

## Isolation, Culture Characteristics, and Identification of Anaerobic Bacteria from the Chicken Cecum

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Studies on the anaerobic cecal microflora of the 5-week-old chicken were made to determine a suitable roll-tube medium for enumeration and isolation of the bacterial population, to determine effects of medium components on recovery of total anaerobes, and to identify the predominant bacterial groups. The total number of microorganisms in cecal contents determined by direct microscope cell counts varied (among six samples) from  $3.83 \times 10^{10}$  to  $7.64 \times 10^{10}$  per g. Comparison of different nonselective media indicated that 60% of the direct microscope count could be recovered with a rumen fluid medium (M98-5) and 45% with medium 10. Deletion of rumen fluid from M98-5 reduced the total anaerobic count by half. Colony counts were lower if chicken cecal extract was substituted for rumen fluid in M98-5. Supplementing medium 10 with liver, chicken fecal, or cecal extracts improved recovery of anaerobes slightly. Prereduced blood agar media were inferior to M98-5. At least 11 groups of bacteria were isolated from high dilutions ( $10^{-9}$ ) of cecal material. Data on morphology and physiological and fermentation characteristics of 90% of the 298 isolated strains indicated that these bacteria represented species of anaerobic gram-negative cocci, facultatively anaerobic cocci and streptococci, *Peptostreptococcus*, *Propionibacterium*, *Eubacterium*, *Bacteroides*, and *Clostridium*. The growth of many of these strains was enhanced by rumen fluid, yeast extract, and cecal extract additions to basal media. These studies indicate that some of the more numerous anaerobic bacteria present in chicken cecal digesta can be isolated and cultured when media and methods that have been developed for ruminal bacteria are employed.

Numerous studies on the intestinal microflora of the domestic fowl, *Gallus domesticus*, have been made (2, 3, 5, 23, 26, 30, 34). In many of these investigations, selective plate media and conventional anaerobic jar methods have been used in an analysis of specific bacterial groups, namely, coliforms, streptococci, lactobacilli, bacteroides, and clostridia. At best only these groups of bacteria have been identified, and unless very good anaerobic techniques were used, some of the predominating anaerobic species have been overlooked. Others, however, have developed nonselective media for the isolation of rumen anaerobic bacteria (9, 12). With these media (containing low levels of energy sources) in conjunction with strict anaerobic techniques, numerous bacterial types are permitted to grow. Relatively few studies on poultry intestinal microflora have been made using such media and methods. Recent work by Barnes et al. (3, 5) on the isolation of anaerobes from the chicken cecum has indicated that 20 to

30% of the direct microscope count can be cultured by using the Hungate technique and a nonselective medium such as medium 10 (medium without rumen fluid devised for rumen anaerobes by Caldwell and Bryant [12]) supplemented with liver and chicken fecal extracts. However, data on comparison of other roll-tube media (e.g., rumen fluid-containing media used for culturing the predominant ruminal species) or the effect of various medium constituents on the enumeration and isolation of chicken cecal bacteria are not available.

Experiments in this laboratory indicate that a rumen fluid medium can be used to isolate a large percentage of the total bacteria from chicken cecal digesta. In addition, our present studies compare the cultural characteristics and isolation efficiency of other nonselective roll-tube media and furnish information on the presumptive identification and relative distribution of the predominant anaerobic bacteria colonizing the cecum of the chicken.

## MATERIALS AND METHODS

**Animals and diet.** In experiments designed to determine colony counts, recovery of anaerobes, and effects of medium components in different nonselective media, cecal samples were obtained from 5-week-old cockerels (White Cornish cockerel  $\times$  White Rock hen) supplied by a commercial broiler facility. In all other experiments on the cecal microflora including isolation and identification of bacterial strains, cecal samples were obtained from 5-week-old, "laboratory-reared" birds described below.

One-day-old cockerels (same breed as above) were housed in temperature-controlled rooms (12-h light cycle and slight negative pressure) on floors covered with autoclaved pine shavings. An initial brooding temperature of 35 C was maintained for young birds and reduced in weekly increments to a constant temperature of 25 C at 3 weeks of age. Throughout the growth period, a commercial broiler grower ration and water were provided without restriction. The diet was supplemented with a coccidiostat continuously and with antibiotic (50 mg of bacitracin per kg of feed) from day 1 to 4 weeks of age. After 4 weeks, antibiotic-free ration of the same composition replaced the medicated ration.

**Anaerobic culture techniques.** Anaerobic techniques employed were similar to those described by Hungate (20, 21) for rumen bacteria with modifications of Bryant (6, 7). Strict anaerobic techniques were maintained throughout all procedures involving the dilution of cecal samples and preparation and inoculation of media.

