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Characterization of Bacteria from a Swine Manure Digester†

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One-hundred thirty bacteria isolated from a swine manure digester were predominately gram-positive anaerobes which were tentatively classified into the following genera: *Peptostreptococcus, Eubacterium, Bacteroides, Lactobacillus, Peptococcus, Clostridium*, and *Streptococcus* plus two unidentified groups. The major fermentation products formed by these organisms included acetate, propionate, succinate, lactate, and ethanol, singly or in various combinations. Acetate was the sole end product of several groups. Few of the isolates (14%) reduced the pH below 6.0. The predominate bacteria appear to differ from the predominate organisms isolated from other anaerobic ecosystems.

Anaerobic digestion of organic matter to carbon dioxide and methane in digesters and other anaerobic ecosystems has been reviewed recently (4, 5, 12, 23, 27, 37). The bacteria in digesters are predominately strict anaerobes. The anaerobic process is carried out by a complex consortium of bacteria. The process and the microbial population are divided into three stages or groups (5, 23). In the first stage, complex molecules such as cellulose, lipids, and proteins are degraded to volatile acids, carbon dioxide, and hydrogen by "acid formers." In the second stage, "acetogenic" bacteria metabolize propionate, butyrate, and possibly other end products of the first stage to acetate, hydrogen, and carbon dioxide. Finally, methane formers, or methanogens, convert the acetic acid, carbon dioxide, and hydrogen to methane. A fourth stage, in which hydrogen and carbon dioxide are metabolized to acetate, can also be included (35).

Many bacteria involved in the process have not been isolated. Usually 12% or less of the total number of bacteria determined by microscopic count can be enumerated and cultured (22, 26; V. H. Varel, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, Q73, p. 231). Much of the work on the acid phase of domestic digesters has been done with partially purified wastes or one segment of the population, or the description of the cultures has been minimal (9, 22, 26, 33, 34). Acetogenic bacteria, except for a few cases, have not been isolated and characterized (3, 5). Methanogens have been studied in greatest detail (2, 27, 35, 37). Little is known about the substrates utilized or the chemical and physical requirements of the microbes.

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We developed a nonselective medium that permits enumeration and isolation of 60% of the organisms in a swine waste digester (17). This recovery is higher than that previously reported for anaerobic digesters and increases the probability that isolates obtained represent most of the functional digester population. This paper reports characteristics of organisms isolated with this medium.

MATERIALS AND METHODS

Bacteria. A total of 138 strains were isolated from the highest dilutions (10^{-7} and 10^{-8}) of an anaerobic swine waste digester (17). Eight strains failed to grow upon subculturing. The remaining strains were maintained on medium 174 (17). The original cultures have been streaked and reisolated.

Media. Preparation and inoculation of media were done with the exclusion of oxygen by the Hungate technique (16) as modified by Bryant and Burkey (6). The medium was tubed under carbon dioxide in 3.5-ml amounts in 13- by 100-mm tubes. The gas phase was changed to 50% hydrogen in carbon dioxide at the time of inoculation.

The basic medium (DFG base) contained Phytone, Casamino Acids, yeast extract, volatile fatty acids, porphyrin-containing compounds, vitamins, minerals, resazurin, reducing agent, bicarbonate buffer, and digester fluid (DF) (18). DFG contained the components in DFG base plus glucose, cellobiose, soluble starch, and pyruvic acid. SMG contained swine manure extract (SME) instead of DF. Substrates were added to DFG or SMG base at 0.5% to test for sugar fermentation. When Phytone (BBL Microbiology Systems, Cockeysville, Md.) was added to DFG or SMG base as the sole substrate, its concentration was increased from 0.2 to 1%.

Casein, with 3.7% brain heart infusion base and 1.5% agar, was added to SMG base without cysteine. Blood agar, egg yolk agar, and milk were made as previously described (15). Casein and blood agar were tubed as slants in 18- by 150-mm tubes under nitrogen.

Strain characteristics. Colony characteristics were

determined by streaking cultures on roll tubes of DFG or long slants of the casein medium with SME. Cellular morphology was determined from wet mounts and Gram stains. Cell size was determined with Gram-stained cells. Flagella were observed after staining with flagella stain (Difco Laboratories, Detroit, Mich.).

Because most of the organisms did not reduce the pH of the medium, substrate utilization was determined by following growth (absorbance at 660 nm) in 13-mm culture tubes in the presence and absence of the substrate. Determinations were made daily or hourly depending on the growth of the organism and until either maximum absorbance was reached or until growth was no longer expected (up to 45 days).

