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Characteristics of a New Cellulolytic *Clostridium* sp. Isolated from Pig Intestinal Tract

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Gram-positive, spore-forming, motile, cellulolytic rods were isolated from 10^7 dilutions of pig fecal samples. The pigs had previously been fed pure cultures of the ruminal cellulolytic organism *Clostridium longisporum*. Isolates formed terminal to subterminal spores, and a fermentable carbohydrate was required for growth. Besides cellulose, the isolates utilized cellobiose, glycogen, maltose, and starch. However, glucose, fructose, sucrose, pectin, and xylose were not used as energy sources. Major fermentation products included formate and butyrate. The isolates did not digest proteins from gelatin or milk. Unlike *C. longisporum*, which has limited ability to degrade cell wall components from grasses (switchgrass, bromegrass, and ryegrass), the swine isolates were equally effective in degrading these components from both alfalfa and grasses. The extent of degradation was equal to or better than that observed with the predominant ruminal cellulolytic organisms. On the basis of morphology, motility, spore formation, fermentation products, and the ability to hydrolyze cellulose, the isolates are considered to be a new species of the genus *Clostridium*. It is unclear whether *C. longisporum* played a role in the establishment or occurrence of this newly described cellulolytic species. This is the first report of a cellulolytic *Clostridium* sp. isolated from the pig intestinal tract.

We have previously reported that some of the predominant fiber-degrading bacterial species found in the rumen are also found in the intestinal tract of pigs in numbers comparable to those seen in the rumen (15, 17). These organisms include *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Prevotella* (*Bacteriodes*) *ruminicola*. With the recent reisolation of the ruminal cellulolytic species *Clostridium longisporum* (16), this organism was fed to pigs in an attempt to ascertain whether this organism can colonize the intestinal tract or transfer cellulolytic activity to one of the clostridial species normally found in the intestinal tract. Clostridial isolates from intestinal contents of pigs have been found at concentrations of 1 to 7% of the total cultured flora, but none have been reported to be cellulolytic (7–10).

Although efforts to establish the ruminal cellulolytic organism *C. longisporum* in the pig intestinal tract were unsuccessful, we did isolate a different cellulolytic *Clostridium* sp. from these pigs. This article describes the characteristics of this new cellulolytic organism.

MATERIALS AND METHODS

Organisms. *C. longisporum* B6405 and OC4 and *Ruminococcus albus* B6403 (16) were obtained from our culture collection. *F. succinogenes* S85, *R. flavefaciens* C94, and *Clostridium cellulovorans* 35296 (13) were obtained from the Microbiology Division, Department of Animal Science, University of Illinois, Urbana. *R. albus* SY3 was obtained from C. S. Stewart, Rowett Research Institute, Aberdeen, United Kingdom. Cultures were maintained on an alfalfa cell wall (neutral detergent fiber) medium and on maintenance agar slant medium (see below).

Media and cultivation conditions. The composition of cellulose agar roll tube medium (per 100 ml) was as follows: clarified preincubated ruminal fluid (5), 15 ml; Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 0.2 g; yeast extract, 0.05 g; mineral S2 (11), 5 ml; cellulose (Whatman no. 1 filter paper ball milled with flint pebbles for 18 h), 0.2 g; resazurin, 0.0001 g; Na_2CO_3, 0.4 g; cysteine-hydrochloride, 0.05 g; and purified agar (BBL), 0.7 g. For alfalfa cell wall medium, alfalfa cell walls (1.0%) (16) were substituted for filter paper cellulose, and no agar was added. The composition of the maintenance agar slant medium (per 100 ml) was as follows: clarified ruminal fluid, 30.0 ml; glucose, cellobiose, maltose, starch, xylose, and glycerol, 0.03 g each; Trypticase, 0.2 g; resazurin, 0.0001 g; mineral S2, 5 ml; Na_2CO_3, 0.4 g; cysteine-hydrochloride, 0.05 g; and purified agar, 1.75 g. The composition of the medium for evaluating growth at various temperatures (per 100 ml) was as follows: clarified preincubated ruminal fluid, 15 ml; Trypticase, 0.2 g; peptone, 0.2 g; yeast extract, 0.1 g; mineral S2, 5 ml; cellobiose, 0.5 g; vitamins (16), 0.5 ml; resazurin, 0.0001 g; Na_2CO_3, 0.4 g; and cysteine hydrochloride, 0.05 g. These media were prepared under CO_2 gas phase by the Hungate anaerobic culturing method as described by Bryant (2).

