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Large Intestine Bacterial Flora of Nonhibernating and Hibernating Leopard Frogs (*Rana pipiens*)

JENNIFER GOSSLING,[1,2]† WALTER J. LOESCHE,[1,3,4] AND GEORGE W. NACE[2,5,6]

Dental Research Institute[1]; Department of Oral Biology, School of Dentistry[3]; Department of Zoology[5]; Amphibian Facility[2]; Center for Human Growth and Development[6]; and Department of Microbiology[4]; University of Michigan, Ann Arbor, Michigan 48109

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The bacteria in the large intestines of 10 northern leopard frogs (*Rana pipiens*) were enumerated and partially characterized. Four nonhibernating frogs were collected in the summer, four hibernating frogs were collected in the winter, and two frogs just emerged from hibernation were collected in the spring. All frogs had about 10^{10} bacteria per g (wet weight) of intestinal contents and about 10^{9} bacteria per g (wet weight) of mucosal scraping, although the counts from the winter frogs were slightly less than those from the other two groups of frogs. Another group of 14 summer frogs, after treatment to induce hibernation, showed a drop in bacterial counts accompanied by a change in the composition of the flora. In most frogs, *Bacteroides* was the dominant organism. Other bacteria repeatedly isolated at high dilutions were strict anaerobes, including butyrigenic and acetogenic helically coiled bacteria; fusobacteria; and acetogenic, small, gram-positive bacilli. These data indicate that the intestinal flora of frogs is similar to that of mammals and birds and that this flora can be maintained at temperatures close to freezing.

The indigenous flora of laboratory mammals has been studied both as a model of host-microbe interactions and also to determine how the flora of experimental animals might influence the results of other investigations (25). Amphibians are widely used laboratory animals, especially when systems tolerant of low or variable temperatures are required (19, 20); however, minimal information is available on the indigenous flora of these ectotherms. It is not known whether the slower metabolism and nutrient turnover of ectotherms can support a dense anaerobic flora such as that found on the mucous membranes of endotherms (12, 17, 25), nor how any such flora is affected by wide variations of temperature and metabolic rate.

Facultative (preferentially aerobic but facultatively anaerobic) bacteria from the intestines of frogs have been investigated as a source of the septicemia, often associated with chilling and hibernation (3, 7), which occasionally kills large numbers of frogs in the laboratory and in the wild (7, 22). Hibernating northern leopard frogs (*Rana pipiens* [34]) and chilled southern bullfrogs (*Rana catesbiana* [3]) had fewer types of facultative bacteria than did control warm frogs, and in the latter case, the total number of bacteria was also reduced. In birds and mammals, potentially pathogenic facultative bacteria in the intestine are controlled by the indigenous anaerobic bacterial flora (2, 25). Strictly anaerobic bacteria have been found in bullfrog intestines (8).

In this investigation, the bacterial flora of the large intestines of nonhibernating and hibernating wild-caught leopard frogs was enumerated and partially characterized. Although this frog flora showed some distinctive features, the general pattern of colonization resembled that found in endotherms, and there was little difference between nonhibernating and hibernating frogs. In the group of frogs monitored during induction of hibernation, we found a temporary reduction and a qualitative change of flora.

MATERIALS AND METHODS

Frogs. Eighteen nonhibernating summer frogs (*Rana pipiens*; snout-vent length, 4.4 to 7.3 cm) were collected by Bioaquatics International, Rochester, Mich., and shipped within 1 day to Ann Arbor, Mich. Hibernating winter frogs (snout-vent length, 7.4 to 8.5 cm) were obtained from J. M. Hazen Co., Alburg, Vt. These hibernating frogs had been collected at the end of the fall migration and, until shipment to Ann Arbor in February, were kept in tanks containing flowing lake water at the temperature of the lake in which they normally hibernated. Two active spring frogs (snout-vent length, 7.9 and 8.5 cm), captured in the wild upon emergence from hibernation and before food had been ingested (it was too cold for prey to be active and no macroscopic food residues were seen in the guts),

† Present address: Division of Microbiology, Washington University School of Dental Medicine, St. Louis, MO 63110.

were also obtained from J. M. Hazen Co.

