-1}, where the temperature was held for 7 min. Concentrations of FAMEs were measured relative to an internal standard with a Hewlett-Packard 3350 data acquisition system.

Mass spectrometry. Gas chromatography-mass spectrometry was performed on a Hewlett-Packard model 5995A gas chromatograph-mass spectrometer equipped with a direct capillary column inlet. A cross-linked methyl silicone fused silica capillary column was used to separate FAMEs. Hydrogen was the carrier gas. The injector temperature was 280°C. Samples were injected in the splitless mode with the vent opening after 30 s. The initial oven temperature was 100°C and was programmed to 300°C at the rate of 4°C min^{-1}. Operating conditions of the mass spectrometer were as follows: electron multiplier, 1,600 V; transfer line, 300°C; source and analyzer, 250°C; autotune file, decafluorotriphenylphosphine (DFTPP) normalized; electron impact energy, 70 eV.

FTIR spectroscopy. Fourier transform infrared (FTIR) analysis with a Nicolet 60 XS instrument was carried out as described previously (23a).

Fatty acid configuration. The fatty acid designation is as follows: number of carbon atoms, number of double bonds, location of double bonds in relation to the omega (methyl) end of the fatty acid. The suffixes c and t for *cis* and *trans* configuration are used. The suffix br indicates that the fatty acid is branched.

RESULTS

Total microbial biomass. The total extractable lipid phosphate (TELP), which is a measure of the viable microbial biomass (32), increased threefold over the input feedstock in slurries recovered from the digesters (Table 1). The two control digester slurries contained essentially the same TELP. The slurries recovered from digesters supplemented with propionate or butyrate contained about 66% of the total microbial biomass measured in the unamended control digester. Slurries from the digesters amended with nitrate had a TELP which was 132% of the slurries from the control digesters (Table 1). The TELP determinations were used to calculate the number of bacteria present in each digester (Table 1).

Microbial community structure. The proportions of PLFAs in the slurries recovered from the digesters and the feedstock are given in Table 2. The PLFAs show the same relative concentrations as the TELP with the exception of the nitrate-amended digester. Slurries from this digester had an increased TELP and equivalent PLFAs when compared with the control digesters (Tables 1 and 2). Statistical analysis of the PLFAs in the slurries recovered from each digester shows significant differences in the community structures of the digesters. Means showing a significant difference ($P < 0.05$) by one-way analysis of variance were compared using the Tukey wholly significant difference test. A map of significant differences between the PLFAs of the feedstock

TABLE 1. Total lipid phosphate from thermophilic, continuously stirred, bench-top digester slurries

Sample	Total lipid phosphate (nmol per mg of sludge)	Bacteria per g (dry wt) ($\times 10^{11}$)[a]
Digester feed	3.47 ± 0.13[b]	0.694
Control digester 1	12.3 ± 1.10	2.46
Control digester 2	12.7 ± 1.90	2.54
Butyrate amended	7.57 ± 0.29	1.51
Propionate amended	8.90 ± 1.12	1.78
Nitrate amended	16.5 ± 0.95	3.30

[a] Assuming 50 μmol of lipid phosphate per g (dry weight) (31) and 1×10^{-12} g (dry weight) per bacterial cell (4).
[b] $\bar{x}$ ± Standard deviation; $n = 3$.

TABLE 2. Proportions of ester-linked fatty acids from the phospholipids from thermophilic, continuously stirred, bench-top digesters[a]