Growth of bacterial cultures was measured in tubes (18 by 150 mm) at 660 nm with a Bausch and Lomb spectrophotometer (Spectronic 88).

Isolation and characterization. Six crossbred pigs (80 kg) were penned separately and fed ad libitum a corn-soybean meal or a 40% alfalfa meal diet for 14 days prior to inoculation with *C. longisporum*. During the 14 days, two fecal samples were obtained from each pig. The cellulolytic organisms were enumerated and tentatively identified by picking colonies or zones of clearing which appeared in roll tubes containing filter paper. Pigs were then fed a culture of *C. longisporum* OC4 grown on alfalfa cell walls (150 ml per pig; 10^7 organisms per ml). At the same time, 150 ml of culture (10^8 organisms per ml grown on cellobiose) was administered by rectal infusion. Fecal samples were monitored on days 3, 5, and 7 and then weekly for 8 weeks for the presence or

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absence of *C. longisporum* by examining cellulose roll tubes for orange-pigmented colonies and picking colonies or zones of clearing. Cellulolytic clostridia were characterized by using the methods of Holdeman et al. (6). Organic acid fermentation products from supernatant fluids (passed through 0.22-μm-pore-size filter) were analyzed with a high-pressure liquid chromatograph (model 6000A; Waters, Milford, Mass.) equipped with a UV detector set at 210 nm and absorbance set at 0.08 (model SPD-6A; Shimadzu Corp., Kyoto, Japan), an integrator (model 760 Series Interface; Nelson Analytical, Cupertino, Calif.), and an organic acid column (HPX-87H; Bio-Rad Laboratories, Richmond, Calif.), operated at 41°C. The eluting solvent was 0.0025 N H_2SO_4 at 0.6 ml/min, and the injection volume was 25 μl. Ethanol was analyzed by flame ionization detector gas chromatography, using a Hewlett Packard model 5840A with a coiled glass column (182.9 by 0.2 cm [inner diameter]) packed with 80/120-mesh Carbopak B-DA–4% Carbowax 20 M (Supelco, Bellefonte, Pa.) (13a). A nitrogen carrier gas was used at a flow rate of 20 ml/min, a sample size of 1 μl, and a temperature gradient from 70 to 170°C, 5°C/min. Hydrogen was analyzed by thermal conductivity detector gas chromatography using a Packard model 428 with a coiled glass column (182.9 by 0.2 cm [inner diameter]) packed with 60/80-mesh Molecular Sieve 5A (Supelco).

Grass and alfalfa cell wall degradation. The new cellulolytic isolate was examined for its ability to degrade alfalfa, bromegrass, and switchgrass cell wall components (cell walls, cellulose, and hemicellulose) at 24, 48, and 72 h in comparison to ruminal cellulolytic organisms and one methanogenic digestor cellulolytic organism, *C. cellulovorans* (13). The forages were ground to pass through a 1-mm-pore-size screen prior to being used to prepare the cell walls. They were boiled for 1 h with neutral detergent, and the insoluble residues (cell walls) were extensively washed to remove detergent before drying (14).