In Ann Arbor, all frogs were housed at the Amphibian Facility (18, 19). Those to be sampled directly were kept for up to 2 weeks. Summer frogs were kept at 21°C in Michigan Environmental Enclosures for Small Animals (MEESA; Keyco Co., Inc., Peach Bottom, Pa. [20]), hibernating frogs were kept at 4°C in deep water in hard-plastic containers (34), and spring frogs were kept 24 h at 4°C in moist, hard-plastic containers. The summer frogs were given live crickets twice a week as feed but were sampled when the guts were empty of gross residues.

Hibernation was simulated in 14 laboratory-conditioned (19) summer frogs housed together initially in a MEESA unit at 21°C with a diurnal cycle of 12 h of light and 12 h of dark. During week 1, the temperature was maintained, the day length was gradually reduced (by about 12 min/day), and crickets were supplied daily. During week 2, the temperature was gradually reduced to 10°C, the day length continued to be shortened, crickets were supplied daily until the frogs stopped feeding, and deep (10-cm) water was made available in the MEESA unit. During week 3, when the temperature was gradually reduced to 4°C and the day length was reduced to 8 h, the frogs moved to and remained in the deep water. Thereafter, the frogs were housed in deep water in the same manner as the hibernating frogs.

Bacteriological sampling. Frogs were sampled two at a time. Each frog was pithed, and its abdominal cavity was opened aseptically. A liver sample was aseptically excised, weighed, and placed in 5 ml of reduced transport fluid (RTF [28]) without EDTA. The large intestine, from the ileocolonic constriction to just craniad to the urethral entry, was aseptically removed and weighed, and the contents were expressed directly into 5 ml of RTF; the empty intestine was then weighed, and the weight of the contents was calculated by subtraction. The intestine was slit open longitudinally, and its mucosa was aseptically scraped off directly into 3 or 5 ml of RTF; the remaining intestinal wall was weighed, and the weight of the mucosal sample was calculated by subtraction. These procedures were completed in less than 15 min for each frog, and the actual samples were exposed to air for less than 10 s.

The samples in RTF were immediately transferred to an anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.) and dispersed for 20 s with a tissue homogenizer (model SDT; Tekmar Co., Cincinatti, Ohio) and for 20 s with a microtip sonifier (Kontes Co., Vinewood, N.J.). Serial 10-fold dilutions in RTF were prepared from these suspensions.

Direct counts. The dispersed particles in suitably diluted suspensions of the intestinal contents from the summer nonhibernating frogs were counted with an electronic particle counter (model ZBI; Coulter Electronics, Inc., Hialeah, Fla.) (16).

The bacteria in suitably diluted suspensions of the intestinal contents and mucosal scrapings from all frogs were counted microscopically, using safranin to stain dried smears (10).

Bacteriological media. Two general growth media were used: Schaedler agar (BBL Microbiology Systems, Cockeysville, Md.) to which 5 g of maltose, 2 ml of sodium lactate syrup (about 60%), and 0.5 g of KNO_3 per liter were added; and a modified medium 10 (MM10-F) containing the following ingredients per liter: NaCl, 114 mg; $(NH_4)_2SO_4$, 114 mg; $MgSO_4 \cdot 7H_2O$, 23.8 mg; $CaCl_2 \cdot 2H_2O$, 11.4 mg; K_2HPO_4, 57 mg; KH_2PO_4, 57 mg; KNO_3, 500 mg; glucose, 250 mg; maltose, 250 mg; soluble starch, 250 mg; yeast extract (Difco Laboratories, Detroit, Mich.), 250 mg; hemin, 5 mg; Trypticase (BBL), 500 mg; sodium formate, 250 mg; sodium fumarate, 250 mg; sodium succinate, 250 mg; sodium lactate syrup, 0.5 ml; pyruvic acid, 0.125 ml; acetic acid, 0.082 ml; propionic acid, 0.029 ml; butyric acid, 0.019 ml; valeric acid, 0.005 ml; isobutyric acid, 0.005 ml; isovaleric acid, 0.005 ml; alpha-methylbutyric acid, 0.005 ml; enough NaOH to neutralize; hog gastric mucin (Sigma Chemical Co., St. Louis, Mo.), 1 g; agar, 15 g; menadione (Nutritional Biochemical Corp., Cleveland, Ohio), 0.5 g; dithiothreitol (Sigma), 200 mg; $NaCO_3$ (anhydrous), 400 mg (solutions of these last three ingredients were filter sterilized and added to the mixture of previous ingredients after it was autoclaved); and sterile sheep blood, 20 ml.

All water used was deionized and glass distilled. All media were prereduced by storage in the anaerobic chamber for at least 24 h before inoculation.