Acetylmethylcarbinol and indole production and casein, esculin, starch, and gelatin hydrolysis were determined by standard methods (15). Short-chain acids, alcohols, methane, and hydrogen were determined by gas chromatography (19); most cultures used for the end product determinations were grown in SMG base with the carbohydrate promoting the highest level of growth. Group 8 was cultured in DFG base with a single carbohydrate. All cultures were incubated for at least 1 month before these determinations were made.

Fiber digestion. Ball-milled cellulose slurry (2%) was prepared from Whatman no. 1 cellulose (11). Hemicellulose was purchased (Xylan; Sigma Chemical Co., St. Louis, Mo.). Holocellulose was chemically prepared from alfalfa (24). Particles from swine manure were obtained by washing the manure through a series of sieves. The fraction that passed through the 147-μm screen but not the 88-μm screen was collected and washed in distilled water at 4°C for 72 h with periodic mixing and decanting. The volatile solids of the particles were 82% of the total solids.

Cellulose (2.5 or 5.0 ml of the slurry in 100 ml) and xylan (0.5%) were added to DFG base with 0.05% soluble starch. Tubes were examined for clearing over a 4-month period. Cellulose, alfalfa holocellulose, sieved particles, and thin turnip slices (8) were added to SMG base with 0.05% soluble starch. The added cellulose, holocellulose, and sieved particles increased the absorbance at 660 nm of the medium 0.45, 0.51, and 0.67 U, respectively, over controls with SMG base containing 0.5% soluble starch. After inoculation, the absorbances of the tubes were determined daily for 2 weeks, then at approximately 2-week intervals for 2 months. The turnip slices were checked for disintegration.

Oxygen tolerance. Oxygen tolerance was determined by inoculation of broths, slants (1.5% agar), and deeps (2% agar). The broths and slants were made with standard methods medium, blood agar, SMG, and DFG and tubed in 4-ml quantities in 13- by 100-mm screw-cap tubes. The tubes were sealed as they were removed from the autoclave and after inoculation. SMG and DFG deeps were tubed anaerobically in rubber-stoppered 13- by 100-mm tubes. At the time of use, the latter tubes were steamed, cooled at 47°C, inoculated with 1 drop of culture, mixed, and cooled to room temperature. After solidification of the agar, the culture was capped with 1 ml of 2% agar and incubated with a sterile aluminum foil closure. The media used to test oxygen tolerance did not contain cysteine or sodium carbonate. Slants were examined under high-intensity light. The cultures had been carried in culture for 6 to 12 months with monthly transfer before these tests were carried out.

pH studies. The DF added to DFG for determination of final pH was treated to remove bicarbonate buffer by reducing the pH to 4 with HCl and then neutralizing with NaOH. The treatment, medium preparation, and inoculation for pH studies was carried out under nitrogen unless stated otherwise.

Analysis of data. Grouping the organisms was done with a computer program developed for numerical taxonomy (1, 25). The program was obtained from Rita Colwell (University of Maryland, College Park). For this analysis, characteristics in which most organisms were positive or negative were excluded; the Joucard coefficient was used. Data analyzed included the results from studies of requirements for crude extracts and known growth factors, stimulation by electron acceptors, and inhibition by Tween 80 (18).

RESULTS

Identification of digester bacteria. The 130 isolates were placed into 11 groups and 15 miscellaneous strains (Table 1). Group 1 contained the largest number of isolates (20 strains). These strains were gram-negative pleomorphic rods with rounded ends. The cell size was 0.5 by 0.75 to 15 μm, with most being 1 to 2 μm long. Occasionally, the cells were curved; chain formation was rare. Twitching movements were observed, but flagella were not detected. On a medium with DF, colonies were circular, convex, entire, translucent, and cream colored; the colonies were opalescent with reflected light. On the medium with casein and SME, the colonies were circular, entire, and convex to raised with white centers and almost clear margins. Broth cultures had dense growth and a ropy sediment.

This group produced acetic (25 ± 3 mM), propionic (22 ± 5 mM), and succinic (28 ± 8 mM) acids in medium DFG and utilized a variety of soluble substrates including amygdalin, arabinose, cellobiose, esculin, fructose, galactose, glucose, lactose, maltose, mannose, ribose, salicin, starch, and trehalose. The utilization of melibiose, pyruvate, raffinose, rhamnose, sucrose, and xylose was variable. Erythrose, glycerol, lactose, mannitol, sorbitol, and succinate were not used.

Starch, casein, esculin, and gelatin were hydrolyzed by most of the strains. Growth was increased by increasing the concentration of Phytone in a medium without added carbohydrate; there were increases in branched-chain fatty acids indicative of protein hydrolysis. Blood agar and milk was not changed. Lipase, lecithinase, and indole were not produced. Traces of hydrogen were detected.

