Studies on the Cecal Microflora of Commercial Broiler Chickens

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A study was made of the cecal microflora isolated from broilers (5-week-old) reared under typical commercial husbandry conditions. Three hundred and twenty-five bacterial strains (randomly isolated from colonies representing 49 to 81% of the microscopic count) were isolated from cecal digesta of six animals on a rumen fluid roll tube medium (M98-5). Seventy-seven percent of these strains consisted of strict anaerobes: gram-negative, pleomorphic cocci (5.2%), Peptostreptococcus (1.5%), gram-positive rods (36.1% as Propionibacterium acnes and Eubacterium sp.), gram-negative rods (18.6% as Bacteroides clostridiiformis, B. hypermegas and B. fragilis) and sporeforming rods (15.7% as Clostridium sp.). Two types of facultatively anaerobic bacteria (gram-positive cocci and Escherichia coli) were also isolated and constituted 17.5% of the remaining flora. The distribution of the bacterial groups isolated from six cecal samples varied considerably. Data on the growth requirements of anaerobic strains indicated that many could be cultured in a simple medium consisting of an energy source, minerals, reducing agent, Trypticase, and yeast extract (or a vitamin mixture in place of yeast extract). The growth of some of these bacteria was also enhanced by CO_2 and rumen fluid. These preliminary data suggest that some of the more numerous anaerobes isolated from the chicken cecum may not require complex nutrients for growth and, in fact, may be nutritionally similar to rumen anaerobes.

There is little information available describing the predominant kinds of bacteria occurring in the intestinal tract of the commercial broiler. Studies on layer and broiler chickens have shown that the cecum contains the largest number of bacteria, most of which are strict anaerobes (2, 4, 18, 22, 23). Barnes and Impey (2, 3) have isolated from poultry ceca (chickens, turkeys, pheasants, and ducks) several groups of anaerobic streptococci as well as gram-negative and gram-positive nonsporeforming anaerobes including species of Bacteroides, Fusobacterium, Eubacterium, Propionibacterium, and Bifidobacterium. In the few remaining studies concerned with chicken microflora, characterization of the cecal bacteria was limited to only a few groups which were cultured on selective media. In contrast, we previously observed that using nonselective rumen fluid media and strict anaerobic methods devised for rumen bacteria (20) allowed a large percentage of anaerobes to be isolated from the cecum of "laboratory-reared" broilers (5-week-old). Ninety percent of the microflora cultured in this prior study was composed of facultatively anaerobic cocci and streptococci and strictly anaerobic species of Peptostreptococcus, Propionibacterium, Eubacterium, Clostridium, Bacteroides, and unidentified gram-negative organisms. The work reported here extends our initial studies on the major groups of bacteria isolated from the chicken to include the cecal microflora of broilers which were reared under commercial husbandry conditions. Data on variability in microbial populations among samples and some growth requirements of the predominant anaerobes are also presented.

MATERIALS AND METHODS

Animals. Five-week-old cockerels (White Cornish cockerel × White Rock hen) maintained on as antibiotic-free grower ration (containing a coccidiostat) were obtained from a commercial broiler facility (Foster Poultry Frms, Livingston, Calif.). Cecal samples (3 to 4 g wet weight) were taken from animals (0.9 to 1.0 kg) which had been killed by CO_2-asphyxiation.

Culture methods and isolation of cecal bacteria. Direct microscopic counting, anaerobic culture techniques, sampling procedure, and nonselective anaerobic roll tube media used in this study were the same as

previously described (20). Portions of 10^{-9} dilutions of the cecal samples were inoculated into roll tubes of M98-5 media. After 6 days of incubation at 37 C, 300 colonies were picked from roll tubes (50 to 60 colonies per tube) inoculated with cecal samples from six individual birds. The colonies were subcultured to slant media of similar composition (20). Mixed culture isolates were purified by restreaking on M98-5 media (15). Initial criteria used for grouping pure cultures of strains were morphology (phase contrast microscopy), Gram stain reaction, oxygen sensitivity (facultative anaerobes or strict anaerobes), growth on glucose, and fermentation products formed from glucose. Media and methods used for these tests have been given (20).