Cultural counts. Suitable dilutions of the RTF suspensions were plated with a spiral plater (Spiral Systems Inc., Cincinnati, Ohio). Four sets of MM10-F plates (each set consisting of one plate from each dilution plated) were inoculated from each sample. One set was incubated at room temperature in the anaerobic chamber for 6 days, a second set was incubated in a refrigerator (4°C) in anaerobic jars (GasPak; BBL) with resazurin as an indicator of anaerobiosis for 21 days, a third set was incubated at room temperature in air for 3 days, and a fourth set was incubated in a refrigerator in air for 7 days. The anaerobic atmospheres were 5% carbon dioxide and, initially, 10% hydrogen in nitrogen. The jars in which cultures were incubated in the refrigerator were checked daily for appearance of color in the indicator. If the indicator was pink, as occurred in a few jars, the jar was returned to the anaerobic chamber and set up again with a fresh GasPak and indicator, without exposing the plates to air, and the inoculated plates never became obviously oxidized.

Specimens from some nonhibernating frogs were also plated in the same manner on Schaedler agar and minimal media (the inorganic salts as in MM10-F, menadione, dithiothreitol, agar, and water, with and without mucin) to compare these media with MM10-F.

The colonies growing within a defined area (15) of suitable plates were counted so as to give a total of 100 to 1,000 colonies per count.

Enumeration, isolation, and characterization of different bacterial types. Differential counts by colony morphology were made on plates that had been incubated anaerobically. Colonies representative of the predominant colonial types were subcultured to Schaedler agar slants in an anaerobic chamber. Subsequently, in calculating counts of individual bacterial types, all colonies of the same morphology were assumed to belong to the same bacterial type as that of the representative colony picked for characterization.

Isolates were examined by Gram stain and tested for growth on Schaedler agar in air and in a refrigerator (or at room temperature, in the case of isolates obtained from the refrigerator cultures). Peptone-yeast extract

TABLE 1. Counts of bacteria from frog large intestines

Source and method of counting	Mean count ± SEM from following type of frog (no.)[a]:		
	Active, summer (4)	Hibernating, winter (4)	Active, spring (2)
Contents			
Electronic particle	10.5 ± 0.1		
Microscopic	10.4 ± 0.1	9.7 ± 0.2[b]	10.4 ± 0.2
Anaerobic culture at room temp	10.3 ± 0.1	9.9 ± 0.2[b]	10.5 ± 0.2
Anaerobic culture at 4°C	7.2 ± 0.5[c]	9.5 ± 0.1[b,c]	10.2 ± 0.1[d]
Mucosa			
Microscopic	8.8 ± 0.3	8.6 ± 0.2	9.3 ± 0.2
Anaerobic culture at room temp	8.9 ± 0.3	8.7 ± 0.2	9.1 ± 0.1
Anaerobic culture at 4°C	6.6 ± 0.6	8.2 ± 0.1[b]	8.9 ± 0.01[d]

[a] Log_{10} geometric mean ± standard error of the mean bacteria per gram (wet weight).
[b] Differs significantly from mean count in summer frogs ($P < 0.05$ by the unpaired t test).
[c] Differs significantly from mean count from same frogs made at room temperature ($P < 0.05$ by the paired t test).
[d] Differs significantly from mean counts in summer and winter frogs ($P < 0.05$ by the unpaired t test).

and peptone-yeast extract-glucose-maltose broths (10) were inoculated and incubated for 5 days, the pH was measured, and the peptone-yeast extract-glucose-maltose broth was analyzed for volatile fatty acids by gas-liquid chromatography (30) with a flame ionization detector. Some isolates did not grow well in the broths, and Schaedler agar slants which had supported growth were analyzed for volatile fatty acids by extracting them in the same way as the broth cultures.

Certain isolates were examined further for production of indole, catalase, and oxidase (14); production of lactic and succinic acids (10); hydrolysis of gelatin and esculin (29); reduction of nitrate (29); growth in the presence of 2% bile; motility in a wet mount of the peptone-yeast extract culture; and position of growth in semisolid Schaedler agar deeps incubated in air.

The purpose of characterizing the isolates was to distinguish the major types of bacteria and to compare them with the types which predominate in the intestines of endotherms.