**Sampling procedure.** Five-week-old cockerels were taken at midday and sacrificed by CO<sub>2</sub> asphyxiation. Immediately, cecal samples (2 to 4 g, wet weight) from both ceca of a single animal were placed in a sterile, stoppered tube and flushed with oxygen-free

CO<sub>2</sub>. Cecal contents were weighed and processed through serial 10-fold dilutions in tubes of the anaerobic mineral solution of Bryant and Burkey (7). Fractions (0.2 and 1.0 ml) of a dilution (up to 10<sup>-9</sup>) were added to pre-reduced and melted agar tubes of the various media maintained at 48 C. Within 10 min after inoculation, roll tubes were made by rapidly rotating tubes in a spinner (Bellco Glass, Inc.) and simultaneously cooling with cold water. Solidified agar roll tubes were incubated up to 14 days at 37 C.

**Roll-tube media and media preparation.** The compositions of different nonselective roll-tube media compared in this study are given in Table 1. Medium 98-5 is an improved rumen fluid medium for culturing ruminal bacteria and has been described by Bryant and Robinson (9). Modified 98-5 (M98-5) medium is one used by R. Williams and M. P. Bryant for culturing bacteria from anaerobic sludge digesters (unpublished data) and is different from 98-5 medium in that glycerol, Trypticase, and hemin are included and mineral solution S2 is substituted for mineral solutions 1 and 2. Mineral solution S2 components and their final concentrations in the medium were as follows: KH<sub>2</sub>PO<sub>4</sub>, 0.3 mM; NaCl, 15.5 mM; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.37 mM; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.2 mM; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.1 mM; MnCl<sub>2</sub>·4H<sub>2</sub>O, 1.0  $\mu$ M; and CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.08  $\mu$ M.

Medium 10 (M10) was developed by Caldwell and Bryant (12) and contains a mixture of volatile fatty acids, hemin, Trypticase, and yeast extract in place of rumen fluid. Supplemented M10 as described by Barnes and Impey (3) contains liver extract (5%, vol/vol) and chicken fecal extract (10%, vol/vol) added to M10. Liver extract (3) was prepared by heating a 13.5% (wt/vol) solution of dehydrated liver (Difco Laboratories) at 50 C for 1 h. The extract was clarified by centrifugation at 10,000  $\times$  g, adjusted to neutral pH, sterilized by autoclaving, and stored

TABLE 1. Composition of nonselective roll-tube media<sup>a</sup>

Component <sup>b</sup>	Percent in medium <sup>c</sup>			
	98-5	M98-5	M10	Supplemented M10
Rumen fluid, clarified (vol/vol) .....	40	40		
Glucose .....	0.03	0.03	0.03	0.03
Cellobiose .....	0.03	0.03	0.03	0.03
Maltose .....		0.03		
Glycerol .....		0.03		
Starch .....	0.05		0.05	0.05
Minerals S2 (vol/vol) .....		5		
Minerals 1 and 2 (vol/vol; 3.75 of each) .....	7.5		7.5	7.5
Trypticase .....		0.2	0.2	0.2
Yeast extract .....			0.05	0.05
Volatile fatty acids (vol/vol) .....			0.31	0.31
Hemin .....		0.0002	0.0002	0.0002
Liver extract (vol/vol) .....				5-20
Cecal extract (vol/vol) .....				5-30
Fecal extract (vol/vol) .....				10

<sup>a</sup> Final pH 6.8 to 7.0; 100% CO<sub>2</sub> gas phase.

<sup>b</sup> Additional components added to all media (percent wt/vol): resazurin (0.0001), Na<sub>2</sub>CO<sub>3</sub> (0.4), cysteine-hydrochloride-water (0.025), Na<sub>2</sub>S·9H<sub>2</sub>O (0.025), and agar (2).

<sup>c</sup> Final percent as wt/vol or as indicated.

refrigerated. Chicken fecal and cecal extracts were prepared by autoclaving (15 psi, 15 min) equal quantities (wet weight) of feces and cecal digesta, respectively, and water. The preparations were clarified by centrifugation ( $10,000 \times g$ , 10 min), neutralized, again autoclaved (15 psi, 15 min), and stored refrigerated.

In addition to these culture media, two types of blood agar media were evaluated with respect to recovery of total anaerobes from cecal material: VL blood (oxalated horse blood) agar as described by Barnes and Impey (3) and Schaedler blood (defibrinated rabbit blood) agar of Starr et al. (32). These media were equilibrated and tubed under  $\text{CO}_2$  gas phase. Blood agar, prepared in this way, supported hemolysis of known hemolytic bacteria and therefore contained intact red blood cells.

In the preparation of all roll-tube media, components except  $\text{Na}_2\text{CO}_3$  buffer, reducing agent (cysteine or cysteine-sulfide), and blood were diluted to volume in round-bottom flasks, equilibrated with  $\text{CO}_2$  gas by gentle boiling, fitted with rubber stoppers, and sterilized by autoclaving (15 psi, 15 min). After autoclaving, a sterile reducing agent, buffer, or blood was added to media held at 48 C and then dispensed (9-ml amounts) anaerobically into sterile, rubber-stoppered, disposable tubes (18 by 150 mm, Bellco Glass, Inc.).