Carbohydrate fermentation, physiological tests, and identification of isolates. Tests to determine the ability of representative strains of anaerobes from bacterial groups in Table 1 to ferment several carbohydrates, to hydrolyze esculin and starch, to reduce nitrate, and to produce indole, H_2, and H_2S were performed. The basal media and methods used in these tests have also been described (20). Identification of strains was based on comparison of these various physiological features with those of known poultry isolates (2, 3), classification schemes of Holdeman and Moore (15) and other published data on anaerobes.

Analytical method for fermentation acids. Fermentation acids (formic, acetic, propionic, butyric,

lactic, and succinic) elaborated in glucose-containing media were analyzed by a gas chromatographic procedure adapted from the method of Lambert and Moss (16) for the preparation of butyl esters. An estimate of the amount of each acid formed was determined from standard curves of authentic butyl esters (obtained from Pfaltz & Bauer, Inc.). The gas chromatograph used was a Hewlett-Packard model 5754B equipped with flame ionization detector an digital integrator.

Ethanol in culture media was determined enzymatically with alcohol dehydrogenase (Sigma Kit no. 331-UV).

Experiments on growth stimulatory factors. Strains of anaerobic bacterial groups listed in Table 1 were subjected to growth stimulatory tests. For all experiments, strains were initially cultured in an inoculum medium (IM) of the following composition and final concentrations: 7.5% (vol/vol) each of mineral solution 1 (0.6% K_2HPO_4) and mineral solution 2 (0.6% KH_2PO_4, 0.6% $(NH_4)_2SO_4$, 1.2% NaCl, 0.245% $MgSO_4 \cdot 7H_2O$, and 0.159% $CaCl_2 \cdot 2H_2O$), 0.4% (wt/vol) glucose, 0.2% (wt/vol) Trypticase (BBL), 0.2% (wt/vol) yeast extract (Difco), 0.0002% (wt/vol) hemin, 0.0001% (wt/vol) resazurin indicator, 0.06% (wt/vol) Na_2CO_3 buffer, and 0.026% (wt/vol) each of cysteine-hydrochloride and Na_2S. The pH was adjusted to 6.8, equilibrated and tubed under 10% CO_2-90% N_2 in 5-ml amounts in rubber-stoppered tubes (17 by 100 mm, Corex). Pure cultures of strains were grown in this medium for 24 to 48 h at 37 C,

TABLE 1. *Some identifying features of major groups of facultatively anaerobic and anaerobic bacteria isolated from cecal digesta[a]*

Morphological group	Isolated strains (%)	Gram reaction	Oxygen tolerance	Terminal pH on glucose	Fermentation products from glucose	Tentative identification
I. Gram-negative pleomorphic cocci and streptococci	5.2	−	A	5.9–6.7	fab	unknown
II. Gram-positive cocci	12.6	+	F	4.7–5.5	La	*Staphylococcus*
III. Streptococci	1.5	+	A	5.3	laf(p)	*Peptostreptococcus*
IV. Gram-positive rods	36.1					
a		+	A	4.8–5.4	PAs(l)	*Propionibacterium acnes*
b		+	A	4.9–5.7	Lba(f)	*Eubacterium*
c		+	A	5.7–6.4	Lb	*Eubacterium*
d		+ or V	A	4.9–5.7	AS(f)	Unknown
V. Gram-negative rods	18.6					
a		−	A	5.0–6.0	Fa(Le)	*Bacteroides clostridiiformis*
b		−	A	4.8–5.0	Pa(ls)	*B. hypermegas*
c		−	A	5.1–5.3	SA	*B. fragilis*
VI. Sporeforming rods	15.7					*Clostridium*
a Subterminal spores		+	A	4.7–5.3	Bl	
b Motile with subterminal and terminal spores		+	A	6.0–6.6	Bal(f)	
c Subterminal spores		+	A	6.3–7.1	b	
VII. Gram-negative motile rods	4.9	−	F	4.9–5.2	LAs	*Escherichia coli*
VIII. Miscellaneous strains	5.4					

[a] Data compiled from 325 strains isolated from six chickens. Symbols: oxygen tolerance, A (anaerobic), F (facultatively anaerobic); +, positive, −, negative, V, variable reaction; products from glucose: e (ethanol); F,f (formic); A,a (acetic); P,p (propionic); B,b (butyric); L,l (lactic); S,s (succinic); upper case letters refer to acids formed in amounts of 10 μmol/ml of medium or greater, and lower case letters refer to amounts less than 10 μmol/ml. Products in parentheses are variable and formed by a few strains.

washed once in anaerobic dilution solution (6), adjusted to an optical density (OD) of 0.2 to 0.3 at 600 nm, and used as an inoculum (0.05 ml) for 4 ml of the various media.