The composition of the basal in vitro digestibility medium (per 100 ml) was as follows: clarified preincubated ruminal fluid, 20 ml; Trypticase, 0.1 g; yeast extract, 0.05 g; mineral S2, 5 ml; resazurin, 0.0001 g; Na_2CO_3, 0.4 g; and cysteine hydrochloride, 0.05 g. Twenty-seven milliliters of this medium was added to 0.5 g of cell walls (dry sterilized for 30 min) in glass tubes (25 by 142 mm) with rubber stoppers. The inocula for this medium were either 3 ml of ruminal fluid passed through two layers of cheesecloth from a fistulated steer fed a grass-alfalfa haylage diet or 3 ml of cellulolytic culture grown with cell walls from the respective forage and transferred every 30 to 36 h for three consecutive times before being used as inocula. Three replicate tubes for each inoculum and for each time interval of 0, 24, 48, and 72 h were incubated at 37°C in tube presses (Bellco, Vineland, N.J.). The samples were vigorously shaken at 0800 and 1600 h daily. As each digestion period was completed, samples were centrifuged at 2,500 × *g* for 20 min. The residue pellet was frozen and later analyzed for fiber content by the sequential detergent system (14). Sulfuric acid (72%) was used to solubilize cellulose and isolate crude lignin plus ash.

Total cell wall was defined as neutral detergent fiber. Hemicellulose and cellulose were calculated by weight difference as follows: neutral detergent fiber minus acid detergent fiber equaled hemicellulose, and acid detergent fiber minus acid detergent lignin equaled cellulose. Degradation was calculated as the disappearance of the component during fermentation relative to the initial concentration. Correction was made for the addition of the components in the inoculum. Each result plotted is the mean of three replicate tubes.

G+C content. Cell suspensions from late log phase were mixed with an equal volume of glass beads (0.2-mm diameter) and disrupted in a Braun cell homogenizer. Chromosomal DNA was isolated by using a modification of the method of Berns and Thomas (1). Cell lysates were gently extracted four times with Tris-phenol. The DNA was precipitated with an equal volume of ethanol, resuspended in TE buffer (0.01 M Tris-HCl [pH 8.0], 0.001 M EDTA) and treated with 20 μg of DNase-free RNase per ml and 100 μg of protease per ml at 37°C for 1 h. The DNA solution was then gently extracted twice more with Tris-phenol and twice with phenol-chloroform. The DNA was precipitated with 0.6 volume of isopropanol, resuspended in TE buffer, and dialyzed extensively against TE buffer. DNA purity was checked by scanning the absorbance of the DNA samples between 200 and 400 nm and monitoring the A_{260}/A_{280} and A_{260}/A_{230} ratios.

G+C contents of chromosomal DNAs were calculated from the buoyant density values measured by cesium chloride density centrifugation (12). Determinations were made with a model E analytical ultracentrifuge (Beckman Instruments, Inc.) equipped with an electronic scanner and were based on three or more separate determinations. *Micrococcus luteus* (synonym, "*Micrococcus lysodeikticus*") and *Escherichia coli* DNAs were used as the reference DNAs and have buoyant densities of 1.7311 and 1.7104 g/ml, respectively.

RESULTS

Isolation and morphological features. One week after six pigs were fed and given rectal infusions of *C. longisporum* OC4 cultures, 10^7 dilutions of fecal samples from two of the pigs not only produced zones of clearing (diameter, 2 to 4 mm) typical of *F. succinogenes* and *R. flavefaciens* but also large zones of clearing (diameter, 10 to 20 mm) in cellulose agar roll tubes (Fig. 1). Both of these pigs were fed the corn-soybean meal diet. Ten to 15 of the cultures producing the larger zones per tube readily hydrolyzed all of the filter paper cellulose in 1 week. None of these large zones of clearing were observed in any of the six pigs before *C. longisporum* was fed to the pigs. Numerous attempts failed to generate a pure cellulolytic culture from these zones of clearing. Finally, three isolates were obtained in pure culture by scraping the outer border of the clearing. One of these isolates, 54408, was representative of the other isolates. The isolates required subculturing every 3 to 5 days when maintained on agar slants or viability was lost. Storage at −70°C maintained viability for 6 months and possibly much longer.