Group 1 was aerotolerant. Growth varied in aerobic broth medium. If growth was initiated, total growth was approximately one-tenth that in the corresponding anaerobic medium. Growth

TABLE 1. Characteristics of 130 isolates from a swine manure digester

Group	No. of strains[a]	Morphology	Gram reaction	Chains	Aerotolerance	Motility	Fermentation products[b]	Tentative identification
1	20	Rod	−	−	(±)[c]	−	A,P,S	*Bacteroides* I
2	16	Rod	−	−	−	+	a	*Bacteroides* II
3	6	Coccus	+	+	(±)	−	A	*Peptostreptococcus* I
4	13	Coccus	+	+	(±)	−	A	*Peptostreptococcus* II
5	4	Coccus	+	+	−	−	a(e,p,b,l)	*Peptostreptococcus* III
6	3	Coccus	+	−	−	−	−	*Peptococcus*
7	18	(Coccus)	(+)	−	−	−	A,E,l	Unknown
8	11	Rod	+	+	−	−	a(p,b,l)	*Eubacterium*
9	14	Rod	(−)	+	−	−	−	Unknown
10	7	Rod	±	−	−	−	L (a)	*Lactobacillus* I
11	3	Rod	+	−	−	+	L,e,a	*Lactobacillus* II

[a] Fifteen miscellaneous strains were not included in this table.

[b] Abbreviations: A, acetate; B, butyrate; P, propionate; E, ethanol; L, lactate; and S, succinate. Capital letters refer to acids produced in amounts of 10 μmol/ml of medium or greater; lowercase letters refer to amounts less than 10 μmol/ml. Products in parentheses are not detected in all strains or under all conditions.

[c] Growth under reduced oxygen only.

was not detected on slants of DFG, SMG, or standard methods medium. When the caps were sealed immediately after autoclaving and again after inoculation, growth was detected in the butts of the slant. When tubes with blood agar were sealed in the same manner, microcolonies were detected on the agar surface; however, the colonies were much smaller and required a much longer period for growth to be seen than colonies on the same medium treated anaerobically. Colonies were first seen in the reduced parts of agar deeps with crude extracts.

Group 1 consisted of 20 strains. Four strains were distinguished from the others by lower maximum absorbance in most media, weaker hydrolytic capability, or greater stimulation by the addition of growth factors. This group was tentatively identified as *Bacteroides* (7, 15).

Cells of group 2 (16 strains) were gram-negative, thin, pleomorphic rods occurring singly, in pairs, and occasionally in short chains. The cells were normally 0.3 to 0.5 by 5 to 8 μm; but cells from 3 to 100 μm long were occasionally found. Cells were often highly curved, resulting in pairs forming donut and horseshoe-like shapes. Motility, when detected in any one strain, was seen only after many observations. Motile cells moved slowly. Flagella were rarely seen. When flagella were detected, they were most often polar or subpolar; although in two cells they appeared peritrichous.

The colonies were punctiform or circular to irregular, transparent to translucent, and colorless. At times, the center of the colony appeared mottled. On the medium with SME and casein, they were small, translucent, and gray. No growth was detected on aerobic media.

Acetate (6.6 ± 4.5 mM) was produced. The growth of these strains was stimulated by pyruvate, peptone, oil, and several soluble carbohydrates including glucose; however, the extent of stimulation was small. The low level of growth in all media made measurements difficult. This group was tentatively identified as *Bacteroides*.

Group 3 organisms (six strains) were gram-positive coccobacilli that occurred in chains, clumps, and singly; however, distinct rods were frequently observed in the middle of chains. The most common size was 0.75 by 1.0 μm; 5 to 20% of the cells of any one strain were rod-like; 25 to 85% were cocci, and the rest were coccobacilli. Colonies on DFG were punctiform to circular, convex, entire, transparent, and honey to cream colored. On the medium with SME and casein, the colonies were circular with slightly irregular and raised edges. Moderate to heavy growth was found in broth culture.

Organisms in group 3 produced a high concentration of acetate (134 ± 10 mM). Fructose, galactose, glucose, maltose, mannose, pyruvate, and Phytone promoted good growth. Increasing the concentration of Phytone increased the level of growth. Small increases of normal and branched-chain volatile fatty acids indicated that the peptone was degraded. Amygdalin, arabinose, cellobiose, erythrose, esculin, lactate, lactose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, starch, succinate, sucrose, and xylose were not used. Small increases in acetate were observed in a medium with succinate compared to that without. Traces of hydrogen were detected. Indole was produced. Casein, esculin, gelatin, and starch were not hydrolyzed. Egg yolk and blood agar and milk were not changed. Group 3, like group 1, was aerotolerant.

Group 3, which consisted of six very similar strains, has morphological characteristics simi-

lar to *Peptostreptococcus* and *Eubacterium* (7, 15). One strain was grown in a medium with penicillin (1, 10, or 100 U/ml); normal and swollen forms resembling aberrant cocci were observed in old cultures. These strains were tentatively identified as *Peptostreptococcus*.

