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Characterization of the Cecal Bacteria of Normal Pigs

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One hundred ninety-two isolates from cecal contents of three normal weaned pigs were obtained by means of anaerobic roll tube methods and were characterized. Seventy-eight percent of the isolates were gram-negative. The most numerous species isolated from each of the pigs was *Bacteroides ruminicola*. This species accounted for 35% of the isolates that were characterized, and *Selenomonas ruminantium* accounted for 21% of the isolates. Other gram-negative bacteria isolated from all three pigs were *Butyrivibrio fibrisolvens* (6.0%) and *Bacteroides uniformis* (3.0%); predominant gram-positive isolates were *Lactobacillus acidophilus* (7.6%), *Peptostreptococcus productus* (3.0%), and *Eubacterium aerofaciens* (2.5%). The other 42 isolates were placed in 14 other species, and 5 additional isolates that did not fit well into existing species were not placed taxonomically. Fifteen of the isolates (representing nine species) produced urease.

Gastrointestinal microbes are important factors influencing animal health in both positive and negative ways, but our knowledge of these microbes and their activities in swine is meager. The best known gastrointestinal ecosystem is the rumen, and there is evidence that methods and media devised to study ruminal microbes are among the best available for studies of predominantly anaerobic microbial populations of the lower bowel of other animals, including swine (3, 22, 29, 30). In an earlier study, we used anaerobic roll tube methods and rumen fluid-based media to determine the relative proportions of bacterial populations of the pig cecum and colon that use specific substrates (3). The present report is a continuation of that study and describes the taxonomic characterization of the predominant bacteria in cecal contents from three pigs.

MATERIALS AND METHODS

Isolation of cecal bacteria. Cecal samples were obtained from weaned Yorkshire pigs from a closed herd at the National Animal Disease Center, Ames, Iowa (3). Cecal samples from pigs 17, 18, and 20 were diluted and cultured on roll tubes of medium CCA. The composition of medium CCA and the colony counts from these same cecal samples, as well as other details, such as diets of the animals and methods used to obtain and culture cecal samples, have been described (3).

After incubation for 7 days, colonies were picked at random from roll tubes of medium CCA that had been inoculated with dilutions that contained 2 or 0.2 ng (wet weight) of cecal contents. As many colonies as possible were picked from individual roll tubes into CCA medium prepared as slants. Gram stains and wet mounts of each isolate prepared from the water of syneresis of slant cultures (24 to 48 h) were examined for morphology, motility, and purity. Cultures were streaked on CCA roll tubes, and three colonies were reisolated and compared with the original isolate. If more than one morphotype was observed, each was characterized.

Culture media and methods. Prereduced anaerobic media were prepared and used according to Holdeman et al. (12) except as otherwise indicated. Growth, terminal pH, and biochemical tests were determined on cultures incubated at 37°C for 7 days.

The basal medium for fermentation studies and for determination of various other physiological characteristics was a modification of the peptone-yeast (PY) basal medium (12). This medium was modified (MPY) for some studies by addition of 0.05% (wt/vol) $(NH_4)_2SO_4$ and 0.31% (vol/vol) of a volatile fatty acid mixture (acetic acid, 17 ml; propionic acid, 6 ml; *n*-butyric acid, 4 ml; *n*-valeric acid, isovaleric acid, isobutyric acid, and DL-α-methylbutyric acid, 1 ml of each). Inocula (0.1 ml/10 ml of medium) were from cultures grown in MPY basal medium with 0.5% (wt/vol) glucose (MPYG). The following substrates (0.5%, wt/vol) were added singly to MPY for fermentation studies: arabinose, cellobiose, esculin, fructose, galactose, glucose, glycerol, inositol, inulin, lactose, sodium lactate, maltose, mannitol, mannose, raffinose, rhamnose, ribose, salicin, sorbitol, starch, sucrose, trehalose, and xylose. Tests for starch hydrolysis and esculin hydrolysis, acetoin production, bile tolerance, catalase, and gelatin liquefaction were as described previously (12), except that MPYG was used as the basal medium. MPYG containing 1.5% agar in tubes closed with cotton plugs was used to determine gas production and oxygen tolerance. Previously described methods were used to test for production of H_2S (5), urease (31), alcohols (2), and hydrogen (8).