Vegetative cells from strain 54408 were gram-positive, motile rods, 1 μm wide by 3 to 8 μm long (Fig. 2a). Clostridial cells were 1 to 2 μm wide by 7 to 10 μm long and contained subterminal to terminal spores 1 μm wide by 1 to 2 μm long (Fig. 2b). Spores were rarely observed.

Biochemical characteristics. After comparing several characteristics of *C. longisporum* OC4 and the new isolate, 54408, which appeared to belong to the genus *Clostridium*, we concluded that the new isolates appear to be a new species. Besides lacking the bright orange pigment of *C. longisporum*, *Clostridium* sp. strain 54408 differed in that it did not utilize glucose, fructose, pectin, salicin, or sucrose; nor did it digest proteins from gelatin or milk. It did metabolize glycogen, maltose, and starch, which *C. longisporum* did not. Other substrates not utilized by the new

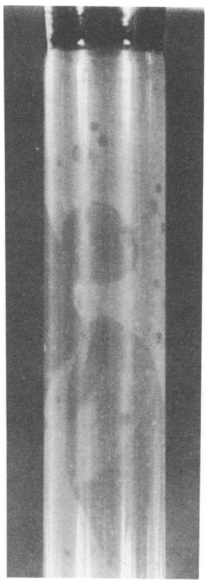

FIG. 1. Cellulose agar roll tube (18 by 150 mm) which was inoculated with a pig fecal sample. Small (2- to 4-mm-diameter) zones of clearing were typical of *F. succinogenes* or *R. flavefaciens* isolates, while the large (10- to 20-mm diameter) zones of clearing were typical of the new cellulolytic isolate.

isolate were amygdalin, arabinose, erythritol, inositol, lactose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, sorbitol, trehalose, and xylose. Esculin and lecithin were not hydrolyzed, and indole production and nitrate reduction were negative. *Clostridium* sp. strain 54408 produced formate (28 mM) and butyrate (12 mM) as major fermentation products when cultured with cellobiose. Ethanol and hydrogen were produced. When cultured with filter-paper cellulose, acetate was assimilated. *C. longisporum* OC4 produced ethanol, acetate, formate, butyrate, and hydrogen as fermentation products. Anaerobic conditions were always essential for growth. The optimum temperature for growth was between 39 and 42°C. No growth occurred at 25 or 55°C. Cultures could be adapted to grow at 45°C. *Clostridium* sp. strain 54408 and *C. longisporum* OC4 both grew on cellulose and cellobiose.

The G+C contents of the DNA were 37.5, 38.9, and 38.2 mol% for strains 54408, 1406-A1-A, and 412-C1-K, respectively.

Degradation of forages. The new swine isolate, *Clostridium* sp. strain 54408, was able to degrade cell wall fractions of alfalfa at rates comparable to those of ruminal cellulolytic organisms (Fig. 3). After 72 h, the extent of degradation by the new isolate exceeded that of the ruminal isolates. Approximately 35, 40, and 45% of the alfalfa cell walls, cellulose and hemicellulose, respectively, were degraded by the swine isolate after 72 h compared with 33, 36, and 50%, respectively, degraded by a mixed population of organisms in ruminal fluid. The swine isolate was equally effective in degrading cell wall fractions from two grasses, bromegrass and switchgrass. After 48 and 72 h, the swine cellulolytic isolate 54408 degraded more of the bromegrass fiber fractions than did the ruminal organisms (Fig. 4). The swine isolate degraded 40, 40, and 47% of the bromegrass cell walls, cellulose, and hemicellulose, respectively, after 72 h compared with 35, 35, and 45%, respectively, for a mixed population of ruminal organisms.