FAME	Proportion of FAMEs from the following sources (%):					
	Digester feed	Control digester 1	Control digester 2	Butyrate amended	Propionate amended	Nitrate amended
Iso 14:0	ND[b]	2.1 ± 0.24	1.6 ± 0.19	ND	ND	0.60 ± 0.17
14:0	ND	2.3 ± 0.17	1.4 ± 0.08	1.3 ± 0.25	2.4 ± 0.43	2.3 ± 0.51
Iso 15:0	Trace	5.5 ± 0.26	5.3 ± 0.16	4.3 ± 0.32	5.0 ± 0.39	3.4 ± 0.42
Anteiso 15:0	ND	4.0 ± 0.22	4.1 ± 0.08	1.4 ± 0.12	2.4 ± 0.19	1.7 ± 0.16
15:0	ND	2.0 ± 0.01	1.6 ± 0.03	2.8 ± 0.21	4.7 ± 0.22	2.2 ± 0.17
Iso 16:0	Trace	15 ± 0.47	18 ± 0.42	13 ± 0.18	6.3 ± 0.16	21 ± 0.11
16:1ω7t	ND	ND	ND	ND	1.0 ± 0.12	0.45 ± 0.03
16:1ω5c	3.0 ± 0.64	1.1 ± 0.10	1.0 ± 0.09	0.88 ± 0.11	1.6 ± 0.10	0.97 ± 0.06
16:0	82 ± 1.4	35 ± 0.44	36 ± 0.86	39.0 ± 0.86	42 ± 0.76	36 ± 0.56
17:1br	ND	2.3 ± 0.07	2.0 ± 0.11	6.1 ± 0.31	3.2 ± 0.10	2.9 ± 0.06
Iso 17:0	ND	4.6 ± 0.07	4.9 ± 0.12	10 ± 0.35	9.6 ± 0.33	9.6 ± 0.32
Anteiso 17:0	Trace	5.1 ± 0.11	6.2 ± 0.20	6.3 ± 0.25	4.7 ± 0.14	4.5 ± 0.10
17:0	1.4 ± 0.07	1.5 ± 0.02	1.5 ± 0.02	2.1 ± 0.05	2.4 ± 0.09	1.3 ± 0.02
18:3ω6	ND	0.13 ± 0.11	Trace	0.7 ± 0.02	0.6 ± 0.06	ND
18:3ω3	ND	1.5 ± 0.15	1.7 ± 0.03	1.9 ± 0.15	0.47 ± 0.06	1.3 ± 0.05
Iso 18:0	ND	0.45 ± 0.03	0.31 ± 0.02	ND	ND	ND
18:1ω9c	ND	0.6 ± 0.6	0.23 ± 0.04	ND	0.55 ± 0.10	Trace
18:1ω7c	Trace	11 ± 0.49	8.1 ± 0.42	4.5 ± 0.82	3.9 ± 0.17	6.5 ± 0.28
18:1ω7t	ND	Trace	0.68 ± 0.03	Trace	Trace	1.2 ± 0.07
18:0	7.8 ± 1.2	3.5 ± 0.06	3.8 ± 0.05	4.7 ± 0.36	5.0 ± 0.21	3.5 ± 0.22
20:0	1.2 ± 0.07	0.65 ± 0.10	0.60 ± 0.02	0.80 ± 0.02	1.2 ± 0.04	0.51 ± 0.04
22:0	1.0 ± 0.49	0.40 ± 0.05	0.42 ± 0.04	0.64 ± 0.05	0.88 ± 0.04	0.34 ± 0.02
23:0	ND	0.25 ± 0.07	0.24 ± 0.01	Trace	0.60 ± 0.02	0.23 ± 0.02
24:0	1.0 ± 0.14	0.36 ± 0.08	0.37 ± 0.03	Trace	0.44 ± 0.38	0.34 ± 0.02

[a] Total FAME (picomoles per milligram of sludge): digester feed, 675; control digester 1, 2,967 ± 705; control digester 2, 2,715 ± 176; butyrate-amended digester, 1,167 ± 110; propionate-amended digester, 1,722 ± 182; nitrate-amended digester, 2,905 ± 483.
[b] ND, Not detected.

and the digester slurries is given in Table 3. Homogeneous subsets are indicated by underlining. The feedstock contained few PLFAs and was composed of predominantly 16:0 and 18:0 PLFAs. The absence of polyunsaturated fatty acids indicated that the feedstock had undergone some degradation during storage. The slurries from the unamended digesters showed significantly higher proportions of iso and anteiso 15:0, iso 18:0, and 18:1ω7c PLFAs. In addition, the PLFAs in the slurries recovered from the unamended digesters were in homogeneous subsets for 17 of the 22 PLFAs detected. The remaining five PLFAs were in adjacent subsets. The butyrate-amended digester slurry had significantly higher proportions of 17:1br and iso 17:0 PLFAs whereas the slurry from the propionate-amended digester had higher proportions of 15:0, 17:0, and 16:1ω7t PLFAs with lesser proportions of iso 16:0 PLFA. The nitrate-amended digester slurry contained significantly higher proportions of iso 14:0 and iso 16:0 PLFAs and a lower proportion of 17:0 PLFA.

Microbial nutritional status. The ratio of the bacterial endogenous storage product PHB to the TELP is a measure of microbial nutritional status (25). The microbiota of the feedstock had the lowest TELP and an intermediate level of PHB (Table 4). The slurries from the control digesters and those supplemented with butyrate or propionate contained high ratios of TELP to PHB, indicating balanced growth. The microbes in the slurry from the digester supplemented with propionate had the lowest PHB level. The microbes in the slurry from the nitrate-amended digester showed a 13-fold increase in PHB with only a 3.8-fold increase in biomass (TELP) relative to the feedstock, which resulted in the lowest ratio of TELP to PHB. The lowered ratio indicated that the microbes in the slurry from this digester were not in balanced growth.