Growth characteristics of the various strains were determined in media containing basal medium plus added components. The basal medium consisted of mineral solutions 1 and 2, glucose, Na_2CO_3, cysteine-hydrochloride, and Na_2S at the same concentrations as given for IM. The following constituents were included to give the final concentrations (wt/vol, vol/vol, or mM): hemin (0.0002%), Trypticase (0.2%), yeast extract (0.2%), rumen fluid (CRF 2, 20%), volatile fatty acid mixture (29.5 mM acetic, 8.1 mM propionic, 4.3 mM butyric, 1.1 mM isobutyric, and 1 mM each of isovaleric and 2-methylbutyric), vitamin mixture (mg/100 ml: thiamine-hydrochloride, Ca-pantothenate, nicotinamide, riboflavin, and pyridoxal-hydrochloride, each 0.2; p-amino benzoic acid, 0.1; biotin, 0.01; folic and DL-thioctic acids, 0.005 each; vitamin B_{12}, 0.002), liver extract (5%), or cecal extract (5%). Preparations of liver and cecal extracts were similar to those mentioned previously (20). Each medium was prepared by combining appropriate ingredients, equilibrating with 10% CO_2-90% N_2 gas, adjusting the pH to 6.8, and dispensing (4-ml amounts) under the same gas phase into sterile, rubber-stoppered tubes (13 by 100 mm, Bellco Glass), and autoclaving (15 psi, 15 min). These media varied in pH from 6.7 to 6.9 after autoclaving. Growth of cultures at 37 C was monitored at 24-h intervals by using a Bausch & Lomb Spectronic 20 colorimeter set at 600 nm.

RESULTS AND DISCUSSION

Isolation of cecal bacteria from broiler chickens. Initially, 300 anaerobic strains were picked. Some morphologically heterogeneous isolates were then reisolated, thus increasing the total number of strains examined to 325. The percent of the total microflora (direct microscopic counts) cultured in M98-5 medium varied among the six samples from 49.3 to 80.9% (mean of 59.6%). A comparison of different media for the isolation of cecal anaerobes from commercial broilers was part of a previous study (20).

Strains were tentatively classified on the basis of morphology, carbohydrate fermentation, fermentation products, and other physiological and biochemical features (Tables 1 and 2). A description of the predominant groups of cecal bacteria isolated follows. With the exception of two groups of facultatively anaerobic cocci and rods (group II and VII, Table 1), the majority of these bacteria were gram-positive and strict anaerobes.

Facultatively anaerobic bacteria. Two types of facultative anaerobes were isolated: gram-positive cocci (group II) and gram-negative, motile rods (group VII) comprising 12.6

and 4.9% of the total isolated microflora, respectively. The gram-positive cocci, which were also isolated from chicken cecal contents in a previous study (20), produce lactic acid as a major fermentation product. Under anaerobic conditions they form acid from glucose, fructose, lactose, maltose, and sucrose. All of the gram-positive cocci examined (eight strains) hydrolyze gelatin and tributyrin and reduce nitrate to nitrite. Catalase activity could be demonstrated in cultures grown aerobically on plate media but not in cultures grown anaerobically in prereduced media (20). Although these organisms were not identified previously (20), we now believe they may be related to the genus *Staphylococcus*. Tentative identification is based on the fact that they are facultatively anaerobic and produce acid from glucose anaerobically and aerobically (1). In addition, they produce major amounts of lactic acid from glucose (anaerobically) as do other strains of *Staphylococcus* (*S. aureus* and *S. epidermidis*) we have examined.

Facultatively anaerobic, gram-negative rods of group VII were presumptively identified as *Escherichia coli* in tests with the improved Enterotube (Roche Diagnostics). These bacteria were present in five of the six cecal samples (Table 3) and constituted 2 to 13% of the isolated strains.