**Direct microscope counts, colony counts, and statistical analysis.** Direct microscope cell counts were made on cecal samples employing a Petroff-Hauser bacterial counting chamber and a phase-contrast microscope following the method of Meynell and Meynell (25). For microscope counts, cecal sample dilutions of  $10^{-3}$  were made in 0.9% saline containing 10% Formalin.

Colony counts in roll tubes were determined after 3, 6, and 14 days of incubation with the aid of a Quebec colony counter. Colony counts from eight roll tubes (prepared from a single dilution) were determined using the colony-counting criteria of Bryant and Robinson (9). Mean counts and standard errors from several cecal samples were obtained, and the data were subjected to an analysis of variance and differences between means evaluated with the 5% least significant difference (31). In some cases, colony counts were converted to percent recovery of the direct microscope counts.

**Isolation of cecal bacteria and presumptive identification of strains.** Fifty isolated colonies were picked from single roll tubes of M98-5 (containing usually 50 to 100 colonies) inoculated with  $10^{-9}$  dilutions of cecal contents and incubated for 6 days. About 300 colonies were isolated from single cecal samples of six different birds. Isolates were subcultured onto maintenance slant medium (composition as modified 98-5, Table 1, except glucose, cellobiose, and maltose were at 0.05% wt/vol concentration). Wet mounts of each isolate, prepared from the water of syneresis of slant media (24- to 48-h cultures), were observed for morphology, motility, and purity with phase-contrast microscopy. These same cultures were gram stained according to the Kopeloff modification

(19). Isolates containing more than one morphological type were separated, and strains were reisolated from roll streaks (19) of M98-5 medium. Pure cultures of strains were grouped and presumptively identified by using media and methods described by Caldwell and Bryant (12). Oxygen relations (facultatively anaerobic or anaerobic) of isolated strains were determined on aerobic plate media. Cultures (by loopful) were streaked onto brain heart infusion-blood agar described by Holdeman and Moore (19), and surface growth was observed at 1, 3, and 6 days of incubation at 37 C. In addition to these preliminary tests, fermentation products elaborated in glucose medium (11) were analyzed for volatile and nonvolatile acids.

Selected strains from each of the presumptively identified groups were then processed through carbohydrate fermentation and biochemical tests. Organisms were identified according to classification schemes of Holdeman and Moore (19) and by comparison with other published data on anaerobic bacteria. The basal medium for carbohydrates and substrates fermented in these studies was that of Bryant (11) and contained the following components: 20% (vol/vol) rumen fluid, CRF2; 7.5% (vol/vol) each of mineral solutions 1 and 2; 0.5% (wt/vol) Trypticase; 0.05% (wt/vol) cysteine-hydrochloride; 0.06% (wt/vol)  $\text{Na}_2\text{CO}_3$ ; and 0.0001% (wt/vol) resazurin. Most carbohydrates were prepared as 10% wt/vol solutions, filter sterilized, equilibrated with  $\text{CO}_2$ , and added (0.5% final concentration) aseptically to the basal medium. Medium and tests for esculin hydrolysis, gelatin liquefaction, nitrate reduction, and indole production were as described by Bryant and Doetsch (8). All of these media were prepared by the method of Bryant and Burkey (7) and tubed in 4-ml amounts in sterile, rubber-stoppered tubes (13 by 100 mm, Bellco Glass, Inc.) under 10%  $\text{CO}_2$ -90%  $\text{N}_2$  gas mixture. Growth, terminal pH, and biochemical tests were determined on cultures incubated for 7 days at 37 C. The inoculum medium in these studies was the same as M98-5 (Table 1) except glucose, cellobiose, and maltose were at 0.3% (wt/vol), and agar was omitted. All tests were inoculated with 3 drops of a 24- to 48-h inoculum medium culture under 10%  $\text{CO}_2$ -90%  $\text{N}_2$  gas phase.

**Analysis of fermentation products.** Volatile (acetic, propionic, butyric, and valeric) and non-volatile (lactic and succinic) acids formed in glucose-containing media were analyzed by gas chromatographic methods. The gas chromatograph used was a Hewlett-Packard model 5754B equipped with a flame ionization detector. Fermentation media samples (1 ml) were acidified with 0.1 ml of 6 N HCl, and any precipitate formed was removed by centrifugation. Acidified samples were analyzed for volatile acids on a column packed with 20% Carbowax 20 M TPA on Chromosorb W (Varian, 60 to 80 mesh, A/W, DMCS). Nonvolatile acids were methylated in acidified media and analyzed by a modification of the procedure of Hautala and Weaver (18).

Formic acid was determined enzymatically by the method of Rabinowitz and Pricer (28). Hydrogen gas produced in gas glucose medium (12) was analyzed by

gas chromatography on a Porapak Q (60 to 80 mesh, Applied Science) column and using a thermal conductivity detector.