Group 4 (13 strains) had cellular and colony morphology identical to that of group 3, but differed in that only maltose was fermented, indole was not produced, and oxygen was not tolerated. Pyruvate and peptone promoted the highest levels of growth. Traces of hydrogen were produced. These strains produced 59 ± 18 mM acetic acid. Group 4 were tentatively identified as *Peptostreptococcus*.

Group 5 consisted of four strains of gram-positive cocci with occasional coccobacilli. The frequency of coccobacilli and chains was less than with groups 2 and 3. On DFG, the colonies were similar to groups 2 and 3; on the medium with casein and SME they were punctiform to circular, entire, convex, and transparent. Two strains formed grey colonies, and two formed white colonies. The strains did not grow on slants incubated aerobically. The principle end product was acetate (7.3 ± 4.7 mM). At times, traces of ethanol, propionate, butyrate, and lactate were found. Pyruvate and peptone supported the highest levels of growth. Carbohydrates, especially cellobiose, glucose, and maltose, slightly stimulated growth. Esculin was hydrolyzed. Indole production was variable. Traces of hydrogen were detected. Group 5 was tentatively identified as *Peptostreptococcus*.

Group 6, comprised of three strains, were large gram-variable cocci. Coccobacilli forms were found occasionally in older cultures. Colonies were circular, convex, and entire. On DFG, they appeared colorless; on the medium with SME and casein, they were white. In broth cultures, growth was sporadic. Lag phases from 3 to 35 days were observed. When growth was initiated, as little as 3 days was required for maximum absorbance. Growth was observed only in the reduced parts of agar deeps and not on slants incubated aerobically. Pyruvate stimulated growth. Fermentation end products were not detected. Group 6 was tentatively identified as *Peptococcus*.

With 18 strains, group 7 was the second largest group. When examined by phase-contrast microscopy, the cells were typically spheroid bodies resembling L forms, cells with defects in the cell wall, or mycoplasmas. The spheroids were 2 to 7 μm in diameter, although forms more than 10 μm were observed. The cytoplasm was very light and stained gram-negative; at times darker areas were found near the outside of the cell. Occasionally , normal coccoid cells which stained gram-positive were observed. The rate of growth and maximum absorbance of the one strain that was tested were identical in media with 1, 10, or 100 U of penicillin per ml to that in the control. In media with and without penicillin, many normal cocci were observed in the middle of the log phase, whereas spheroids were seen at other times including the early and late log phase. Cellular morphology was determined for most of the strains after 2 to 4 h, at approximately 18 h, and after full growth. Since these times correspond with early and late log phases, the time of maximum numbers of cocci may have been missed.

I. M. Robinson (National Animal Disease Center, Agriculture Research Service, U.S. Department of Agriculture, Ames, Iowa) examined several strains of this group and concluded that they were not identical to the anaerobic mycloplasma isolated from the rumen (28, 29). Most of the cells did not have cell walls when examined with an electron microscope. Again occasional gram-positive organisms were present.

Group 7 formed circular, convex, colorless colonies with darker, gray centers on DFG. The culture did not form colonies on the medium with SME and casein. No growth was found on the surface of agar slants with crude extract, standard methods medium, or blood agar medium. Four strains initiated growth in aerobic broths when the tubes were sealed immediately after autoclaving. Growth was found in deeps of the medium with SME from just above the line of resazurin oxidation to the butt of the tube.

Group 7 produced ethanol (14 ± 9.8 mM), acetate (11 ± 7.7 mM), and small amounts of lactate (4 ± 1.8 mM). Approximately half of the 7 mM propionate added to the medium as a possible growth factor and with the crude extracts was utilized during growth.

Growth was poor in broth culture with the best growth occurring in multi-substrate medium (DFG). Pyruvate, ribose, trehalose, and xylose slightly stimulated growth. Esculin was hydrolyzed. Several of the strains with higher growth levels hydrolyzed gelatin and were the strains that grew with reduced oxygen tension. We believe that this group is a member of family *Peptococcaceae* because of the gram-positive cocci observed; nevertheless, identification depends upon development of better growth conditions.

Group 8 (11 strains) consisted of gram-positive, lancet-shaped bacilli that existed as long chains, pairs, and singly. Cell length was normally 0.5 to 1 μm; although with some cultural conditions, longer cells were observed. Colonies were circular, convex, entire, colorless, and transparent. Broth cultures had low levels of growth.