FTIR analysis. Figure 1 shows subtraction spectra produced by diffuse reflectance FTIR spectroscopy of lyophilized digester slurries. The subtraction spectra were obtained by subtracting the spectrum for the control digester from the spectra for each amended digester. Subtracting the spectrum of the control digester slurry from itself resulted in a flat spectrum (Fig. 1a). The peak near wavenumber 1,750 shown in the subtraction spectrum for slurries from the nitrate-amended digester (Fig. 1d) was identified as PHB from spectra determined previously (23a).

DISCUSSION

Viable biomass. Phospholipids are found in the membranes of all cells and under the growth conditions found in fermentative communities contain a relatively constant proportion of their biomass as phospholipids (29). The phospholipids have been related to various measures of microbial biomass (30). Phospholipids are not found in storage lipids and have a relatively rapid turnover in both living and dead cells in natural environments (29, 32). The data of Table 1 indicated that the formation of microbial slurries in these thermophilic fermentors was associated with a significant increase in the total phospholipids. This increase in extractable lipid phosphate indicated the development of the fermentative microbial consortia.

The calculation, based on total extractable lipid phosphate, of bacterial numbers in the control digesters produced results (Table 1) that agree with the following direct counts previously reported for other digestion systems: 2.88×10^{11} bacteria per g of total solids (18); ca. 3×10^{11} bacteria per g of total solids (20); 5.2×10^{10} to 6.8×10^{10} bacteria per g of wet weight (28); and ca. 1×10^{10} bacteria per ml (22). The use of this technique may result in an automated procedure for continuous monitoring of viable microbial biomass in fermentation systems.

TABLE 3. Significant difference map[a] of the FAMEs from Table 2

FAME	Low					High
Iso 14:0	B		P		F	N
14:0	F	B	C2	N	C1	P
Iso 15:0	F	N	B	P	C1	C2
Anteiso 15:0	F	B	N	P	C1	C2
15:0	F	C2	B	C1	N	P
Iso 16:0	P	B	C1	C2		N
16:1ω7t	C1	C2	B	F	N	P
16:1ω5c	B	N	C2	C1	P	F
16:0	N	C1	C2	B	P	F
17:1 br	F	C2	C1	N	P	B
Iso 17:0	F	C1	C2	N	P	B
Anteiso 17:0	F	N	P	C1	C2	B
17:0	N	F	C2	C1	B	P
18:3ω6	N	F	C2	C1	P	B
18:3ω3	F	P	N	C1	C2	B
Iso 18:0	B	P	N	F	C2	C1
18:1ω9c	Not significantly different					
18:1ω7c	F	P	B	N	C2	C1
18:1ω7t	Not significantly different					
18:0	C1	N	C2	B	P	F
20:0	F	N	C2	B	F	P
22:0	N	C1	C2	B	P	F
23:0	F	B	C1	N	C2	P
24:0	B	N	C1	C2	P	F

[a] SPSS, Cyber 750 computer, Florida State University was used. Values of means, in moles percent, are ranked from lowest to highest for each reported FAME with homogeneous subsets indicated by being joined with a line. The familywise error rate was 0.05. C1, control digester 1; C2, control digester 2; B, butyrate-amended digester; P, propionate-amended digester; N, nitrate-amended digester; F, digester feed.

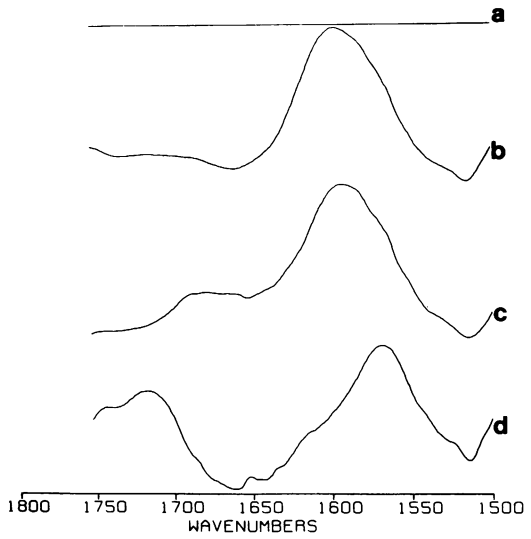

FIG. 1. Subtraction spectra for diffuse reflectance infrared Fourier transformation spectroscopy of lyophilized slurries from thermophilic methane-producing digesters. The spectra represent the spectral differences when the spectrum of the slurry from the control digester was subtracted from the spectrum of the slurry from each digester. Spectra: (a) control − control; (b) butyrate-amended digester − control; (c) propionate-amended digester − control; (d) nitrate-amended digester − control.