Tests for indole and nitrate reduction were as described (12), except indole was determined both on chopped-meat cultures and on cultures grown on indole-nitrate medium that was prepared by adding 0.1% potassium nitrate to MPYG. Cysteine and resazurin were deleted from MPYG for these tests. Fermentation acids (formic, acetic, propionic, isobutyric, butyric, branched-chain C_5, valeric, lactic, caproic, phenylacetic, fumaric, and succinic) were identified as their butyl esters by gas chromatography (23). For characterization of lactobacilli, concentrations of L(+)- and D(−)-lactic acids were determined (12) with L(+)- and D(−)-lactate dehydrogenases (Boehringer Mannheim Corp., New York, N.Y.).

RESULTS

Most of the isolates from high dilutions of cecal contents of the three pigs were obligate anaerobes. Of the 250 colonies picked from roll tube cultures, 224 grew on primary isolation, but 27 of these were subsequently lost. One hundred ninety-two isolates were identified as belonging to previously described species (Table 1), whereas five isolates did not appear to fit well into any previously identified species. These isolates are described in Table 2.

Isolates characterized as either *Bacteroides ruminicola* or *Selenomonas ruminantium* accounted for more than 50% of the isolates obtained from each pig (Table 1). Cecal samples from all three pigs also yielded isolates that were characterized as *Lactobacillus acidophilus*, *Butyrivibrio fibrisolvens*, *Peptostreptococcus productus*, *Bacteroides uniformis*, and *Eubacterium aerofaciens*. Isolates characterized as *Lactobacillus fermentum*, *Bacteroides multiacidus*, and *Fusobacterium prausnitzii* were isolated from two of the pigs, and the other 11 species listed in Table 1 were represented by isolates from cecal samples from only one of the pigs.

TABLE 1. *Relative frequencies of bacterial species in cecal contents from three pigs*

Species	No. of isolates from pig no.:			% of flora (three pigs)
	17	18	20	
Bacteroides ruminicola	19	25	26	35.5
Selenomonas ruminantium	10	9	22	20.8
Lactobacillus acidophilus	2	6	7	7.6
Butyrivibrio fibrisolvens	4	2	6	6.1
Peptostreptococcus productus	1	1	4	3.0
Bacteroides uniformis	2	2	2	3.0
Eubacterium aerofaciens	1	2	2	2.5
Lactobacillus fermentum	0	2	3	2.5
Bacteroides multiacidus	1	0	3	2.0
Fusobacterium prausnitzii	0	2	2	2.0
Megasphaera elsdenii	0	0	4	2.0
Fusobacterium necrophorum	0	0	3	1.5
Staphylococcus epidermidis	0	0	3	1.5
Bacteroides capillosus	0	3	0	1.5
Peptostreptococcus anaerobius	0	2	0	1.0
Leptotrichia buccalis	2	0	0	1.0
Eubacterium tenue	0	2	0	1.0
Eubacterium cylindroides	0	0	2	1.0
Eubacterium lentum	0	0	1	0.5
Bacteroides furcosus	0	0	1	0.5
Streptococcus intermedius	1	0	0	0.5
Unclassified	1	3	1	2.5

Thirty-five percent of the isolates were classified as *B. ruminicola*. This was thus the largest group of isolates. Seven percent of the isolates were placed in four other species of the genus *Bacteroides*. All isolates in the *B. ruminicola* group were gram-negative, nonmotile, nonsporing rods that fermented glucose and produced succinate and acetate as major growth products on MPYG medium. The mean quantity of succinate produced by the 70 isolates on MPYG medium was 24.3 ± 6.2 μmol/ml, and the mean molar ratio of succinate to acetate produced was 1.15 ± 0.29. Formate was detected as a measurable fermentation product from 84% of these isolates, and lactate, fumarate, and propionate were detected as products from 13, 10, and 7% of the isolates, respectively. None of these isolates produced butyrate, indole, or hydrogen in detectable quantities; three isolates reduced nitrate to nitrite. All isolates identified as *B. ruminicola* fermented glucose and fructose. Arabinose, starch, galactose, lactose, maltose, sucrose, and xylose were fermented by 69 of the 70 isolates in this group, and 67 fermented cellobiose and inulin. Growth of 57% of the isolates in the *B. ruminicola* group was markedly inhibited by bile, and a further 20% were weakly inhibited by bile. Other characteristics of the isolates not inhibited by bile were very similar to those of the bile-inhibited isolates, and no attempt was made to separate these isolates on this basis.