Anaerobic cocci. Bacteria in group I were gram-negative, pleomorphic cells with many club, dumbbell and budding forms in pairs and streptococcal-like chains. It is uncertain whether these spherical to elongated cells are truly cocci or rods because of their extreme pleomorphism. We have temporarily designated them as budding cocci. Strains similar to this unnamed species have been isolated from the chicken cecum (4, 20), human feces (J. Gossling, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 81, 1972) and human uterus (12). In a rumen fluid basal medium these bacteria do not ferment sugars but do hydrolyze esculin (Table 2). Fermentation products from glucose include minor amounts of formic, acetic, and butyric acids and some H_2 gas (0 to 2.6%, vol/vol).

Strains in group III are large (1.5 to 2.0 μm) gram-positive cocci found in pairs and chains which are probably related to species of *Peptostreptococcus* Kluyver and Van Niel (19). These anaerobic streptococci differed from *Peptostreptococci* cultured from chicken cecal contents in a previous study (20) inasmuch as they fermented several sugars. These strains resembled *P. intermedius* as described by Holdeman and Moore (15) except for the production of indole and hydrolysis of esculin. Group III

TABLE 2. *Fermentation and physiological reactions of predominant cecal anaerobes*[a]

Determination	Group (no. of strains tested)											
	I 3	III 2	IVa 15	IVb 6	IVc 6	IVd 3	Va 12	Vb 3	Vc 4	VIa 2	VIb 12	VIc 4
A. Fermentation												
Arabinose	−	−	−	a	−	−	−^{a}	a	a	−	−	−
Cellobiose	−	a	−	−	−	−	−^{a}	a	−	−	−	−
Erythritol	−	−	a⁻	−	−	−	−	−	−	−	−	−
Esculin (hydrolysis)	+	−	−$^+$	+⁻	+	+	+	+	+^{w}	w	−	−
Fructose	−	a	a	a	w	a	a^w	a	a	a	w⁻	−
Glucose	−	a	a	a	w	a	a^w	a	a	a	w⁻	−^{w}
Lactose	−	a	−	w	−	a	−^{w}	a	a	−	−	−
Maltose	−	a	−	a	−	a	a^w	a	a	w	−	−
Mannitol	−	−	−^{a}	−	−	a	−^{a}	a	−	−	−	−
Mannose	−	a	a	w	−	−	a⁻	a	a	a	−	−
Melibiose	−	a	−	−	−	a	w^a	a	a	w	−	−
Raffinose	−	a	−	−	−	a	w^a	a	a	w	−	−
Ribose	−	−	a⁻	−	−	−	−^{w}	a	a^w	−	−	−
Salicin	−	a	−^{a}	−	a	a	−^{w}	w	a⁻	−	−	−
Sorbitol	−	−	V	−	−	a	−	a	−	−	−	−
Starch (hydrolysis)	−	−	−	−	−	−	−	−	+	−	−	−
Sucrose	−	a	−^{a}	−	a	a	a^w	a	a	w	−^{w}	−
Trehalose	−	a	−	a	−	a	a^w	a	−	w	−^{w}	−
Xylose	−	−	−	w	−	−	−^{a}	a	a	−	−^{w}	−
B. Physiological												
Gelatin liquefaction	−	−	+	−	−	−	−	−	w	−	−	−
Indole production	−	+	+⁻	−	+⁻	−	−$^+$	−	−	−	−	−
Nitrate reduction	−	−	−$^+$	−$^+$	−	−	−$^+$	−	−	−	−	−
Catalase	−	−	+⁻	−	−	−	−	−	−	−	−	−
Hydrogen production	+	+	−	+	−	+	+	−	+	+	V	+
H₂S production	−	−	−	+	−	+	−	−	+	−	V	−
NH₃ production	−	+	+	w	−	w	−	V	+	−	V	−
Tributyrin hydrolysis	−	−	+	−	−	−	−	+	−	−	−	−
Fermentation products from:[b]												
Pyruvate	f	af	PA	Ab	−(fab)	As	Af	p	Aps	FAb	B(ab)	−
Lactate	f	−	PA	−	−(f)	−	−(af)	p	aps	B	b	B
Threonine	−	−	p⁻	−	−	−	−(p)	p	P	−	p(−)	−

[a] Media and methods for most tests are given in references. Production of ammonia was determined with Nessler reagent in media used for indole production. Lipolysis was determined in basal media containing 1% (vol/vol) tributyrin (Sigma Chemical Co.) as fermentable substrate. Reactions given are for most strains without a group. Symbols: −, no fermentation (terminal pH 6.3 to 7.0) or no reaction; a, acid reaction (terminal pH 5.5 or less); w, weak reaction (terminal pH 5.6 to 6.2); +, positive reaction; V, variable reaction. Superscripts refer to reactions of a few strains. None of the strains fermented amygdalin or inositol and only 1% of all isolates fermented melezitose. Glycogen and rhamnose were fermented only by group Vc. Motile strains were observed in group VIb.