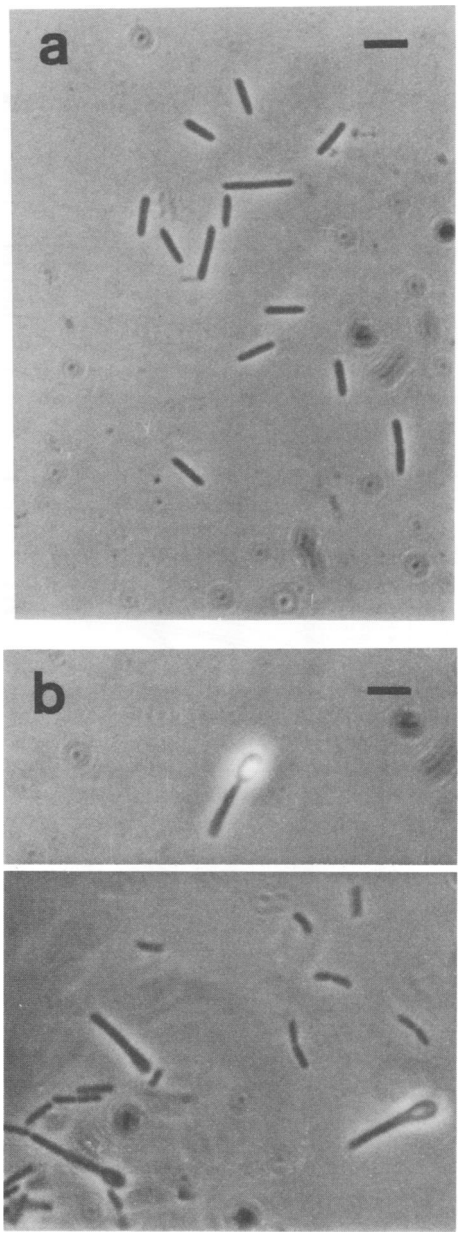

FIG. 2. Phase-contrast photomicrographs of vegetative cells (a) and clostridial cells with spores (b) from cellulolytic strain 54408. Bars, 5 μm.

DISCUSSION

This is the first report of a cellulolytic *Clostridium* sp. isolated from the intestinal tracts of pigs. Phenotypically, the swine cellulolytic *Clostridium* sp. strain 54408 is very different from *C. longisporum* OC4. *Clostridium* sp. strain 54408 will metabolize maltose, starch, and glycogen, which *C. longisporum* OC4 does not; strain 54408 does not utilize glucose, fructose, pectin, salicin, or sucrose, whereas *C. longisporum* OC4 does. Strain 54408 effectively degrades cell wall components from bromegrass and switchgrass, whereas *C. longisporum* appears to have limited ability to degrade structural carbohydrates from grasses (Fig. 4) (18). The G+C content of the DNA from the swine isolate is 38 to 39 mol%, whereas for *C. longisporum* OC4, it is 23 mol%

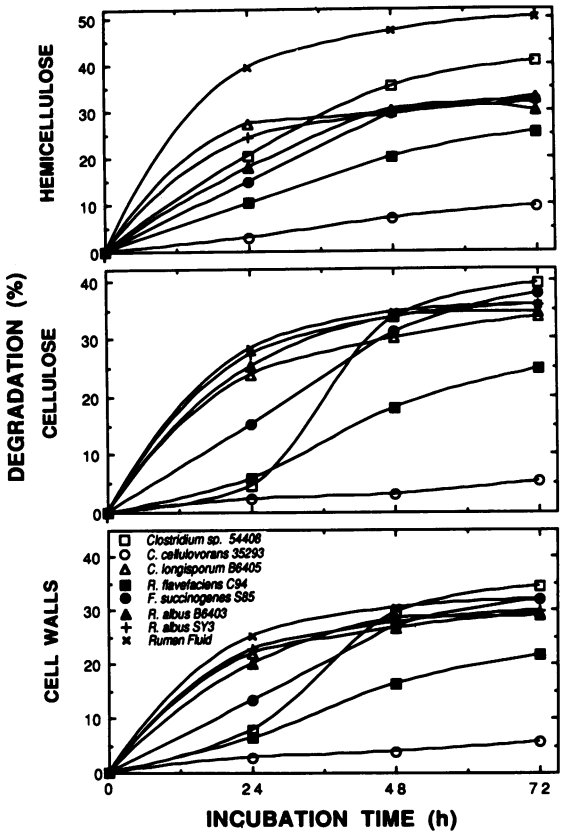

FIG. 3. Comparison of in vitro degradation of alfalfa fractions by *Clostridium* sp. strain 54408, mixed-culture ruminal fluid, and other ruminal or digestor cellulolytic strains.