When hemin was deleted from MPYG medium, 51 of the 70 *B. ruminicola* isolates failed to grow. Since procedures to remove trace amounts of hemin from the medium were not used, we are not certain that the 19 isolates which grew without added hemin were indeed hemin independent, and we have thus not separated this group into the two subspecies that are presently recognized.

The *B. ruminicola* group, at present, thus includes a continuum of phenotypes (6). The heterogeneity of this group is also indicated by the finding that there was no deoxyribonucleic acid homology among several of these isolates from the pig cecum or between these isolates

TABLE 2. *Characteristics of isolates that were not identified as particular species*

Isolate no.	Gram reaction	Motility	Rod shape	Cellobiose	Esculin hydrolysis	Fructose	Galactose	Glucose	Lactose	Maltose	Mannose	Raffinose	Rhamnose	Ribose	Starch	Starch hydrolysis	Sucrose	Xylose	Milk	Nitrate	Hemolysis	Gas	H_2S	Acids from glucose[b]
18-14	−	+	Curved	−	−	−	−	w	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	fa2
18-63	−	+	Curved	a	−	a	−	a	a	a	a	a	−	−	a	+	a	−	c[c]	−	−	−	−	A2
17-26	Variable	−	Straight	−	−	w	w	a	−	−	−	−	w	w	−	−	−	w	−	−	−	+	+	AL
18-89	−	−	Straight	−	+	−	−	w	−	−	−	−	−	−	−	+	−	−	−	+	−	+	−	B
20-52	Variable	−	Straight	−	+	−	−	a	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	Laf

Characteristic[a]

[a] Symbols are similar to those used by Holdeman et al. (12): a, acid (pH below 5.5); w, weak acid (pH 5.6 to 6.0). None of the isolates produced acid from arabinose, esculin, glycerol, inulin, mannitol, salicin, sorbitol, or trehalose; none hydrolyzed gelatin, produced indole or urease; and all were inhibited by bile.

[b] Products are formic (F, f), acetic (A, a), butyric (B), and lactic (L) acids and ethanol (2). Capital and lowercase letters indicate, respectively, major and minor products.

[c] c, Curd.

and *B. ruminicola* strains from the bovine rumen (J. Johnson, personal communication).

Isolates characterized as *S. ruminantium* subsp. *lactilytica* were the second most numerous group of swine cecal bacteria. All 41 of these isolates fermented lactate and glycerol and had typical selenomonad morphology and tumbling motility (4). Only five of these isolates produced H_2S, six reduced nitrate, and three produced urease.

The *Selenomonas* strains were separated into four groups based on quantities of acids produced during growth on MPYG and MPY-lactate media (Table 3). Groups 1 and 2 were similar, except group 2 isolates produced considerably higher concentrations of formate, acetate, and propionate than did group 1 isolates during growth on MPY-lactate medium. Group 3 isolates produced lactate and no formate on MPYG medium, whereas group 4 isolates produced neither lactate nor formate during growth on MPYG medium. One of the selenomonad isolates did not fit into any of the four groups, and two isolates were not placed in these groups because of incomplete data.

Succinate was produced ($\geq$2 μmol/ml) by 25% of the selenomonads during growth on MPYG; only 5% produced succinate on MPY-lactate medium, but 90% (26 of the 29 tested) produced succinate during growth on MPY-glycerol medium.