[b] Designation of amounts of fermentation products is similar to that given in the footnote of Table 1. Products in parentheses are formed by a few strains.

strains constituted a small part of the total microflora (Table 1) and were observed in only two out of six cecal samples.

Anaerobic gram-positive rods. As in a previous study (20), this group of gram-positive, nonsporeforming rods represented the largest portion of the cecal bacteria isolated. Half of the strains in group IV (18%) were identified as *Propionibacterium acnes* (IVa). These belonged to serotypes I and II on the basis of sorbitol

fermentation (15). *P. acnes* was also isolated from poultry cecal material by Barnes and Impey (3). Groups IVb and IVc resembled species of *Eubacterium* as they were anaerobic, gram-positive rods primarily producing lactic and butyric acids from glucose (15). Group IVb strains were oval-shaped cells or rods with tapered ends (1.5 by 2.5 to 5.0 μm) occurring as singles, pairs, and chains. We could not establish any similarity of group IVb strains with

TABLE 3. *Growth requirements of anaerobic strains from bacterial groups*[a]

Medium addition[b]	Culture turbidity, maximum OD × 100										
	I	III	IVa	IVb	IVc	IVd	Va	Vb	Vc	VIa	VIb
Trypticase (T)	0[c]	0	0	0	0	0	0	0	21 (48)	0	0
T + hemin (H)	0	0	0	0	0	0	0	0	65 (48)	0	0
Vitamins (V)	0	0	0	0	0	0	0	30 (24)	0	0	0
Rumen fluid (R)	16 (24)[d]	21 (24)	0	38 (24)	0	51 (48)	23 (72)	42 (48)	53 (48)	58 (48)	49 (48)
T + H + yeast extract (Y)	0	71 (48)	106 (48)	51 (24)	68 (48)	43 (48)	62 (48)	53 (48)	90 (48)	57 (48)	38 (48)
R + T + H + Y	48 (48)	72 (48)	120 (48)	54 (48)	68 (48)	60 (96)	47 (48)	51 (48)	120 (72)	75 (24)	20 (48)
R + T + H + V	65 (48)	—[e]	120 (96)	54 (48)	48 (48)	66 (48)	40 (72)	42 (48)	98 (72)	64 (48)	30 (48)
Volatile fatty acids + T + H + Y	0	0	120 (48)	27 (48)				42 (48)	65 (48)	—	
Liver Extract	0	0	110 (96)	0	30 (96)	46 (96)	33 (96)	73 (48)	75 (72)	—	0
Cecal extract	0	0	0		0	0	47 (48)	72 (48)	120 (72)	—	0
Representative strains tested	Q32, S26, S41	N1, P6, P37	N9, N44a, O11, P22	Q39a, Q46, R43, S19	N44b, Q41, S47	N8, N30a, R4	Q4, Q16, R49, S50	Q15, Q27, Q47	R1, R35, R48a	P32, P38, R33, R44	N37b, P2, S40b

[a] From groups of anaerobes given in Table 1. Turbidity measurements were based on the average determinations with three to four strains from each group.

[b] The basal medium to which additions were made consisted of minerals 1 and 2 (7.5% each, vol/vol), glucose (0.4%, wt/vol), Na_2CO_3 (0.06%, wt/vol), and cysteine HCl-Na_2S (2.5% each, wt/vol). Concentrations of the different components added are given in Materials and Methods.

[c] Little or no growth in 168 h of incubation (maximum OD × 100 was 0 to 10).

[d] Figures in parentheses indicate hours of incubation required to reach maximum optical density.