Statistical analyses. To evaluate the plating media for effectiveness in supporting growth of the many different types of bacteria present, the arithmetic means of the numbers of colonies on the plates from the same dilution were compared. In all other cases, the number of bacteria per gram (wet weight) of original sample was calculated from the counts and dilution factors and expressed as log_{10} for further analysis. All means were listed with the standard error of the mean. Differences between the means of different counts made on the same frogs were tested for significance with the paired t test. Differences between the means of counts made by the same method on different frogs were tested with the unpaired t test or, where the standard deviations differed significantly, by the F test, the Dixon and Massey approximation (5). In comparing frequencies, χ^2 was calculated from contingency tables. Only differences for which $P < 0.05$ are reported.

RESULTS

Choice of medium. The intestinal contents and mucosa of nonhibernating frogs yielded about 12% more colonies on MM10-F agar than on Schaedler agar. The colonies on MM10-F agar were easier to differentiate because they were more chromogenic and there were fewer large colonies overgrowing small colonies.

In a separate experiment, there was no significant difference among MM10-F agar; a minimal medium containing only the inorganic salts, menadione, dithiothreitol, and agar; and minimal medium with mucin in the number of colonies grown from contents and mucosa of four nonhibernating frogs, but the colonies on both minimal media were very small and transparent and thus not suitable for differentiation.

Total bacterial counts. The large intestines of both nonhibernating and hibernating frogs had about 10^{10} bacteria per g (wet weight) in the contents and 10^9 bacteria per g (wet weight) in the mucosa as determined microscopically or by anaerobic cultures incubated at room temperature (Table 1). Particle counts of the intestinal contents of the nonhibernating summer frogs also gave totals of 10^{10}/g (wet weight).

Although in the same range, counts from the hibernating frogs were significantly less than those from the nonhibernating summer frogs by both microscopic and room temperature culture methods. The two frogs which had just emerged from hibernation had counts similar to those of the nonhibernating summer frogs (Table 1).

Cultures incubated at 4°C gave lower and more variable counts (Table 1); counts from the

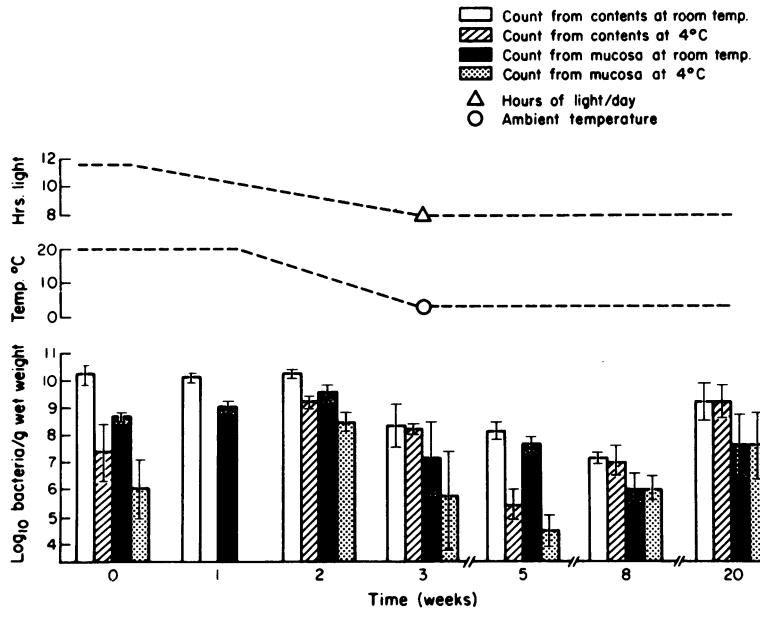

FIG. 1. Counts of bacteria in large intestines of frogs going into hibernation. Geometric means and ranges for pairs sampled from a group being induced to hibernate in the laboratory are shown.

nonhibernating summer frogs were the lowest. The growth on plates from jars in which anaerobiosis had failed briefly was similar to that on comparable plates from jars where the indicator had been continuously reduced.

Figure 1 shows the average culture counts obtained from the intestinal contents and mucosa of frogs undergoing simulated hibernation. The total number of bacteria decreased during the change in environmental conditions but recovered while the frogs were held at 4°C. The ratio of bacteria growing at 4°C to bacteria growing at room temperature was highest in the frogs which had been housed longest at the low temperature.

The livers of all frogs had less than $10^{4.5}$ bacteria per g (wet weight) as determined microscopically and by culture.