## RESULTS AND DISCUSSION

**Effect of incubation time on colony counts with different roll-tube media.** The data in Table 2 show the effect of incubation time on colony counts in 98-5, M98-5, and M10 media. Although the number of colonies continued to increase throughout the 14-day incubation period, the colony counts at 14 days were not significantly higher ( $P = 0.05$ ) than those at 6 days with M98-5 and M10. In contrast, there was a significant ( $P = 0.05$ ) increase in colony counts at each incubation time with 98-5. At all intervals of incubation, colony counts were significantly higher in M98-5 than in either 98-5 or M10 (Table 2, last column). Six-day colony counts in 98-5, M98-5, and M10 represented 79%, 92%, and 85%, respectively, of the 14-day counts. When 6-day counts were converted to percent recovery of the direct microscope cell count, 42.8% (98-5), 59.6% (M98-5), and 45.6% (M10) of the total bacterial population in cecal contents could be cultured.

**Deletion, addition, or substitution of components in M98-5 medium.** Omission of rumen fluid from M98-5 reduced colony counts by 50%,

whereas deletion of hemin alone made no significant difference in the number of colonies observed (Table 3). Colony counts in M98-5 lacking rumen fluid were similar to those obtained with M98-5 without rumen fluid and hemin. These results indicate that rumen fluid enhances the growth of many cecal bacteria when Trypticase is the only other source of organic growth factors with the exception of the carbohydrate energy sources. From the data, it is not possible to determine whether hemin is a nutritional requirement for cecal anaerobes because rumen fluid would be expected to contain heme (13). Preliminary nutritional studies on representative isolates (48 strains) from each group given in Table 6, however, indicate that few if any of these bacteria require hemin for growth or are stimulated by it. Hemin does augment the growth of most strains of *Bacteroides* isolated from the bovine rumen (10, 13, 22), human oral cavity, and intestine (16, 24).

Colony counts in medium M98-5 were not significantly ( $P = 0.05$ ) altered when the following single changes were made (data not shown): (i) substitution of minerals S2 with minerals 1 and 2, (ii) deletion of Trypticase or substitution of Trypticase with Casamino Acids (0.2%), (iii) addition of yeast extract (0.05%), and (iv) deletion of glycerol. The reason for M98-5 yielding higher colony counts than 98-5 is not clear, particularly because deletion of Trypticase from M98-5 has no effect on colony counts. Perhaps the added energy source, maltose, or the combined addition of maltose, Trypticase, and glycerol in M98-5 enhances the growth of additional strains of cecal bacteria.

Recent unpublished work by E. Barnes of the Food Research Institute, Norwich, England, (personal communication) suggests that several

TABLE 2. Comparison of 98-5, modified 98-5, and medium 10 roll-tube media on colony counts and incubation time

Expt	Incubation (days)	Mean <sup>a, b, c</sup>			Statistical significance between media <sup>b, d</sup>
		98-5	M10	M98-5	
1	2	1.93x		3.06x	S <sup>e</sup>
	6	2.54y		3.63xy	S
	14	3.23z		4.07y	S
2	3		1.84x	2.66x	S
	6		2.48y	3.20y	S
	14		2.94y	3.49y	S

<sup>a</sup> Mean colony counts times  $10^{10}$  per gram, wet weight, of cecal contents of four (expt 1) or six (expt 2) cecal samples. Different preparations of modified 98-5 medium were used in experiments 1 and 2.

<sup>b</sup> Means not followed by the same letter are significantly different at the 5% level of probability.

<sup>c</sup> Within each medium, comparison of significance is made among incubation times.

<sup>d</sup> At each incubation time, comparison of significance is made between media; in experiment 1 between 98-5 and modified 98-5 and in experiment 2 between M10 and modified 98-5.

<sup>e</sup> S, counts are significantly different.

TABLE 3. Effect of deletions from modified 98-5 medium on colony counts

Deletion	Mean <sup>a</sup>	Statistical significance <sup>b</sup>
None	3.20	x
Hemin	2.85	x
Rumen fluid	1.66	y
Hemin and rumen fluid	1.94	y

<sup>a</sup> Mean colony counts times  $10^{10}$  per gram, wet weight, of cecal contents obtained from six cecal samples after 6 days of incubation. Colony counts on all samples were made from a single batch of medium prepared with and without the noted deletions.

<sup>b</sup> Means not followed by the same letter are significantly different at the 5% level of probability.

chicken cecal anaerobes require liver extract to grow in carbohydrate-containing media. In our experiments, addition of liver extract (5 to 20%) to M98-5 (Table 4) did not significantly affect colony counts.

To test the possibility that chicken cecal extract might replace rumen fluid for the growth of cecal bacteria, M98-5 (minus rumen fluid) was supplemented with 5 to 30% cecal extract. No increase in colony counts was noted (Table 4). Also supplementing M98-5 with varying amounts (2.5 to 20%) of cecal extract did not increase counts above the nonsupplemented M98-5 medium.