This group was divided into three subgroups with nine, one, and one strains. Growth of the latter two strains as measured by maximal absorbance was two- and threefold greater than growth of the first subgroup. The nine-strain subgroup produced small amounts of acetate (6.6 mM) and at times trace amounts of propionate, butyrate, and lactate. The two one-strain subgroups produced more than 10 mM acetate and small amounts (<10 mM) of propionate, butyrate, and lactate. Growth was stimulated by the addition of cellobiose. The strain with the highest level of growth was also stimulated by xylose. Increasing the level of Phytone increased the level of growth. No growth was detected with reduced oxygen or on aerobic slants. Group 8 was tentatively identified as *Eubacterium*.

The cellular and colony morphology of group 9 (14 strains) was identical to that of group 8, except that the Gram reaction was negative. They were grouped because of the inability to measure growth on most media. Cultures in both solid and liquid media have been transferred repeatedly. Growth along the stab in solid media could be seen with high-intensity light, and the number of cells, as observed microscopically, increased in broth media. However, the level of growth was always too small to measure. Most of the results, therefore, were negative; esculin hydrolysis, which was weakly positive, was one of the few exceptions. Even the Gram stains may have been inaccurate since a long growth period was necessary to obtain a sufficient number of cells.

The swine waste digester should contain organisms that metabolize propionate and butyrate and which would be inhibited by the buildup of hydrogen. One strain from this group was found to produce low levels of hydrogen. However, when this strain was cultured in a medium with propionate, butyrate, and constant sparging with carbon dioxide, the final absorbance was not increased, and the concentrations of the acids were not reduced.

Group 10 (seven strains) consisted of gram-variable, thin, pleomorphic rods that occurred singly, in pairs, and rarely in short chains. They were easily decolored during Gram staining. The colonies appeared punctiform to circular, convex, entire, translucent, and colorless. There was no growth on aerobic media and growth only in reduced parts of agar deeps. Broth cultures had low levels of growth.

The strains produced lactic (35 ± 24 mM) and acetic (4 ± 3 mM) acids in the media promoting the highest level of growth. Acetate was not detected as an end product in all media. The highest levels of growth were obtained with glucose, fructose, mannose, and trehalose. Cellobiose, soluble starch, maltose, pyruvate, lactic acid, salicin, and esculin also stimulated growth. Esculin was hydrolyzed. There was a net decrease in branched-chain acids that might have been due to synthesis of branched-chain lipids or amino acids. Some of the strains weakly hydrolyzed gelatin. There was no growth on casein medium. Hydrogen could not be detected. Group 10 was tentatively identified as *Lactobacillus*.

Group 11 were motile, thin, gram-variable rods that formed clusters and occasionally filaments. Colonies were circular, convex, entire, transparent, and colorless; colony centers often had an uneven texture. Broth cultures were typically clumped. No growth was detected in the presence of oxygen.

Group 11 produced ethanol (5.9 ± 3.3 mM), acetate (9.3 ± 5.9 mM), and lactic acid (23 ± 7.9 mM). Growth was stimulated by various substrates, but the clumping made quantitation difficult. Gelatin and esculin were hydrolyzed. This group was tentatively identified as *Lactobacillus*.

In addition to the above strains, there were 15 miscellaneous organisms. These strains included four gram-positive and two gram-negative anaerobic non-spore-forming rods, three gram-positive, anaerobic, spore-forming rods, one gram-positive, anaerobic coccus, and five facultative anaerobes. One of the gram-positive rods was the only organism producing a large amount of butyric acid. The coccus produced 60 mM of lactic acid and 9 mM of acetic acid and was identified as *Streptococcus*. The three strains of *Clostridium* were proteolytic and produced a mixture of normal and branched-chain acids. Two produced subterminal spores; the other produced terminal spores. The facultative organisms differed from the aerotolerant strains described above because they rapidly grew on the surface of aerobic media. The miscellaneous organisms could be distinguished by fermentation end products, substrate utilization, and conditions producing maximal growth.

Final pH. Only 14% of the strains produced sufficient acid to reduce the pH of unbuffered media below 6.0. The 14% included strains from *Bacteroides* I and two miscellaneous organisms. The DF added to DFG for these determinations was treated to remove the bicarbonate buffer. The ability to decrease the pH was also examined under carbon dioxide, in single-substrate media (1% starch or glucose), or in media in which sodium chloride or possible sources of ammonia (Phytone, Casamino Acids, and yeast extract) were deleted. Only *Bacteroides* I had a lower pH in a medium other than DFG with treated DF. The final pH was 5.3 or 5.4 with starch and 5.5 or 5.6 with glucose.

Cellulose and hemicellulose hydrolysis. We have not been able to demonstrate cellulose or hemicellulose utilization by clearing of these substrates in broth media inoculated with any of these isolates. We have tried several batches of ball-milled cellulose, hemicellulose, holocellulose, sieved particles from swine waste, and turnip slices, all to no avail. The cellulose and hemicellulose were examined in a medium with DF and one with SME. An exception was a miscellaneous bacterium that cleared the commercial hemicellulose. Other organisms formed a mucoid mass with the fiber, but did not clear the medium.