[e] No data signified by a dash.

known *Eubacterium* species (15). Group IVc included short and long slender rods (0.5 to 1.0 by 2.5 to 5.0 μm) in chains. Although these strains appear to be closely related to *E. tortuosum* described by Holdeman and Moore (15), most of our strains ferment salicin and produce indole. Bacteria in group IVd were gram-positive to gram-variable rods (1.0 by 2.5 to 5.0 μm) with rounded ends found in long chains. These bacteria differed from any previously described nonsporeforming, gram-positive anaerobes as they characteristically produced acetic and succinic acids and H₂ gas from glucose. They may be related to species of *Eubacterium* on the basis of presumptive criteria for this genus (15). Similar strains of gram-positive, nonsporeforming, succinate-producing bacteria have been isolated by E. M. Barnes from poultry cecal material (personal communication).

Anaerobic, gram-negative rods. Group V was composed of gram-negative rods and totaled 18.6% of the isolated microflora. Strains in group Va were variable with respect to the fermentation tests given in Table 2. Representative strains were short and long fusiform-shaped rods (1.5 to 2.5 by 2.5 to 5.0 μm) as singles, pairs, and chains. Fermentation of fructose, glucose, maltose, sucrose, and trehalose as well as formation of formic and acetic acids as major products from glucose and pyruvate were some predominant characteristics of the strains tested. Morphological features of these organisms and their products from glucose were similar to *Bacteroides biacutus* and *Bacteroides clostridiiformis* (15). They differed from *B. biacutus* in that melezitose and lactose were not fermented. Phase contrast microscopy revealed that a few strains appeared to have small spores or vacuoles located centrally and subterminally. It is not known whether these are true spores since none of the strains survived a spore heat test (15). Identification of this group is provisional until characteristics of additional strains are examined. Strains similar to group Va were isolated and identified as *B. clostridiiformis* from chicken cecal contents (20) and from poultry ceca (2).

Group Vb were large, blunt-ended rods (2.0 by 5.0 to 7.5 μm) occurring mainly as singles and pairs. These species were identified as *Bacteroides hypermegas* since many fermentation reactions agreed well with those of Holdeman and Moore (15). Our organisms fermented a wide variety of sugars, hydrolyzed tribytyrin, and produced major amounts of propionic and acetic acids from glucose. Strains of *B. hypermegas* were first described by Harrison and

Hansen (14) and later isolated by Goldberg et al. (13) and Barnes and Impey (2) from poultry ceca.

Bacteria in group Vc appear to be similar to *Bacteroides fragilis* as described by Holdeman and Moore (15). These gram-negative rods (measuring 0.5 to 1.0 by 1.5 to 3.0 μm) produced succinic and acetic acids and H₂ gas (2.1 to 4.8%, vol/vol) from glucose fermentation. All four isolates we examined differed from known *B. fragilis* strains in that our isolates converted threonine to propionate. They differed from *B. ruminicola* in that H₂ gas was produced in the culture media (5). Preliminary studies by M. P. Bryant have indicated that most strains of *B. fragilis* can be distinguished from *B. ruminicola* on the basis of H₂ production (unpublished observations). It is possible, therefore, that the succinate-producing, gram-negative rods isolated in this study may be variants of *B. fragilis* which can convert threonine to propionate. Barnes and Impey (2) have also isolated *B. fragilis* from poultry ceca.

Anaerobic, sporeforming rods. Group VI comprised 16% of the isolated cecal microflora and consisted of three types of *Clostridium* species which did not liquefy gelatin. Group VIa was represented by large, nonmotile rods measuring 1.5 to 2.5 by 5.0 μm (some as long as 20 μm) with blunt ends and subterminal spores. The two strains tested produced acid from fructose, glucose, and mannose and formed butyric and lactic acids as well as copious amounts of H₂ gas (9 to 13%, vol/vol) from glucose. These strains may be similar to *C. tyrobutyricum* on the basis of sugars fermented, products from glucose (15), and lactate fermentation (7). However, our strains appeared to be nonmotile. Strains in group VIb were pleomorphic, motile rods (1.0 by 2.5 to 5.0 μm) with terminal and subterminal spores; some cells were typically fusiform- and spindle-shaped arranged as singles, pairs, and a few chains. These organisms weakly fermented fructose and glucose and formed primarily butyric acid from glucose. These strains were similar to those isolated in a previous study (20). Group VIc consisted of oval-shaped rods (1.5 to 2.0 by 2.5 to 5.0 μm) with subterminal spores occurring as singles, pairs, or chains. These bacteria were nonreactive in several tests (Table 2), producing minor amounts of butyric acid and H₂ gas from glucose but large amounts of butyric acid from lactate fermentation. None of the organisms in groups VIb and VIc could be identified as being similar to known species of *Clostridium* described by Holdeman and Moore (15) on the basis of the few isolates studied.