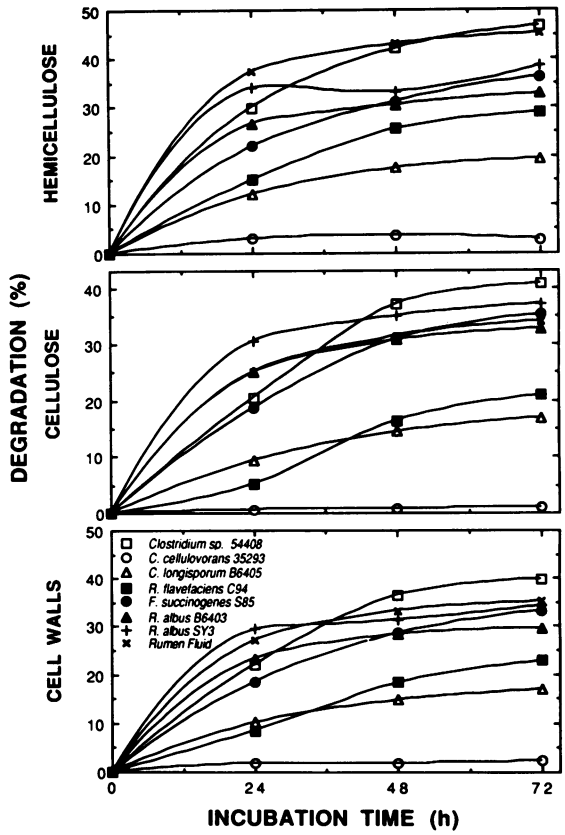

FIG. 4. Comparison of in vitro degradation of bromegrass fractions by *Clostridium* sp. strain 54408, mixed-culture ruminal fluid, and other ruminal or digestor cellulolytic strains.

(16). Thus, from these very different phenotypic characteristics and the G+C content, it is unlikely that strain 54408 is a modified species of the original *C. longisporum* fed to these pigs. It also seems unlikely that the genes for an entire cellulase enzyme complex would be transferred from *C. longisporum* to a resident clostridial species in the pig large intestine, even though 7% of the cultured flora from the pig gut can be clostridia (11).

Characteristics of *Clostridium* sp. strain 54408 do not fit those of other known cellulolytic clostridia (3, 4), and thus, it is considered a new species. Its ecological significance is uncertain because it was found in only two of the six pigs sampled. It is also unclear whether feeding *C. longisporum* to pigs is a factor contributing to the occurrence or establishment of the new cellulolytic species.

We have not observed these large zones of clearing (diameter, 10 to 20 mm) in cellulose roll tubes in our previous efforts to characterize the cellulolytic or hemicellulolytic organisms in the pig intestinal tract (15, 17). It is possible that cellulolytic clostridia are part of the normal flora, in low numbers, and once we feed and rectally infuse cultures of *C. longisporum* we stress the pigs enough that the normal flora is modified to permit colonization of cellulolytic clostridia at 10^7 populations. However, we were able to isolate cellulolytic cultures from these two pigs 8 weeks after our initial isolations, with similar phenotypic characteristics, suggesting that the cellulolytic clostridia were part of the stable flora.