Since hemicellulose and cellulose are major substrates in the digester (19), we assumed that the corresponding lytic organisms were not isolated from the original nonselective medium. Most probable number determinations with enrichment cultures with cellulose and hemicellulose as substrates indicated that the populations hydrolyzing these compounds comprised less than 0.1% of the total digester population. These determinations were scored for total clearing. There were small visual differences in the amount of cellulose and hemicellulose remaining in the tubes. Since the uninoculated tubes had a consistent concentration of the polysaccharide, we felt the differences were due to partial substrate degradation. When the initial concentration of cellulose and hemicellulose was reduced, we were not able to distinguish between low levels of settled substrate and microbial cells after growth.

To test the possibility that a partial breakdown did occur in pure culture, the concentration of cellulose, hemicellulose, holocellulose, and sieved particles was reduced to permit absorbance determinations. After 2 months, there were differences in absorbance and concentration of fermentation products that might indicate degradation of these substrates; however, the differences were small. We believe that a limiting factor, either a nutrient or toxic compound, prevented growth and valid tests.

DISCUSSION

The 130 bacterial strains isolated from a swine manure digester were predominantly gram-positive anaerobes. Only 4% readily grew on aerobic media; 30% grew in the area of reduced oxygen tensions found below the surface of freshly prepared media with a high organic content; 56% of the bacteria were cocci or coccobacilli. If some of the *Bacteroides* (total, 27%) were included with these organisms, the proportion of cocci or coccobacilli in the pure cultures would be close to that observed in the total population (74%) (17).

The gram-positive cocci or coccobacilli could be excluded from several genera by the absence of propionate, butyrate, lactate, or succinate as sole or major end products, but they could not be excluded from the genera *Peptostreptococcus*, *Peptococcus*, or *Eubacterium* (7, 15). The isolates did not require added carbohydrates and were stimulated by the addition of peptone and pyruvate. All three of the above genera have species with similar metabolism (7, 15). The groups tentatively identified as *Peptostreptococcus* or *Peptococcus* were placed in these genera because large numbers of cocci were found in all growth phases. The distinction between the *Peptostreptococcus* and the *Peptococcus* was based on the tendency to form long chains. *Eubacterium* species were normally coccobacilli; cocci were not observed and occasionally longer forms are seen. There were two groups that we did not try to identify: the unidentified cocci (group 7) and the gram-negative, chain-forming rods (group 9). We feel that these groups may be identified as *Peptococcaceae* and *Eubacterium* as the culture conditions are improved. This is based on the presence of small numbers of gram-positive cocci in cultures of the former group and the similarity of the morphology of the latter to strains identified as *Eubacterium*.

The strains identified as *Lactobacillus* are similar to other lactobacilli since lactic acid was the only major end product (7, 15). Also, the isolates did not form gas in agar deeps, and hydrogen was not detected. The strains differ from the majority of *Lactobacillus* in that the final pH was not reduced significantly; small amounts of acetate and ethanol were also detected. *Lactobacillus* species that do not reduce the pH of the medium and that form products other than lactate, especially when the pH remains near neutrality, have been described previously (7, 15).

A high concentration of gram-positive organisms appears to be characteristic of swine manure and swine manure handling systems; nevertheless, the organisms differ from system to system. Salanitro et al. (30) reported that 90% of the isolates from swine manure were gram positive, including 44% *Streptococcus*, fermentative and nonfermentative *Eubacterium*, *Propionibacterium*, and *Clostridium*. A majority of these organisms, unlike those in this study, reduced the pH of the media below 6.0. Hobson and Shaw (14) examined two samples of manure that had been collected as a slurry under a slatted floor for up to 2 weeks and found *Streptococcus* (69 and 40%), *Clostridium* (20 and 16%), and *Bacteroides* (20 and 40%). The anaerobic population with a composition closest to what we found in the present study was in a swine manure anaerobic pit (32). Two-thirds of the isolates were gram positive, saccharolytic, and

non-saccharolytic *Peptostreptococcus*, and one-third were *Bacteroides*.

Hobson and Shaw (14) reported on the bacterial population in two swine manure digesters. One was started with the contents of a domestic waste digester, and the other was started with dilute swine manure only. In both, the dominant organisms were facultatively anaerobic *Streptococcus* (79 and 70%, respectively). These organisms were identical to those found in their influent and were nonreactive since they were not amylolytic, cellulolytic, or hemicellulolytic. They were α-haemolytic. The remaining strains included *Clostridium*, *Lactobacillus*, and unidentified gram-negative and gram-positive organisms.