Characteristics of the bacteria. Several (11 to 43; mean, 26) isolates from each frog were characterized; the number depended on the variety of colonial types seen and the number of isolates lost on subculture. In all, 625 isolates were characterized, 90% of those picked from room temperature cultures and 60% of those picked from 4°C cultures. A total of 5 to 11

TABLE 2. Estimated counts of certain bacterial types isolated from frog large intestines

Bacterial type	Mean count isolated from following type of frog (no.)[a]:					
	Active, summer (4)		Hibernating, winter (4)		Active, spring (2)	
	Contents	Mucosa	Contents	Mucosa	Contents	Mucosa
Bacteroides	10.0[b] (4[c])	8.5 (4)	9.4[d] (4)	8.0 (4)	10.1[e] (2)	8.4 (2)
Coiled butyrigens	(0)	9.1 (1)	8.6 (4)	7.4 (3)	9.7 (2)	8.5 (2)
Fusobacteria	9.3 (2)	8.0 (2)	(0)	7.9 (3)	9.5 (2)	8.3 (2)
Gram-positive acetogens	9.5 (1)	7.8 (3)	8.4 (2)	7.9 (1)	8.3 (1)	(0)
Facultative[f]	8.4 (4)	6.5 (4)	8.9 (4)	7.2 (4)	9.4 (2)	7.6 (2)

[a] Log_{10} geometric mean bacteria per gram (wet weight).

[b] Significantly more than for fusobacteria, gram-positive acetogens, and facultative in the same group of frogs ($P < 0.05$ by the paired t test).

[c] Number of frogs from which this bacterial type was counted.

[d] Significantly more than the mean for coiled butyrigens and facultative in this group of frogs ($P < 0.05$ by the paired t test).

[e] Significantly more than the mean for facultative in this group of frogs ($P < 0.05$ by the paired t test).

[f] All bacteria which grew in air.

(mean, 8) different types of bacteria were distinguished on the room temperature cultures of each frog, with no significant variation in the number of distinguishable types among the nonhibernating, hibernating, and emerging frogs.

The bacteria most commonly isolated were (i) *Bacteroides* sp; (ii) butyrigenic, helically coiled bacilli; (iii) butyrigenic fusiforms; and (iv) acetogenic, small, gram-positive bacilli. The frequency of isolation and the estimated counts of these four types are shown in Table 2. The facultative bacteria formed a significantly larger proportion of the flora in the hibernating frogs as compared with that in the nonhibernating summer frogs. The same types of bacteria were isolated from the frogs which underwent simulated hibernation, except there was a reduction in the number of bacterial types and facultative bacteria predominated in one of the frogs examined 8 weeks after the start of the process and in both of the frogs examined 20 weeks after the start. One of these latter frogs showed no strict anaerobes.

Bacteroides were the most numerous bacteria in all 10 of the samples of contents and in 6 of the samples of mucosa from the frogs not manipulated in the laboratory (Table 2). The isolates resembled the *Bacteroides fragilis* group in producing acetate, lactate, succinate, small amounts of propionate, and variable amounts of isobutyrate and isovalerate from peptone-yeast extract-glucose-maltose broth. They were short, thick, gram-negative rods (averaging 0.6 by 1.4 μm) with rounded ends and, in older cultures, a tendency to stain at the ends only. Many exhibited catalase activity; none of those tested produced indole from tryptophan.

The helically coiled bacilli morphologically resembled the helically coiled clostridia which have been isolated from the intestines of various animals (13). However, most of these isolates differed biochemically in that they produced milliequivalent quantities of butyrate from peptone-yeast extract-glucose-maltose broth; also, they did not show any bifurcation. In both the original samples and culture, they were seen as single, semicircular rods, as circular pairs of rods, or as coiled filaments which might be tight and regular or large and loose. These rods varied from 0.3 to 1 μm in diameter, with the smaller ones being more common. The tight coils of the smallest forms were very regular and could be mistaken for large bacilli. The smaller forms stained gram-negative except in very young cultures, but the larger ones were generally gram-positive. Acetogenic coiled bacteria (not shown in Table 2), which resembled those previously described (13) in having occasional bifurcations, were isolated from nine frogs.

The other two groups of anaerobic bacteria listed in Table 2 were less homogeneous and were defined only as butyrigenic fusiform bacilli and as acetogenic, small, gram-positive bacilli.

Other bacterial types, including clostridia, *Campylobacter*-like bacteria, non-butyrigenic fusiforms, and facultative bacilli, were isolated in large numbers from some frogs.