Microbial community structure. Examination of the ester-linked fatty acids of the phospholipids provides insight into the community structure of the microbial community (2, 3, 29, 31). Specific PLFAs or groups of fatty acids can be utilized as signatures of specific components of the microbial communities (3, 29). Interpretations of the significance of shifts in the proportions of PLFA have been correlated with changes in microbial morphology of biofilms; the responses of consortia to specific inhibitors and antibiotics; the effects of light, toxins, and predation; and comparison with the compositions of isolated microbial monocultures for validation (29). The patterns of PLFA from the digester slurries (Tables 2 and 3) show that incubation in the digesters induces significantly higher concentrations of the short-branched saturated and monounsaturated fatty acids typical of bacteria as well as the ω7 unsaturation of monoenoic fatty acids formed by the microbial anaerobic desaturase pathway. The significantly higher proportions of 18:3ω3, a PLFA recently detected in a mesophilic syntrophic acetogenic bacterium (Henson, McInerney, and White, unpublished data), indicated the possible presence of similar organisms in this thermophilic digester. A thermophilic, syntrophic bacterium was recently isolated from the control digesters (15).

TABLE 4. PHB found in thermophilic bench-top digesters operating under various nutritional conditions and ratio of total lipid phosphate (TLP) to PHB

Digester	PHB (pmol per mg of sludge)[a]	TLP/PHB ratio
Digester feed	64.7	53.6
Control digester 1	74.4 ± 41.6	165
Control digester 2	54.1 ± 11.8	235
Butyrate amended	60.0 ± 7.8	127
Propionate amended	38.6 ± 0.65	230
Nitrate amended	821 ± 62	20.1

[a] $\bar{x}$ ± Standard deviation; n = 2 (except digester feed, where n = 1).

Digester microbial structure. The addition of propionate, butyrate, or nitrate to the anaerobic digesters significantly altered both the total microbial biomass and the community structure of the microbiota (Tables 1 through 3). The detailed significance of the shifts in PLFA awaits the analysis of more monocultures isolated from the digester slurries. From these data it is clear that the input feedstock contains PLFA possibly from the residual microbiota or the plant material itself (high proportions of 16:0, 16:1ω5c, and 18:0). Some degradation of the plant material, however, appeared to have occurred because of the absence of long-chain polyunsaturated fatty acids.

The nitrate-amended digester, compared with the control digesters, had greater microbial biomass as indicated by the higher levels of TELP (Table 1), but an equal concentration of PLFA (Table 2). This may indicate a shift in the microbial community toward bacteria that contain non-ester-linked PLFA, such as plasmalogens (14) or sphingolipids (21). The higher biomass may have resulted from the greater quantities of energy available to the microbes when using nitrate as a terminal electron acceptor.

The biochemical technique for microbial community structure analysis should be valuable in comparing microbial communities in large-scale digestion systems inoculated

from small-scale fermentation systems. Results of fermentability measurements, fermentation rates, and other studies obtained from small-scale digesters could be extrapolated to large-scale digesters if the microbial community structures were known to remain similar.

Nutritional status. The lipid analysis also provided insight into the nutritional status of the bacteria. Some of the organisms form the endogenous storage polymer PHB under conditions of unbalanced growth when the organisms can accumulate substrates but not undergo cellular division (10). Other organisms in the digesters may utilize PHB formation as a terminal electron acceptor (McInerney, personal communication). The ratio of total microbial biomass (TELP) to PHB ranges between 100 and 200 for the control digester slurries (Table 4). These digesters have a stable output of ca. 3 liters of methane per day (J. M. Henson, F. M. Bordeaux, C. J. Rivard, and P. H. Smith, submitted for publication). Supplementation with butyrate depressed the total microbial biomass, with little effect on the total PHB. Methane production in the butyrate-supplemented digester was 150% of that in the control digester (Henson et al., submitted for publication). Propionate supplementation induced a marked decrease in PHB with a total biomass similar to that of the butyrate-supplemented digester and shifted the community structure as indicated in the proportions of PLFA. This microbial consortium was stable, although it may have been slightly inhibited, and produced 120% of the methane which was produced in the control digester (Henson et al., submitted for publication). Supplementation with nitrate stimulated the growth of the microbial community and induced a large increase in PHB synthesis. Nitrate supplementation resulted in a stable digestion system with a decreased production of methane (64% of control) with increased pool sizes of acetate and propionate. (C. J. Rivard, Ph.D. thesis, University of Florida, Gainesville, 1983.) The nitrate was converted to ammonia, which was not inhibitory to this digester (Rivard, Ph.D. thesis) and which may be the preferable source of nitrogen in the digester as has been shown for the rumen (6). This digester, therefore, was not nitrogen limited. The reason for the increased PHB production and accumulation in the nitrate-amended digester is unknown. To our knowledge, the detection of PHB in these digesters is the first report of this storage polymer in digestion systems.