Little similarity exists between the organisms reported by Hobson and Shaw (14) and those in this study. The differences may have been due to the methods of isolation and to the operation of the digesters. The percentage of the organisms recovered (viable count/microscopic count) was not reported for their study; however, we found that their medium cultured only 5 to 10% of the isolates in our digester, compared with 60% for our medium (M174) (17). Their digester was operated at a longer retention time (38 versus 15 days used here) and was lightly loaded with manure that was collected as a slurry under a slatted floor where the manure stayed for up to 2 weeks after deposition (13). Since the studies of Hobson and Shaw, it was found that a swine manure digester can be more heavily loaded and operated at a reduced retention time (10). The 4-g/liter loading rate and 15-day retention time used in our study are now recommended. In our study, the manure was collected fresh from a concrete floor and refrigerated until use. The combined differences in methods and operations have resulted in large differences in the organisms cultured; we found 7×10^9 bacteria per ml compared to between 6×10^5 and 2×10^7 bacteria per ml (14, 17).

Gram-negative, nonsporeforming, anaerobic rods predominate in domestic sludge and thermophilic cattle waste digesters (22, 26, 33, 34; V. H. Varel, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, I31, p. 89). Kirsh (22) reported that the largest group of gram-negative rods in sewage sludge had unique spinning motility and produced acetate from glucose and acetate, propionate, and butyrate from sludge, whereas Salanitro and Bryant (unpublished data, see reference 4) found high numbers of a bacterium similar to *Bacteroides ruminicola* in a domestic waste digester. Although in lower concentration than gram-positive bacteria, two large groups in the swine manure digester were *Bacteroides*. *Bacteroides* I was similar to *B. ruminicola* in utilizing a variety of substrates, but it had different fermentation patterns. *Bacteroides* II was similar to the group of Kirsh (22) because it was motile and formed only acetate; however, a unique spinning motility was not observed.

The major fermentation products of the pure cultures were acetate, propionate, succinate, lactate, and ethanol. Acetate was produced in the greatest quantities and was the sole end product of many of the isolates. Only one strain produced butyrate as a major product. The proportion of fermentation products complements information on the flow of carbon in the total population of digesters. Of the carbon in methane, 70% or more is derived from the methyl group of acetate (20, 31); also, of the various intermediates, this step is the most rate limiting in well-digested sludge (21). Approximately 10% of the methane in cattle manure and sludge digesters comes from propionate; less comes from butyrate. The digester is probably similar to the rumen; ethanol, lactate, and succinate found in pure culture may not be produced in the ecosystem (36). The interactions between the acid formers and methanogenic bacteria would result in a shift from ethanol and lactate to more acetate, carbon dioxide, and hydrogen. Any succinate produced would be decarboxylated to propionate (36) or metabolized to acetate. Thus, the isolates, when grown with the methanogen as a mixed population, would be expected to form primarily acetate, some propionate, and small amounts of butyrate.

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LITERATURE CITED

1. **Austin, B., and R. R. Colwell.** 1977. Evaluation of some coefficients for use in numerical taxonomy of microorganisms. Int. J. Syst. Bacteriol. **27**:204–210.
2. **Balch, W. E., G. E. Fox, L. J. Magun, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43**:260–296.
3. **Boone, D. R., and M. P. Bryant.** 1980. Propionate-degrading bacterium, *Syntrophobacter wolinii* sp. nov. gen. nov., from methanogenic ecosystems. Appl. Environ. Microbiol. **40**:626–632.
4. **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., Elsmford, N.Y.
5. **Bryant, M. P.** 1979. Microbial methane production—theoretic aspects. J. Anim. Sci. **48**:193–201.
6. **Bryant, M. P., and L. A. Burkey.** 1953. Cultural methods and some of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci. **36**:205–217.
7. **Buchanan, R. E., and N. E. Gibbons.** 1974. Bergey's manual of determinate bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
8. **Cato, E. P., W. E. C. Moore, and M. P. Bryant.** 1978. Designation of neotype strains for *Bacteroides amylophilus*, Hamlin and Hungate 1956, and *Bacteroides succinogenes*, Hungate 1950. Int. J. Syst. Bacteriol. **28**:491–495.