**Comparison of various media on percent recovery of anaerobes.** In Table 5, the percent recovery of anaerobes in different nonselective media is compared. Although considerable variation (10 to 30%) was observed among cecal samples with all media tested, M98-5 medium gave consistently higher percent recoveries (mean of 60%) than M10, supplemented M10, or blood agar media. Barnes and Impey (3) have found that addition of liver and chicken fecal extracts to M10 is necessary for the isolation of many fastidious anaerobes from the chicken cecum. It may be concluded from our studies that supplementation of M10 with liver extract and/or chicken fecal or cecal extracts yielded 46 to 54% of the direct microscope cell count. These results indicate that such additions only marginally improve the recovery of bacteria

from that of M10 alone. Moreover, we have consistently observed higher percent recoveries (15 to 25% higher) with M10 or supplemented M10 than those reported by Barnes and co-workers (3, 4).

With the blood agar roll-tube media tested, 25% of the microflora could be cultured. The high partial pressure exerted by CO<sub>2</sub> (in the absence of added Na<sub>2</sub>CO<sub>3</sub>), however, altered the pH of VL blood agar to 6.5 and of Schaedler blood agar to 5.9 and, therefore, may have inhibited the growth of some bacteria. In subsequent experiments, six cecal samples were tested on VL blood agar and Schaedler blood agar buffered with Na<sub>2</sub>CO<sub>3</sub> (0.4% vol/vol) to a pH of 6.8 to 6.9. The median percent recovery with VL blood agar was 33% (range 21 to 44%) and with Schaedler blood agar was 44% (range 33 to 52%) of the total microscope cell count. In contrast, the median percent recovery with M98-5 medium in these experiments was 81% (range 72 to 96%). These data indicate that for primary isolation of cecal anaerobes, pre-reduced blood agar media are inferior to rumen fluid media (M98-5). Also, adequate buffering of blood agar media in roll tubes is important when CO<sub>2</sub> is used as a culture gas.

In contrast to the culture counts in anaerobic roll tubes, plate counts of total aerobes on Eugonagar or of fungi on Sabouraud agar represented only 2% of the direct microscope counts. Most of the strains isolated on Eugonagar were identified as *Escherichia coli* and facultatively anaerobic streptococci in further studies.

**Characterization of cecal strains.** From M98-5 medium, 298 strains of bacteria from six cecal samples were isolated. Strains were initially grouped according to a few morphological and physiological features, as well as fermentation products formed from glucose (Table 6). Identification of selected strains was also based on a comparison of their reactions in various fermentation and biochemical tests to those of known species (Table 7). The predominant microflora is represented by at least 11 groups of bacteria, most of which are gram positive and strictly anaerobic, although two groups of facultatively anaerobic cocci were isolated.

Group I was composed of gram-negative cocci or budding cocci (1 by 1.5 to 2 μm), which were club- or dumbbell-shaped cells arranged in pairs and chains. These organisms in a rumen fluid medium (Table 6) were obligately anaerobic and produced small amounts of H<sub>2</sub> gas, and formic, acetic, propionic, and butyric acids from glucose. One strain (E10), further analyzed by the VPI Anaerobe Laboratory on peptone-yeast extract basal medium, was

TABLE 4. Effect of additions to modified 98-5 medium on colony counts

Expt	Medium	Addition (%, vol/vol)	Mean <sup>a</sup>	Statistical significance <sup>b</sup>
1	Complete Complete	None	3.63	x
		Liver extract		
		5	3.76	x
		10	3.85	x
		20	3.52	x
2	Complete Complete Minus rumen fluid	None	3.20	x
		Cecal extract		
		5	2.39	z
		10	2.66	y z
		20	2.95	x y
		30	2.75	x y z

<sup>a</sup> Mean colony counts (6 days of incubation) times 10<sup>10</sup> per gram, wet weight, of cecal contents of four (experiment 1) or six (experiment 2) cecal samples. Different preparations of modified 98-5 medium were used in experiments 1 and 2.

<sup>b</sup> Means not followed by the same letter are significantly different at the 5% level of probability.

TABLE 5. Summary of comparisons of various roll-tube media on percent recovery of anaerobes from cecal samples

Medium	Recovery (%) <sup>a</sup>							Statistical significance <sup>b</sup>
	1	2	3	4	5	6	Mean	
Rumen fluid (modified 98-5) .....	51 <sup>c</sup>	81	60	49	63	53	60	x
Medium 10 supplemented with (% vol/vol) .....	37	63	44	45	41	45	46	y
Liver extract (5) .....	40	58	48	38	44	43	45	y
Cecal extract (10) .....	41	66	52	33	50	48	48	y
Fecal extract .....	35	92	51	46	49	49	54	x y
Liver (5) + cecal (10) extracts .....	45	64	56	43	50	48	51	x y
Liver (5) + fecal (10) extracts .....	47	77	51	40	46	46	51	x y
VL blood agar .....	31	— <sup>d</sup>	—	18	24	—	27	z
Schaedler blood agar .....	19	—	—	25	31	—	25	z

<sup>a</sup> Cecal samples from six individual birds direct microscope count times 10<sup>10</sup> of cecal contents per gram was 7.00, 3.83, 2.68, 6.82, 5.67, and 7.64, respectively; mean was 5.59.