The lipid methods outlined in this study can be utilized to characterize the microbial biomass, community structure, and possibly the nutritional status of the complex fermenter microbiota as they relate to the conversion of biomass to methane. These methods are destructive and require a chemical analysis. The fact that these digester slurries have distinctly different infrared spectra when examined by diffuse reflectance FTIR spectroscopy (23a) and the indication of PHB by diffuse reflectance FTIR spectroscopy in this study, suggests that the chemical methods to define the structure and activity of the community can be correlated with the nondestructive FTIR analysis. This combination of methods may offer insight into the functioning of complex anaerobic communities that eventually could be developed into nondestructive on-line monitoring techniques.

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LITERATURE CITED

1. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of lipid extraction and purification. Can. J. Biochem. Physiol. **35:** 911–917.
2. **Bobbie, R. J., J. S. Nickels, G. A. Smith, S. D. Fazio, R. H. Findley, W. H. Davis, and D. C. White.** 1981. Effect of light on biomass and community structure of estuarine detrital microbiota. Appl. Environ. Microbiol. **42:**150–158.
3. **Bobbie, R. J., and D. C. White.** 1980. Characterization of benthic microbial community structure by high-resolution gas chromatography of fatty acid methyl esters. Appl. Environ. Microbiol. **39:**1212–1222.
4. **Brock T. D.** 1979. Biology of microorganisms, 3rd ed., p. 121. Prentice-Hall, Inc., Englewood Cliffs, N.J.
5. **Bryant, M. P.** 1959. Bacterial species of the rumen. Bacteriol. Rev. **23:**39–66.
6. **Bryant, M. P.** 1977. Microbiology of the rumen, p. 287–304. *In* M. J. Swenson (ed.), Duke's physiology of domestic animals, 9th revised edition. Cornell University Press, Ithaca, N.Y.
7. **Bryant, M. P.** 1977. The microbiology of anaerobic degradation and methanogenesis with special reference to sewage, p. 107–117. *In* H. G. Schlegel and J. Barnea (ed.), Microbial energy conversion. Pergamon Press, Inc., Elmsford, N.Y.
8. **Bryant, M. P.** 1979. Microbial methane production-theoretical aspects. J. Anim. Sci. **48:**193–201.
9. **Buhr, H. O., and J. F. Andrews.** 1977. The thermophilic anaerobic digestion process. Water Res. **11:**129–143.
10. **Dawes, E. A., and P. J. Senior.** 1973. The role and regulation of energy reserve polymers in micro-organisms. Adv. Microb. Physiol. **10:**135–266.
11. **Findley, R. H., and D. C. White.** 1983. Polymeric beta-hydroxyalkanoates from environmental samples and *Bacillus megaterium*. Appl. Environ. Microbiol. **45:**71–78.
12. **Findley, R. H., and D. C. White.** 1984. *In situ* determination of metabolic activity in aquatic environments. Microbiol. Sci. **1:**90–95.
13. **Gillan, F. T., and R. W. Hogg.** 1984. A method for the estimation of bacterial biomass and community structure in mangrove-associated sediments. J. Microbiol. Methods **2:** 275–293.
14. **Goldfine, H., and P. O. Hagan.** 1972. Bacterial plasmalogens, p. 329–350. *In* F. Synder (ed.), Ether lipids: chemistry and biology. Academic Press, Inc., New York.
15. **Henson, J. M., and P. H. Smith.** 1985. Isolation of a butyrate-utilizing bacterium in coculture with *Methanobacterium thermoautotrophicum* from a thermophilic digester. Appl. Environ. Microbiol. **49:**1461–1466.
16. **Hungate, R. E.** 1966. The rumen and its microbes. Academic Press, Inc., New York.
17. **Hungate, R. E.** 1975. The rumen microbial ecosystem. Annu. Rev. Ecol. Syst. **6:**39–66.
18. **Iannotti, E. L., J. R. Fischer, and D. M. Sievers.** 1978. Medium for the enumeration and isolation of bacteria from a swine waste digester. Appl. Environ. Microbiol. **36:**555–566.
19. **Iannotti, E. L., J. R. Fischer, and D. M. Sievers.** 1982. Characterization of bacteria from a swine manure digester. Appl. Environ. Microbiol. **43:**136–143.
20. **Kirsh, E. J.** 1969. Studies on the enumeration and isolation of obligate anaerobic bacteria from digesting sewage sludge. Dev. Ind. Microbiol. **10:**170–176.
21. **LaBach, J. P., and D. C. White.** 1969. Identification of ceramide phosphorylethanolamine and ceramide phosphorylglycerol in the lipids of an anaerobic bacterium. J. Lipid Res. **10:**528–534.
22. **Mah, R. A., and C. Sussman.** 1968. Microbiology of anaerobic sludge fermentation. I. Enumeration of the nonmethanogenic anaerobic bacteria. Appl. Microbiol. **16:**358–361.