Preliminary findings on the growth requirements of anaerobic strains. Our previous study on cecal bacteria (20) indicated that rumen fluid and yeast extract stimulated the growth of many anaerobes. Since the growth requirements of chicken intestinal bacteria are largely unknown, we attempted a survey of some factors which might enhance the growth of several cecal isolates from the various morphological groups (Table 1). These data are shown in Table 3. Preliminary experiments (data not shown) with culture gas phase indicated that good growth was observed with Na_2CO_3-containing media prepared under CO_2 or CO_2/N_2 atmosphere. Maximun culture density for most strains was achieved with a medium containing Na_2CO_3-CO_2 buffer. Carbon dioxide may be stimulatory to the growth of most chicken cecal anaerobes; however, more rigorous experiments are needed to establish whether particular species of cecal bacteria have an absolute requirement for CO_2. Work by Dehority (11) with ruminal anaerobes has revealed that many strains required or were stimulated by CO_2.

A simple basal medium consisting of minerals, glucose, Na_2CO_3-CO_2, and cysteine-sulfide did not support the growth of any cecal strain examined. The addition of Trypticase and hemin, in particular, to this medium stimulated the growth of strains similar to *Bacteroides fragilis* (group Vc). The growth stimulatory properties of hemin for intestinal anaerobes are well known and have been described for human intestinal isolates of *B. fragilis* (15, 17) and ruminal strains of *Bacteroides ruminicola* (9, 10). When yeast extract was included in the basal medium with Trypticase and hemin, the growth of strains from 8 of 11 groups was enhanced. When a vitamin mixture was added to the basal medium, only strains in group Vb grew (Table 3) as well as one strain each from groups Va and VIa (data not shown). Addition of rumen fluid (20% vol/vol) alone to the basal medium supported the growth of only a few groups (Va, Vb, and VIa). Components in rumen fluid other than volatile fatty acids and hemin appear to be required or highly stimulatory for strains in group I (gram-negative, pleomorphic cocci).

Most strains of the various groups of cecal bacteria grew well in basal media supplemented with rumen fluid, Trypticase, hemin, and yeast extract (or a vitamin mixture in place of yeast extract). Hemin could be deleted from media containing rumen fluid without affecting the growth of hemin-stimulated strains (group Vc). In this respect, Caldwell et al. (10) showed that rumen fluid contains heme. Although a mixture of volatile fatty acids (VFA) could partially reproduce the stimulation of groups IVc and Va brought about by rumen fluid, VFA may not be required for the growth of most cecal anaerobes.

Liver or cecal extract added to the basal medium only enhanced the growth of group Vb strains (*B. hypermegas*). These observations are consistent with our previous findings that most chicken cecal bacteria do not require liver or cecal extract for growth (20). The growth of some strains of chicken cecal anaerobes isolated by E. M. Barnes, however, appears to be enhanced by a factor(s) in liver extract (unpublished observations).

The data in Table 3 suggest that most anaerobic cecal bacteria could be grown in a Na_2CO_3-CO_2 buffered medium containing minerals, cysteine-sulfide (as reducing agent), carbohydrate energy source, rumen fluid, Trypticase, and yeast extract. The fact that yeast extract or a vitamin mixture is highly stimulatory to many cecal bacteria suggests that chicken intestinal anaerobes may have specific vitamin requirements for growth. The growth stimulation by vitamins such as *p*-aminobenzoic acid, biotin, folic acid, and vitamin B_{12} for the cellulolytic rumen anaerobes, *Ruminococcus* species, and *Bacteroides succinogenes* (8, 21), has been established. Not much is known about the vitamin requirements of noncellulolytic anaerobes. However, Varel and Bryant (24) have recently observed a B_{12} requirement (replaced by methionine) for growth with human isolates of *B. fragilis*.