B. fibrisolvens isolates were slender, curved, motile rods with monotrichous, subpolar flagellation. Butyric acid was the major product, with lesser amounts of lactic and acetic acids. All isolates produced hydrogen, and none was viable after heating at 80°C for 10 min. Four of the 12 isolates reduced nitrate, and 1 was urease positive. Although *B. fibrisolvens* cells have walls similar to those of gram-positive organisms (24), cells are gram negative when stained by standard procedures and they are considered as gram-negative organisms here. The remaining groups of gram-negative rods, *F. prausnitzii*, *Fusobacterium necrophorum*, and *Leptotrichia buccalis*, were minor constituents of the total cecal flora.

Gram-positive, nonsporing rods constituted 22.5% of the characterized cecal isolates. Most of these, 10.1%, were placed in the genus *Lactobacillus*. Of the 20 lactobacilli isolated, 15 had characteristics that closely fit fermentation patterns described for *L. acidophilus* (20) and 5 were characterized as *L. fermentum*. Several species of *Eubacterium* were identified, including *E. aerofaciens* (17) and *E. cylindroides* (7). *E. lentum* isolates were characterized by their lack of fermentative ability; they reduced ni-

TABLE 3. *Grouping of S. ruminantium isolates based on acids produced from glucose and lactate*

Medium	Group	Acid produced (μmol/ml)[a]				
		Formate	Acetate	Propionate	Lactate	Succinate
MPYG	1 (n = 15)	13.4 ± 1.9	26.8 ± 4.2	27.1 ± 3.1	—	—
	2 (n = 8)	16.2 ± 2.2	33.7 ± 3.6	31.0 ± 2.3	—	—
	3 (n = 9)	—	18.1 ± 6.5	21.7 ± 4.2	50.3 ± 53	3.1 (8)
	4 (n = 6)	—	25.1 ± 11	31.7 ± 3.4	—	4.6 (2)
MPY-lactate	1 (n = 15)	2.7 ± 6.9	14.6 ± 2.2	20.2 ± 1.8		1.2 (1)
	2 (n = 8)	5.8 ± 1.4	29.8 ± 5.2	52.3 ± 8.2		1.6 (1)
	3 (n = 9)	4.1 (2)	21.9 ± 9.7	36.8 ± 16		2.2 (1)
	4 (n = 6)	—	27.6 ± 8.7	39.8 ± 21		1.8 (2)

[a] Mean ± standard deviation of quantity of acid produced during incubation for 1 week. Numbers in parentheses indicate numbers of isolates in each group that produced each acid; where no such value is given, all isolates in the group produced the acid, and — indicates that no isolates in the group produced the acid.

trate, and growth was enhanced by arginine.

Although the two isolates identified as *Eubacterium tenue* (14) did not produce indole, they were motile, and products from glucose fermentation included major amounts of acetate with smaller amounts of formate, propionate, isobutyrate, isovalerate, and lactate. *Megasphaera elsdenii* was isolated from one pig.

All of the isolates listed in Table 1 were tested for production of urease. Three of 41 isolates of *S. ruminantium* and all 3 isolates of *Staphylococcus epidermidis* produced urease. Two isolates of *B. multiacidus* and two of *P. productus* produced urease, whereas one isolate each of *B. fibrisolvens*, *L. fermentum*, *Streptococcus intermedius*, *B. uniformis*, and *M. elsdenii* also produced urease. Several other isolates of *L. acidophilus*, *B. fibrisolvens*, and *S. ruminantium* were strongly ureolytic when tested initially but had lost this capability when the test was repeated; thus, they were not counted as ureolytic isolates.

DISCUSSION

Concentrations of bacteria in contents of the gastrointestinal tract of normal swine are much higher in the cecum and colon than in more proximal portions of the tract (9, 28, 30). Information about the predominance of bacteria in the cecum and colon of pigs is, however, limited, since many of the early studies of these populations used methods that now appear to be inadequate for culture of some of the anaerobes that are the most numerous members of the populations. In some instances, the total anaerobes were grouped together and counted as such, whereas selective media were used for estimates of particular groups of organisms. The latter procedures characteristically yielded estimates of concentrations of bacteria grouped at family or genus level, but these groups often represented minor components of the total population, since few selective media suitable for enumeration of distinct taxonomic groups that are representative of the predominant population have been developed.