Campylobacter-like bacteria were isolated from eight frogs. These bacteria were helical and motile and showed microaerophilic growth in agar deeps incubated in air.

Helical forms characteristic of *Campylobacter* and *Spirillum* could easily be distinguished microscopically at frequencies as low as 10^{-3} total bacteria (too low to be picked up on culture) and were seen in more frogs than they were isolated from. Microscopic counts on the four active summer frogs showed that helical bacteria constituted a significantly larger (two- to fourfold) proportion of the total bacteria in the mucosa (2 to 24% of the total) than in the contents (1 to 7% of the total). Large, sporebearing, helical bacteria resembling *Sporospirillum* (4) were seen in some of the frogs going into simulated hibernation.

The facultative bacteria isolated were, with one exception (see below), gram-negative bacilli. Most (33 isolates from 14 frogs) were enterobacteria (fermentative, oxidase negative). Pseudomonads (nonfermentative, oxidase positive) were more common from frogs kept at 4°C (29 pseudomonad isolates from seven cold frogs as compared with 9 enterobacteria isolates from four cold frogs). Pseudomonads were not isolated from frogs kept at room temperature.

Veillonella-like cocci were isolated from one frog, and micrococci were isolated from another.

Culture at 4°C. Differential counts and isolations were also made from cultures incubated anaerobically at 4°C. The differentiation was difficult because of small colony size. There were fewer successful isolations (103 as compared with 522 from cultures at room temperature). Sometimes the jars used for incubating these cultures failed to maintain continuous anaerobiosis, as described previously.

No strict anaerobes were successfully isolated at 4°C from the nonhibernating summer frogs. However, from the hibernating frogs and those just emerged from hibernation, the mix of colonies growing at 4°C was the same as that growing at room temperature. *Bacteroides* sp., butyrigenic and acetogenic helically coiled bacteria, and butyrigenic fusiforms were isolated at 4°C.

Strains isolated at room temperature were tested for their ability to grow at 4°C (Table 3), and in this case, all jars maintained anaerobiosis. Many, but not all, of the isolates from cold frogs grew at 4°C, whereas only a few of the isolates from warm frogs grew at this temperature. All

TABLE 3. Growth at 4°C of bacteria isolated at room temperature from frog large intestines

Source of isolates	Wk	Bacterial isolates		
		No. tested	No. growing at 4°C	(%)
Summer frogs		55	4	(7)
Winter frogs		81	48	(59)
Spring frogs		37	23	(62)
Frogs going into simulated hibernation	0	32	2	(6)
	1	48	1	(2)
	2	46	2	(4)
	3	33	6	(18)
	5	40	9	(23)
	8	15	2	(13)
	20	7	7	(100)

strains isolated at 4°C which could be subcultured at 4°C grew at room temperature.

DISCUSSION

The distribution of bacteria found in the frogs (*Rana pipiens*) resembled that found in mammals and birds (12, 25), as there were few bacteria in the liver and dense populations (10^{10}/g [wet weight]) in the large intestine. Also, as in mammals and birds, these dense populations consisted mainly of strict anaerobes, dominated by *Bacteroides* of the *B. fragilis* group. Apart from this general similarity, there were some distinctive tendencies, including lower total counts (10^{10}/g [wet weight] microscopically and by culture, compared with 10^{11} in mice [31] and humans [11]), some distinctive bacteria and some bacteria found in larger numbers than in other animals, and the absence or sparsity of some bacteria found in large numbers in other animals.

Characteristics which distinguish frogs from the herbivorous or omnivorous endotherms previously studied (2, 11, 12, 25) and which might influence their flora are (i) lower temperature and slower metabolism (23); (ii) carnivorous habit, associated with a short intestine and moist, low-residue feces (23); (iii) a more numerous and diverse intestinal protozoal fauna (24); (iv) separation of the adult generations by the larval period with a different mode of life (23); and (v) hibernation in an aquatic environment.

The lower total counts in frogs probably reflected fewer bacteria present, since the counts made by culture, with the particle counter, and with the microscope agreed, although both of these latter methods can miss bacteria less than 0.3 μm in diameter. The bacteria may be less populous in frogs because of less dehydration of intestinal contents and because of space taken up by protozoa (24), which appeared to be numerous on microscopic examination. In addition, all frogs were sampled when their guts were empty of food residues. Larger bacterial populations might have been associated with digesta. It is unlikely that the lower counts in frogs were directly correlated with their lower temperature and metabolic rate; if this were the case, the winter and spring frogs would have had much lower counts than would the summer frogs.