23. **McInerney, M. J., and M. P. Bryant.** 1981. Basic principles of bioconversions in anaerobic digestion and methanogenesis, p. 277–296. *In* S. S. Sofer and O. R. Zaborsky (ed.), Biomass conversion processes for energy and fuels. Plenum Publishing Corp., New York.

23a. **Nichols, P. D., J. M. Henson, J. B. Guckert, D. E. Nivens, and D. C. White.** 1985. Fourier transform-infrared spectroscopic methods for microbial ecology: analysis of bacteria, bacteria-polymer mixtures, and biofilms. J. Microbiol. Methods **4**:79–94.

24. **Nichols, P. D., P. H. Shaw, and R. B. Johns.** 1985. Determination of the double bond position and geometry in monoenoic fatty acids from complex microbial and environmental samples by capillary GC-MS of the Diels-Alder adducts. J. Microbiol. Methods **3**:311–319.

25. **Nickels, J. S., J. D. King, and D. C. White.** 1979. Poly-β-hydroxybutyrate accumulation as a measure of unbalanced growth of the estuarine detrital microbiota. Appl. Environ. Microbiol. **37**:459–465.

26. **Russell, J. B., and M. S. Allen.** 1984. Physiological basis for interactions among rumen bacteria: *Streptococcus bovis* and *Megasphaera elsdenii* as a model, p. 239–247. *In* M. J. Klug and C. A. Reddy (ed.), Current perspectives in microbial ecology. American Society for Microbiology, Washington, D.C.

27. **van den Berg, L.** 1984. Developments in methanogenesis from industrial waste water. Can. J. Microbiol. **30**:975–990.

28. **Varel, V. H.** 1984. Characteristics of some fermentative bacteria from a thermophilic methane-producing fermenter. Microb. Ecol. **10**:15–24.

29. **White, D. C.** 1983. Analysis of microorganisms in terms of quantity and activity in natural environments, p. 37–66. *In* J. H. Slater, R. Whittenbury, and J. W. T. Wimpenny (ed.), Microbes in their natural environments. Cambridge University Press, New York.

30. **White, D. C., R. J. Bobbie, J. S. Herron, J. D. King, and S. J. Morrison.** 1979. Biochemical measurements of microbial mass and activity from environmental samples, p. 69–81. *In* J. W. Costerton and R. R. Colwell (ed.), Native aquatic bacteria: enumeration, activity, and ecology. ASTM STP 695. American Society for Testing and Materials, Philadelphia.

31. **White, D. C., R. J. Bobbie, J. D. King, J. Nickels, and P. Amoe.** 1979. Lipid analysis of sediments for microbial biomass and community structure, p. 87–103. *In* C. D. Litchfield and P. L. Seyfried (ed.), Methodology for biomass determinations and microbial activities in sediments. ASTM STP 673. American Society for Testing and Materials, Philadelphia.

32. **White, D. C., W. M. Davis, J. S. Nickels, J. D. King, and R. J. Robbie.** 1979. Determination of the sedimentary microbial biomass by extractable lipid phosphate. Oecologia **40**:51–62.

33. **Zeikus, J. G.** 1980. Chemical and fuel production by anaerobic bacteria. Annu. Rev. Microbiol. **34**:423–464.