Our future studies will involve direct infusion of *C. longisporum* into the cecum of the pig to determine whether the percentage of the pigs that are colonized by cellulolytic clostridia can be increased. If *C. longisporum* plays a role in establishing the cellulolytic clostridia in pigs, many of the *C. longisporum* cells may not survive the low pH environment of the pig stomach, which may explain in part why the cellulolytic clostridia were found in only two of six pigs. Rectal infusion of *C. longisporum* was considered less than satisfactory because pigs defecated shortly after infusion. Thus, infusion directly into the cecum would circumvent these problems.

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REFERENCES

1. **Berns, K. I., and C. A. Thomas, Jr.** 1965. Isolation of high molecular weight DNA from *Haemophilus influenzae*. J. Mol. Biol. **11**:476–490.
2. **Bryant, M. P.** 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. Am. J. Clin. Nutr. **25**:1324–1328.
3. **Cato, E. P.** 1989. Taxonomy and phylogeny, p. 1–26. *In* N. P. Minton and D. J. Clark (ed.), Biotechnology handbooks, vol. 3. Clostridia. Plenum Press, New York.
4. **Cato, E. P., W. L. George, and S. M. Finegold.** 1986. Genus *Clostridium* Prazmowski 1880, 23^{AL}, p. 1141–1200. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's

manual of systematic bacteriology, vol. 2. Williams & Wilkins, Baltimore.

5. **Dehority, B. A., and J. A. Grubb.** 1976. Basal medium for the selective enumeration of rumen bacteria utilizing specific energy sources. Appl. Environ. Microbiol. **32:**703–710.
6. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
7. **Moore, W. E. C., L. V. H. Moore, E. P. Cato, T. D. Wilkins, and E. T. Kornegay.** 1987. Effect of high-fiber and high-oil diets on the fecal flora of swine. Appl. Environ. Microbiol. **53:**1638–1644.
8. **Robinson, I. M., S. C. Whipp, J. A. Bucklin, and M. J. Allison.** 1984. Characterization of predominant bacteria from the colons of normal and dysenteric pigs. Appl. Environ. Microbiol. **48:** 964–969.
9. **Russell, E. G.** 1979. Types and distribution of anaerobic bacteria in the large intestine of pigs. Appl. Environ. Microbiol. **37:**187–193.
10. **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.
11. **Salanitro, J. P., I. G. Fairchilds, and Y. D. Zgornicki.** 1974. Isolation, culture characteristics, and identification of anaerobic bacteria from the chicken cecum. Appl. Microbiol. **27:**678–687.
12. **Schildkraut, C. L., J. Marmur, and P. Doty.** 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. J. Mol. Biol. **4:**430–433.
13. **Sleat, R., R. A. Mah, and R. Robinson.** 1984. Isolation and characterization of an anaerobic, cellulolytic bacterium, *Clostridium cellulovorans* sp. nov. Appl. Environ. Microbiol. **48:**88–93.

13a. **Supelco.** 1982. Gas chromatography bulletin 790B. Supelco, Bellefonte, Pa.

14. **van Soest, P. J., and J. B. Robertson.** 1980. Systems of analysis for evaluating fibrous feeds, p. 49–60. *In* W. J. Pigden, C. C. Balch, and M. Graham (ed.), Standardization of analytical methodology for feeds. Publication no. IDRC-134e. International Development Research Center, Ottawa, Canada.
15. **Varel, V. H.** 1987. Activity of fiber-degrading microorganisms in the pig large intestine. J. Anim. Sci. **64:**488–496.
16. **Varel, V. H.** 1989. Reisolation and characterization of *Clostridium longisporum*, a ruminal sporeforming cellulolytic anaerobe. Arch. Microbiol. **152:**209–214.
17. **Varel, V. H., S. J. Fryda, and I. M. Robinson.** 1984. Cellulolytic bacteria from pig large intestine. Appl. Environ. Microbiol. **47:**219–221.
18. **Varel, V. H., A. J. Richardson, and C. S. Stewart.** 1989. Degradation of barley straw, ryegrass, and alfalfa cell walls by *Clostridium longisporum* and *Ruminococcus albus*. Appl. Environ. Microbiol. **55:**3080–3084.