<sup>b</sup> Means not followed by the same letter are significantly different at the 5% level of probability.

<sup>c</sup> Colony counts per direct microscope counts times 100%. Calculated from 6-day culture counts.

<sup>d</sup> —, No data.

TABLE 6. Presumptive identification features and distribution of bacterial groups in chicken cecal contents<sup>a</sup>

Group (morphology)	Iso- lated strains (%)	Gram reac- tion	Oxy- gen toler- ance	H <sub>2</sub> S	Glucose fermen- tation (pH)	Cata- lase	Starch hy- droly- sis	H <sub>2</sub> (%) vol/vol	Fermenta- tion products	Tentative identi- fication
I. Gram-negative cocci .....	7	—	A	—	6.2-6.6	—	—	0-1	Apbf	Unknown
II. Gram-positive cocci .....	12	+	F	—	4.8-5.6	—	—	0	Labf(P)	Unknown
III. Streptococci .....	14									
a .....		+	F	—	4.0-5.2	—	—	0	Apb(Lf)	Unknown
b .....		+	A	+	4.9-5.0	—	—	2.1-21.5	Ap(1)	<i>Peptostrepto- coccus</i>
c .....		+	A	—	5.3-6.2	—	-+	2.1-21.5	Apl	<i>Peptostrepto- coccus</i>
IV. Gram-positive rods .....	32									
a .....		+	A <sup>F</sup>	+ <sup>-</sup>	4.7-5.1	- <sup>+</sup>	—	0	PAs(L)	<i>Propionibac- terium</i>
b .....		+	A	+ <sup>-</sup>	4.8-5.2	—	+	4.7-25	Labf(ps)	<i>Eubacterium</i>
c .....		+	A	+ <sup>-</sup>	5.6-7.0	—	—	0-6.1	Apb(Lf)	<i>Eubacterium</i>
V. Gram-negative rods .....	14	—	A	+ <sup>+</sup>	5.0-5.9	—	-+	0-16.3	LAp(fb)	<i>Bacteroides</i>
VI. Spore-forming rods .....	10									
a .....		+	A <sup>F</sup>	+ <sup>-</sup>	4.5-5.0	—	—	0-20.6	LAfp(b)	<i>Clostridium</i>
b .....		+	A	—	6.6-6.8	—	—	0	ap	
VII. Miscellaneous rods .....	11									

<sup>a</sup> Reactions given are those for a majority of the strains within a group. Symbols: oxygen tolerance, A (anaerobic), F (facultative); +, positive reaction; —, negative reaction. Superscripts refer to reactions of a few strains; fermentation products from glucose: f (formic); A, a (acetic); P, p (propionic); b, f (butyric); L, l (lactic); s (succinic). Upper-case letters refer to acids formed in amounts of 10 μmol/ml of medium or greater, whereas lower-case letters refer to amounts less than 10 μmol/ml. Products in parentheses are formed by a few strains. None of the strains tested digested cellulose, and only a few strains in Group VIb were motile.

TABLE 7. Fermentation and physiological characteristics of isolated cecal strains<sup>a</sup>

Characteristic	Group (no. of strains tested)										
	I 4	II 6	IIIa 5	IIIb 3	IIIc 4	IVa 23	IVb 11	IVc 14	V 9	VIa 4	VIb 4
Amygdalin	-	-	a <sup>-</sup>	-	-	-	-	-	-	a <sup>w</sup>	-
Arabinose	-	-	a <sup>-</sup>	a	w	-	a	-	w <sup>-</sup>	-	-
Cellobiose	-	-	a	w	-	-	a	-	w <sup>-</sup>	a <sup>-</sup>	-
Esculin hydrolysis	w	-	+	-	w	-	+	- <sup>+</sup>	+	+	-
Fructose	-	a	a	a	-	a	a	-	- <sup>w</sup>	a	-
Glucose	-	a	a	a	a <sup>-</sup>	a	a	-	a <sup>w</sup>	a	-
Glycogen	-	-	-	-	-	-	a	-	-	-	-
Lactose	-	a	a	a	w	-	- <sup>w</sup>	-	w <sup>a</sup>	a	-
Maltose	-	a	a	-	w	-	a	-	w	a <sup>-</sup>	-
Mannitol	-	-	a <sup>-</sup>	-	-	a <sup>-</sup>	-	-	-	w	-
Mannose	-	-	a	-	-	a <sup>-</sup>	-	-	-	a	-
Melezitose	-	-	-	-	-	-	a	-	-	-	-
Melibiose	-	-	a <sup>-</sup>	w	w	-	a	-	- <sup>a</sup>	w	-
Raffinose	-	-	a <sup>-</sup>	w	-	-	a	-	-	w	-
Rhamnose	-	-	-	-	w	-	a	-	-	-	-
Ribose	-	- <sup>a</sup>	- <sup>a</sup>	a	-	a <sup>-</sup>	-	-	w	a <sup>-</sup>	-
Salicin	-	-	a	-	w	- <sup>w</sup>	a	-	w	a	-
Sorbitol	-	- <sup>a</sup>	- <sup>a</sup>	-	-	a <sup>-</sup>	-	-	-	-	-
Sucrose	-	a	a	-	a <sup>-</sup>	- <sup>a</sup>	a	- <sup>a</sup>	a <sup>w</sup>	a	- <sup>w</sup>
Trehalose	-	- <sup>a</sup>	a	-	w	-	a	-	w <sup>a</sup>	a <sup>-</sup>	-
Xylose	-	-	-	w	w	-	a	-	w <sup>-</sup>	-	- <sup>w</sup>
Gelatin liquefaction	-	- <sup>+</sup>	- <sup>+</sup>	-	-	+	- <sup>w</sup>	+	- <sup>+</sup>	w <sup>-</sup>	-
Indole production	-	- <sup>+</sup>	-	-	-	+	-	-	- <sup>+</sup>	-	-
Nitrate reduction	-	+	- <sup>w</sup>	+	+	+	+	- <sup>+</sup>	- <sup>+</sup>	-	- <sup>+</sup>
Growth stimulated by <sup>b</sup>	YE	YE, RF	YE	YE, RF	YE	YE, RF, T	YE, CE, RF	-	YE, RF	YE, CE, RF	-