In the present study, isolates were obtained with what appears to be among the best of current anaerobic techniques (3). Most of these isolates were then characterized to the level of species. Some isolates would not readily fit into any described species. Cultural reactions of these are presented in Table 2. Isolate 18-14, which produced only small amounts of formate, acetate, and ethanol from glucose, was not presumptively assigned to any genus. Isolate 18-63 probably belongs to the genus *Bacteroides*. It resembles *Bacteroides* K (18) more closely than any of the described species of this genus, but differs from *Bacteroides* K in that H_2S was not produced and esculin was not hydrolyzed. Isolate 17-26 has some characteristics that relate it to species in the genus *Bifidobacterium*; however, growth was inhibited by bile. Isolate 18-89 is tentatively assigned to the genus *Fusobacterium* on the basis of production of butyrate. Isolate 20-52 was presumptively identified as a *Lactobacillus*; however, it fermented fewer substrates than most species of this genus.

Comparisons between our results and those of others are complicated by differences due to culture methods, culture media, animal age, and diet. The isolation medium (CCA) used here was designed to be relatively nonselective and yielded higher counts than were obtained with any of several other media tested (3). Comparisons between culture counts and direct microscopic counts (3) indicate that most of the predominant cecal bacteria are able to grow on medium CCA. There are, of course, organisms, e.g., methanogens, which would not be isolated by procedures used here.

Of the 192 isolates characterized, 78% were

gram negative. With roll tube methods similar to those used here, but with media that were somewhat different, approximately one-half of the cecal isolates in one study (16) and 42 and 76% in another (29) were gram-negative. Our microscopic observations of Gram-stained preparations of cecal contents have not included differential counts based on staining reaction, but suggest that in cecal samples, gram-negative cells are at least as numerous as gram-positive cells.

Gram-positive bacteria, however, markedly outnumbered gram-negative organisms when colonic contents (21, 29) or feces (22) were cultured on media in roll tubes or on plates in an anaerobic chamber (21). Direct microscopic observations of colonic samples (21) also support the cultural results indicating that gram-positive bacteria are predominant in the pig colon. Many of the gram-positive fecal bacteria studied by Salanitro et al. (22) were facultative anaerobes, whereas culture counts (3, 16) and studies with the isolates in the present study indicate that a small proportion of the cecal population is able to grow under aerobic conditions. The results of Vervaeke and Van Nevel (29) support the concept that the pig cecum contains a higher proportion of strict anaerobes than does the colon. Thus, apparently cecal and colonic populations differ, and this finding is in contrast to the conclusion reached on the basis of differential culture counts of groups that now appear to be minor components of the populations (19).

Various workers found bacteria of the genus *Bacteroides* in cecal, colonic, or fecal material from swine (1, 11, 15, 16, 20, 25, 29), but these workers did not make species assignments. *B. ruminicola* has been identified as a minor component of the population of the human colon (10, 13), but is usually a major component of the ruminal population (6). We know of no previous reports indicating that *B. ruminicola* is a predominant species in the pig cecum. There is, however, evidence that this species is the most numerous of the *Bacteroides* isolates in samples from the colon (21) and feces (27) of swine, so the finding that cecal samples contained high concentrations of *B. ruminicola* is not surprising.

Other workers have reported the isolation of organisms belonging to the genus *Selenomonas* from swine cecal contents (16) and from feces (22), but species designations were not made. Our selenomonad isolates fermented lactate and thus are considered to belong to *S. ruminantium* subsp. *lactilytica*. The group separations in Table 3 may not reflect differences that have taxonomic significance, but these results are included here to illustrate the heterogeneity among strains that appear to fit into this subspecies. Further study is needed to clarify their taxonomic status.

Little is known of urease production by intestinal bacteria from pigs. Detection of urease production by intestinal anaerobes is dependent upon use of culture methods that minimize the repression of urease that is found when organisms are grown on media containing high levels of ammonia or organic nitrogen (31). Using such methods, urease production was demonstrated in a variety of intestinal anaerobes (26), and the present results supplement this knowledge. We are not aware of previous evidence for production of urease by *B. uniformis* or *M. elsdenii*.

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