Compositional variations in the cecal microflora of broilers. In this study (Table 1), 83% of the total isolated microflora (325 strains) of 5-week-old broilers consisted of (1) gram-positive, facultative cocci (group II, 12.6%), (2) *Propionibacterium acnes* and *Eubacterium* (group IV, 36.1%), (3) *Bacteroides clostridiiformis*, *B. hypermegas*, and strains similar to *B. fragilis* (group V, 18.6%) and (4) *Clostridium* sp. (group VI, 15.7%). The remaining groups of the flora were made up of gram-negative, budding cocci (group I, 5.2%), *Peptostreptococcus* (group III, 1.5%) and *E. coli* (group VII, 4.9%). Table 4 shows the distribution of the various morphological groups and subgroups isolated and indicates that considerable variation exists among individual cecal samples. The cecal profile of microorganisms from each animal suggests that some species are isolated with (or associated with) certain other species. For example, in samples 1, 2, and 3, the lactic acid-producing, facultatively anaerobic cocci (group II) were isolated with large numbers of other gram-positive organisms (primarily group IVa strains)

TABLE 4. *Distribution of bacterial groups isolated from cecal digesta of six broilers*

Bacterial group[a]	Percent of isolated strains (cecal sample)					
	1	2	3	4	5	6
I.	0	0	3.7	2.1	9.1	17.7
II.	14.7	49.2	8.3	0	0	0
III.	4.4	0	4.2	0	0	0
IV.	64.6	29.1	33.5	37.5	25.4	17.8
(a)	(30.8)	(25.5)	(27.2)	(25.0)	(0)	(2.0)
(b)	(5.9)	(0)	(6.3)	(8.3)	(10.9)	(2.0)
(c)	(17.6)	(0)	(0)	(2.1)	(3.6)	(11.8)
(d)	(10.3)	(3.6)	(0)	(2.1)	(10.9)	(2.0)
V.	1.5	7.2	4.2	41.8	32.8	27.5
(a)	(1.5)	(3.6)	(2.1)	(16.7)	(14.6)	(2.0)
(b)	(0)	(0)	(0)	(23.0)	(0)	(0)
(c)	(0)	(3.6)	(2.1)	(2.1)	(18.2)	(9.2)
VI.	8.9	0	18.9	10.4	32.7	25.8
(a)	(1.5)	(0)	(4.2)	(0)	(3.6)	(2.0)
(b)	(7.4)	(0)	(10.4)	(8.3)	(10.9)	(23.8)
(c)	(0)	(0)	(4.3)	(2.1)	(18.2)	(0)
VII.	5.9	12.7	4.2	4.2	0	2.0
VIII.	0	1.8	23.0	4.0	0	9.2
Total strains isolated	68	55	48	48	55	51
Percent of microflora cultured[b]	51.4	80.9	60.4	49.3	63.0	52.5

[a] Morphological groups given in Table 1.

[b] Culture counts per direct microscope cell counts × 100.

along with relatively smaller numbers of spore-forming rods (group VI) and gram-negative rods (group V). In samples 4, 5, and 6, however, the gram-negative rods in group V were isolated with gram-positive rods (groups IVb, c, and d) and sporeforming rods (group VI). It is interesting to note that *B. hypermegas* (Vb) and *B. fragilis*-like strains (Vc) were isolated in high numbers (23.0 and 18.2% of the isolated microflora, respectively) in only two samples (4 and 5). These two species were not isolated in our initial study (20).

Results of the previous study (20) on chicken cecal microflora of 5-week-old, "laboratory-reared" birds indicated that species of anaerobic gram-negative cocci, facultatively anaerobic cocci and streptococci, *Peptostreptococcus* sp., *Propionibacterium acnes*, *Eubacterium* sp., *B. clostridiiformis*, and *Clostridium* sp. were isolated. The types of anaerobes isolated from cecal material in our two studies were similar to those described by Barnes and Impey (2) from poultry ceca. However, the relative proportions of the various bacterial types differed.

Few studies dealing with intestinal microflora in nonruminants consider the animal to animal variation when describing the predominant bacterial species isolated from intestinal material. Our data indicate that appreciable variation may exist in the cecal microflora among differ-

ent animals (as well as within the same animal) reared under similar growth conditions (environment, food, water). These observations suggest that other factors (host-microbe or microbe-microbe interactions) may have some effect on the occurrence and distribution of the various bacterial species in the cecum. These must be taken into consideration in any study involving the isolation, cultivation, identification, and relative importance of species in a mixed culture habitat such as the chicken cecum.

ACKNOWLEDGMENTS

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