<sup>a</sup> Reactions given are those of a majority of the strains within a group. Symbols: -, no fermentation, terminal pH, 6.3-7.0 or no reaction; a, acid reaction, terminal pH 5.5 or less; w, weak reaction, terminal pH 5.6-6.2; +, positive reaction. Superscripts refer to reactions of a few strains.

<sup>b</sup> Tested in the basal medium (with 0.4% glucose) given in Materials and Methods but with the addition of yeast extract, YE (0.2%), cecal extract, CE (5%), rumen fluid, RF (20%), or Tween 80, T (1%).

shown to ferment amygdalin, glucose, maltose, raffinose, and trehalose, to weakly ferment fructose, lactose, and salicin, and to hydrolyze esculin and produce formic and butyric acids from glucose. In preliminary nutritional studies, yeast extract was found to enhance the growth of bacteria in this group. A vitamin mixture containing thiamine-hydrochloride, nicotinamide, riboflavin, pyridoxine-hydrochloride, biotin, folic acid, and DL-thioctic acid could replace yeast extract as a growth stimulant.

Foubert and Douglas (15), in a taxonomic analysis of anaerobic micrococci, described a strain (U5) that was isolated from the human uterus. This unnamed strain is similar morphologically and in fermentation characteristics (in peptone-yeast extract basal medium) to our Group I. Strains similar to Group I have also been isolated and described by Gossling (Abstr. Ann. Meet. Amer. Soc. Microbiol., p. 81, 1972)

from human feces and by Barnes et al. (5) from chicken cecal contents.

Group II was composed of facultatively anaerobic gram-positive cocci (1- to 2- $\mu$ m diameter) occurring as singles, pairs, or tetrads. Many strains on initial isolation did not grow on aerobic plates. These strains fermented glucose, fructose, lactose, maltose, and sucrose, reduced nitrate, and formed lactic, acetic, butyric, and formic acids from glucose. They were catalase-negative and therefore differed from catalase-positive species of *Micrococcus*, *Staphylococcus*, and *Sarcina* (1). These organisms do not appear to belong to any previously described genus.

Strains in Group III were represented by three different types of gram-positive cocci in chains. Group IIIa was comprised of facultatively anaerobic cocci (1 to 1.5 by 1 to 2  $\mu$ m) in chains of lancet-shaped cells. None of the strains in

Group IIIa were hemolytic, but they fermented a wide variety of sugars and may be related to species of Group D streptococci (14). Some strains in this group did not produce an amount of lactic acid characteristic of fecal streptococci and, therefore, could not be species of the genus *Streptococcus*.

Groups IIIb and IIIc were similar in morphology and consisted of large gram-positive lancet cocci (1 to 1.5 by 1.5 to 2.5  $\mu\text{m}$ ) arranged in pairs and chains. Similar fermentation products from glucose (acetic and small amounts of propionic and lactic acids) with large amounts of  $\text{H}_2$  gas were produced by strains of both groups. Group IIIb produced  $\text{H}_2\text{S}$  and fermented fructose and ribose, whereas Group IIIc was weakly fermentative on most sugars. Yeast extract (Groups IIIb and IIIc) and rumen fluid (Group IIIb) stimulated the growth of these anaerobic streptococcal types. Groups IIIb and IIIc represent species related to *Peptostreptococcus* Kluver and van Niel according to emended descriptions of this genus by Rogosa (29). Group IIIb may be similar to Hare Group VII isolated from human feces, but an insufficient number of characteristics were given in the publication of Thomas and Hare (33) for a suitable comparison. Strains in Groups IIIb and IIIc were not related to anaerobic streptococci isolated by Barnes and Impey (3) from poultry ceca or any of the several species of *Peptostreptococcus* classified by Holdeman and Moore (19) and may constitute new species of *Peptostreptococcus*. Group IIIb strains also appear to be closely related (based on similar fermentation properties) to the chain-forming anaerobic streptococci (strain 21-29) isolated by Harrison and Hansen (17) from turkey cecal feces.