9. **Chynoweth, D. P., and R. A. Mah.** 1977. Bacterial populations and end products during anaerobic sludge fermentation of glucose. J. Water Pollut. Control Fed. **49**:405–412.
10. **Fischer, J. R., E. L. Iannotti, J. H. Porter, and A. Garcia.** 1979. Producing methane gas from swine manure in a pilot-size digester. Trans. ASAE **22**:370–374.
11. **Halliwell, G., and M. P. Bryant.** 1963. The cellulytic activity of pure strains of bacteria from the rumen of cattle. J. Gen. Microbiol. **32**:441–448.
12. **Hobson, P. N., S. Bonsfield, and R. Summers.** 1974. Anaerobic digestion of organic matter. Crit. Rev. Environ. Control **4**:131–191.
13. **Hobson, P. N., and B. G. Shaw.** 1973. The anaerobic digestion of waste from an intensive pig unit. Water Res. **7**:437–449.
14. **Hobson, P. N., and B. G. Shaw.** 1974. The bacterial population of piggery waste anaerobic digesters. Water Res. **8**:507–516.
15. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore.** 1977. Anaerobe laboratory manual. Virginia Polytechnic Institute and State University, Blacksburg.
16. **Hungate, R. E.** 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. **14**:1–49.
17. **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.
18. **Iannotti, E. L., J. R. Fischer, and D. M. Sievers.** 1982. Medium for enhanced growth of bacteria from a swine manure digester. Appl. Environ. Microbiol. **43**:247–249.
19. **Iannotti, E. L., J. H. Porter, J. R. Fischer, and D. M. Sievers.** 1979. Changes in swine manure during anaerobic digestion. Dev. Ind. Microbiol. **19**:519–529.
20. **Jeris, J. S., and P. L. McCarty.** 1965. The biochemistry of methane fermentation using ^{14}C tracers. J. Water Pollut. Control Fed. **37**:178–192.
21. **Kaspar, H. F., and K. Wuhrmann.** 1978. Kinetic parameters and relative turnovers of some important catabolic reactions in digesting sludge. Appl. Environ. Microbiol. **36**:1–7.
22. **Kirsch, E. J.** 1969. Studies on the enumeration and isolation of obligate bacteria from digesting sewage sludge. Dev. Ind. Microbiol. **10**:170–176.
23. **Kirsch, E. J., and R. M. Sykes.** 1971. Anaerobic digestion in biological waste treatment. Progr. Ind. Microbiol. **9**:155.
24. **Knehans, A. W., R. L. Kincaid, W. O. Regan, and B. L. O'Dell.** 1980. An unrecognized dietary factor for guinea pigs associated with the fibrous fractions of plant products. J. Nutr. **109**:418–425.
25. **Lockhart, W. R., and J. Liston.** 1970. Methods for numerical taxonomy. American Society for Microbiology, Washington, D.C.
26. **Mah, R. A., and C. Sussman.** 1968. Microbiology of anaerobic sludge fermentation. I. Enumeration of the nonmethanogenic anaerobic bacteria. Appl. Microbiol. **16**:358–361.
27. **Mah, R. A., D. M. Ward, L. Baresi, and T. L. Glass.** 1977. Biogenesis of methane. Annu. Rev. Microbiol. **31**:309–341.
28. **Robinson, I., M. Allison, and P. Hartman.** 1975. *Anaeroplasma abactoclasticum* gen. nov., sp. nov.: an obligately anaerobic mycoplasma from the rumen. Int. J. Syst. Bacteriol. **25**:173–181.
29. **Robinson, I. M.** 1979. Special features of *Anaeroplasmas*, p. 515–528. *In* M. F. Barile and S. Razin (ed.), The micoplasmas, vol. 1. Academic Press, Inc., New York.
30. **Salanitro, J. P., I. G. Blake, and P. A. Muirhead.** 1977. Isolation and identification of fecal bacteria from adult swine. Appl. Environ. Microbiol. **33**:79–84.
31. **Smith, P. H., and R. A. Hah.** 1966. Kinetics of acetate metabolism during sludge digestion. Appl. Microbiol. **14**:368–371.
32. **Spoelstra, S. F.** 1978. Enumeration and isolation of anaerobic microbiota of piggery waste. Appl. Environ. Microbiol. **35**:841–846.
33. **Toerien, D. F.** 1970. Population description of the nonmethanogenic phase of anaerobic digestion. I. Isolation, characterization and identification of numerically important bacteria. Water Res. **4**:129–148.
34. **Toerien, D. F., and W. H. J. Hattingh.** 1969. Anaerobic digestion I. The microbiology of anaerobic digestion. Water Res. **3**:385.
35. **Wolf, R. S.** 1979. Microbial biochemistry of methane—a study in contrasts. Part 1. Methanogenesis. Int. Rev. Biochem. **21**:267–300.
36. **Wolin, M. J.** 1975. Interactions between the bacterial species of the rumen, p. 134–148. *In* I. W. McDonald and A. C. I. Warner (ed.), Digestion and metabolism in the ruminant. University of New England Publishers, Armidale, Australia.
37. **Zeikus, J. G.** 1977. Biology of methanogenic bacteria. Bacteriol. Rev. **41**:514–541.