The most distinctive bacteria were the butyrigenic, helically coiled bacilli which have not been reported elsewhere, although acetogenic, helically coiled clostridia are found in many animals (13). Another distinctive form was the sporebearing, helical bacterium which has been seen only in anurans (4). The isolation of microaerophilic helical bacteria not commonly isolated from healthy endotherms might be the result of the medium (containing fumarate, succinate, and nitrate) used (26), rather than a difference in the flora, since helical bacteria have been seen in the intestinal mucosa of laboratory mammals (25). The proportion of facultative bacteria was greater than that found in many other surveys, particularly in hibernating frogs, and pseudomonads were easily isolated from hibernating frogs. In this way, these temporarily aquatic animals resembled fish (32) rather than endotherms.

In many animals, nonsporeforming, gram-positive bacteria form a significant proportion (averaging up to 40%) of the colonic or fecal flora (11, 25), but few (less than 4%) were found in frogs, and no gram-positive, anaerobic cocci were isolated. Also, there was no predominant facultative species, such as *Escherichia coli*, on the plates incubated aerobically.

Seasonal chilling and restricted opportunities for transmission of bacteria may limit the flora. Some bacteria may come from food (34); insects, such as crickets, carry *Bacteroides* (33). On the basis of the bacteria characterized from these frogs, it is suggested that the facultative flora could have been derived from that of ancestral fish, but the anaerobic flora was probably derived from that of the insect prey. The history of the indigenous flora is of interest because of the interaction of the flora with the development of the immune system (6, 25).

The hibernating frogs had slightly but significantly lower counts than did the active frogs, but considering the small sample size and geographical differences between the groups, this may not be of any general significance.

The lowest total counts and predominance of a few types of facultative bacteria were found in the frogs subjected to environmental changes in the laboratory. These were the only frogs housed in nonflowing water, but previous studies at this facility indicated that frequency of

water change did not influence the facultative flora (34). Environmental changes have been shown to result in increased numbers of facultative intestinal bacteria in mammals (11, 31). Other studies of chilled (3) and hibernating (34) frogs have also shown overgrowth by a few facultative types of bacteria. Such overgrowth may precede the septicemia that is sometimes associated with hibernation (22). However, there was no overgrowth of facultative bacteria in the naturally hibernating frogs. Cultures of the livers showed no significant parenteral invasion by intestinal bacteria in either naturally or laboratory hibernating frogs at a time when the gut clearance was reduced (9) and some immune processes were inactive (35). Potentially invasive bacteria might be controlled by the activity of *Bacteroides* (2, 25), the secretion of mucus (9), the passage of material through the gut (1, 9), lysozymes (21, 27), and physiological changes peculiar to hibernation.

It was interesting that a typical intestinal flora could be maintained at temperatures near freezing and in the absence of any food supply exogenous to the host, even though material is slowly cleared from the gut (9). This flora is probably made up of reproducing indigenous bacterial populations rather than bacteria repeatedly replaced from the environment, because both the direct culture of samples and the subculture of isolates at 4°C indicated that the bacteria do grow at hibernating temperatures; the high bacterial counts obtained on the minimal media suggest that very low concentrations of nutrients, such as may be provided by slow intestinal secretion and epithelial turnover, are sufficient for the growth of most of the bacteria; and flowing fresh water at 4°C seems an even less likely habitat for fastidious anaerobes such as *Bacteroides* than the gut at that temperature.

This quantitative and qualitative analysis of the bacteria of the large intestines of frogs indicates that frogs have an intestinal flora similar to that found in mammals and birds. Thus, such a flora is not dependent on the high temperature, metabolic rate, and nutrient turnover of endotherms. This general similarity indicates that, in investigating systems in which the activity of the bacterial flora might be significant, the frog would be a suitable experimental animal for comparison with endotherms.

The analysis of the flora of hibernating frogs and frogs entering hibernation indicates that the bacteria may be reduced in total numbers and, in some cases, variety during hibernation, possibly because the low growth rates of bacteria at hibernation temperatures would make recovery from any disturbance on entering hibernation very slow, that is, a matter of weeks or months. However, the typical mucous membrane flora can persist at temperatures near freezing, and the tissues of the host remain immune to degradation or invasion by the flora.

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