Group IVa was one of the largest groups of anaerobes isolated and consisted of gram-positive, irregular, pleomorphic rods (1 by 2 to 3  $\mu\text{m}$ ). Most strains were strict anaerobes, but a few also grew aerobically. These organisms were identified as *Propionibacterium acnes* according to criteria of Holdeman and Moore (19). A number of variants were observed in this group that could be placed into biotypes A, D, and G as described by Pulverer and Ko (27). Barnes and Impey (4) have also isolated *P. acnes* from the chicken cecum. The growth of many of our *P. acnes* strains was enhanced by yeast extract, rumen fluid, and Tween 80.

Group IVb consisted of some of the more active strains isolated. These were gram-positive organisms with rounded ends (1 by 2.5 to 4  $\mu\text{m}$ ) occurring as singles, pairs, and long chains. Strains in this group were identified as *Eubacterium rectale* (19). Group IVb strains were also

similar to strain EBG 1/80 isolated by Barnes and Impey (3) from chicken cecal contents. The growth of these strains was stimulated by yeast and cecal extracts and rumen fluid.

Group IVc included gram-variable (many stained gram negative in older cultures) fusiform and lancet-shaped cells (1.5 by 2.5  $\mu\text{m}$ ) distributed as singles, pairs, and chains. The majority of strains in this group were nonfermentative, but a few hydrolyzed esculin and starch and fermented sucrose. They appear to be related to species of *Eubacterium*.

Group V consisted of large, gram-negative pleomorphic, fusiform-shaped cells (1 to 1.5 by 2.5 to 5  $\mu\text{m}$ ) in pairs and chains. These strains resembled species classified as *Bacteroides clostridiiformis* by Holdeman and Moore (19). They were characterized by production of lactic and acetic acids from glucose and by having a variable and limited fermentation capacity. Recently, known ATCC strains of *B. clostridiiformis* have been observed to form spores and are, therefore, species of *Clostridium* (W. E. C. Moore, personal communication). Spores have not been detected in our cultures of *B. clostridiiformis* even after prolonged incubation (3 weeks) at least in our rumen fluid-glucose liquid medium. Similar strains of *B. clostridiiformis* were isolated from chicken cecal material by Barnes and Impey (3).

Two types of spore-forming rods comprised Group VI. Group VIa contained pleomorphic, nonmotile cells (1 by 2 to 3  $\mu\text{m}$ ) bearing terminal spores. These were similar to known strains of *Clostridium ramosum* in that glucose, fructose, lactose, maltose, mannose, melibiose, and sucrose were fermented (19). Strains in Group VIb were motile, fusiform rods (1 by 3 to 4  $\mu\text{m}$ ) with subterminal spores and were species of *Clostridium* that could not be identified.

**Distribution of bacteria in the cecum.** Results of this work indicate that 90% of all strains isolated from cecal material of six animals (5 weeks old) consist of at least 11 groups and subgroups of facultative and anaerobic bacteria (Tables 6 and 7). Approximately 10% of the strains were made up of unknown species (miscellaneous rods) that could not be identified. The predominant groups that were consistently isolated from high ( $10^{-9}$ ) dilutions of samples were distributed as follows: 7% as gram-negative budding cocci (Group I, unknown species); 12% as gram-positive facultative cocci (Group II, unknown species); 14% as streptococci (Groups IIIa, b, c; *Streptococcus* and *Peptostreptococcus*); 32% as gram-positive rods (Groups IVa, b, c; *P. acnes*, *E. rectale* and *Eubacterium* sp.); 14% as gram-negative rods



(Group V, *B. clostridiiformis*); and 10% as spore-forming rods (Group VIa, b; *C. ramosum* and *Clostridium* sp.).

Barnes and Impey (3), on the other hand, found that the anaerobic cecal microflora of the 5-week-old chicken was composed of 40% gram-positive nonspore-forming rods and bifidobacteria, 40% gram-negative rods (*Bacteroidaceae*), and 15% strains of peptostreptococci and curved rods. Not only are there these differences in the distribution of bacterial types in our study and that of Barnes and Impey (3), but the latter investigators also isolated gram-negative rods such as *B. fragilis* and *B. hypermegas*. Moreover, bifidobacteria and lactobacilli, cultured from chicken intestinal contents by some investigators (26, 30, 34), were not recovered in any sample analyzed in this study, at least in high dilutions ( $10^{-9}$ ) of cecal material. We isolated similar strains of budding cocci, *P. acnes* and *B. clostridiiformis*, as did Barnes and co-workers (4, 5). It may be concluded, therefore, that the relative proportion of the various bacterial groups within the intestinal microbial population of chickens are somewhat variable owing to such differences as breed, diet, growth conditions, and geographic location of animals. The relative proportion of bacterial groups would also be affected by the isolation methods used by different workers.

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