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Classification and Distribution of Large Intestinal Bacteria in Nonhibernating and Hibernating Leopard Frogs (*Rana pipiens*)

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The large intestinal flora of the leopard frog, *Rana pipiens*, was examined to determine whether differences existed between the nonhibernating and hibernating states of the animal and to determine the relative concentrations and proportions of potential frog pathogens. Hibernators had a logarithmic decrease of bacteria per milligram of intestine averaging one, and significantly greater proportions of facultative bacteria and psychrophiles relative to nonhibernators. The predominant anaerobic bacteria were gram-positive *Clostridium* species and gram-negative *Bacteroides* and *Fusobacterium* species. The predominant facultative bacteria were enterobacteria in nonhibernators but *Pseudomonas* species in hibernators. Many species of *Pseudomonas* are pathogenic for frogs, and thus the intestinal flora in hibernators may be a potential source of infectious disease.

The frog is an animal of great value for education and research in a variety of disciplines (5). Despite the importance of frogs to biomedical research, little is known about the mechanisms of pathogenicity of bacterial pathogens for frogs or their effects on amphibian populations (1, 5). Neither have the implications of hibernation on frog-bacterium interactions been extensively studied. Investigations of infectious disease have been limited to the identification of frog pathogens and the treatment of prevention of infections in laboratory-maintained animals.

There is some evidence that bacteria that are commonly isolated from the large intestines of frogs are also pathogenic for these animals. Carr et al. (4) maintained bullfrogs, *Rana catesbeiana*, at low temperatures (3°C) and found that potential pathogens among the genera *Acinetobacter*, *Aeromonas*, *Bacillus*, and *Pseudomonas* survived. Gossling et al. (9) isolated enterobacteria from a predominantly anaerobic flora in room-temperature frogs, *Rana pipiens*, and pseudomonads from frogs maintained at low temperatures (4°C). Glorioso et al. (7) examined septicemic bullfrogs and isolated numerous enterobacteria and *Pseudomonas* species. Many of these isolates, when inoculated into healthy animals intramuscularly or in the dorsal lymph sac, caused the death of the frogs.

The relative concentrations of potential pathogens in the frog intestinal flora during the different seasonal physiological states is not known. Boni and Battaglini (2) described the intestinal flora of nonhibernators as copious and containing species similar to those in a mammalian flora. Gossling et al. (9) characterized intestinal isolates from nonhibernators and hibernators and reported that there was little difference between these groups, with the majority of organisms belonging to the genus *Bacteroides*. However, only a few frogs in each group were examined, and these were obtained from various geographic sources. Standardization of frogs used in laboratory research is difficult, and it is not known to what extent geographic location, age of the animals, and physiologic state, among other factors, may affect results.

In this investigation the large intestinal floras from a larger sample of nonhibernators and hibernators obtained from the same geographic location were characterized. Completely anaerobic isolation conditions were used to maximize bacterial recovery. The concentrations and distributions of the common intestinal isolates were determined and compared between the nonhibernating and hibernating states of the animal.

MATERIALS AND METHODS

Frogs. All frogs were adult *Rana pipiens* purchased from NASCO (Ft. Atkinson, Wis.). Nonhibernating and hibernating frogs were representative of their physiological states when collected. Snout-to-vent length ranged from 6.0 to 9.0 cm. Nonhibernating frogs were housed at room temperature in Michigan Environmental Enclosures for Small Animals (Keyco Co., Inc., Peach Bottom, Pa.) at Amphitech, Inc. (Ypsilanti, Mich.). These frogs were fed live crickets twice a week.

Hibernating frogs were kept in plastic containers (76-liter volume) in a cold room at 0 to 4°C. The containers were modified to include a drain at the bottom, a filter, and an aeration source. The containers were filled three-fourths full with tap water containing 3.2 g of NaCl per liter. This osmolarity was maintained as a preventative measure against the development of a bacterial epidemic. Half the volume of water was changed weekly. Chlorine was allowed to evaporate by opening the water container to air overnight. Light conditions were set to simulate an 8-h day.

Dissection procedure. The dissection procedure was done within an anaerobic chamber (85% N_2, 10% H_2, and 5% CO_2; Coy Manufacturing Co., Ann Arbor, Mich.) to maximize recovery of obligate anaerobes. A frog was sacrificed by pithing and brought inside the chamber. The abdomen was opened aseptically, portions of the liver and thigh muscle

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were excised and weighed, and each portion was placed into 10 ml of reduced transport fluid (21) without EDTA. The entire large intestine was removed and weighed, and the contents were expressed by the rolling action of cotton-tipped applicator sticks. The intestinal contents were placed into 10 ml of reduced transport fluid. After weighing, the remaining intestinal tissue, referred to as the intestinal lining, was cut into small pieces and placed into 10 ml of reduced transport fluid.

All samples in reduced transport fluid were homogenized for 20 s with a tissue homogenizer (Tekmar Co., Cincinnati, Ohio), and clumps of bacteria were dissociated by dispersion for 20 s with a microtip sonifier (Kontes Co., Vinewood, N.J.). Serial 10-fold dilutions were made from these suspensions.

The liver and thigh muscle were sampled to look for bacterial contamination in tissues that would normally be considered sterile. Both have been reported to be contaminated in sick and dead frogs during epidemics (6, 11, 18, 19).

Plating and cultural counts. Suitable dilutions of each suspension were plated onto Schaedler agar (BBL Microbiology Systems, Cockeysville, Md.) to which was added (per liter) 4 g of NaCl, 3 g of agar (Difco Laboratories, Detroit, Mich.), 1 g of potassium nitrate, filter-sterilized menadione (2.0 ml of a 0.5 mg/ml solution), 0.4 g of sodium carbonate, 0.5 g of cysteine hydrochloride contained in 25 ml of distilled water, and 20 ml of sheep blood. Suspensions were also plated on Schaedler agar containing metronidazole (10 mg/liter) to allow the selection of organisms that were facultatively anaerobic. All media were prereduced by storage in the anaerobic chamber for at least 24 h before use.

Plating was done with a spiral plater (Spiral Systems Inc., Cincinnati, Ohio). Duplicate sets of plates were incubated within the anaerobic chamber for 1 week at room temperature (22 to 25°C). A second set of plates were incubated in anaerobic jars for 1 week at 4°C. The anaerobic jars were sealed within the anaerobic chamber and thus contained the same atmosphere as the room-temperature plates. Colony counts were determined by using a template designed for use with the spiral plater (12). The recovery on Schaedler agar was taken as the total count; colonies on Schaedler agar plates containing metronidazole were assumed to be facultative anaerobes; colonies growing at 4°C were assumed to be psychrophilic.

Bacterial characterization. All colonies from a random area on a Schaedler agar plate were selected for further characterization. Approximately 32 colonies from each sample were selected. In some instances total counts from 4°C plates were less than 32 colonies, and in these cases all colonies on the plate were tested.

All colonies were subcultured anaerobically on Schaedler agar. The organisms were Gram stained, tested for catalase production, and restreaked on Schaedler agar plates containing metronidazole. Those isolates that grew were considered facultative anaerobes, and those that did not were considered obligate anaerobes.

A heavy suspension of each isolate was prepared in 2.5 ml of sterile saline, and 2 drops of these suspensions was used to inoculate Minitek plate wells (BBL) containing reagents for the following tests: esculin hydrolysis (100 μl of esculin [17]), nitrate reduction (100 μl of 0.1% potassium nitrate [10]), glucose utilization (100 μl of 5% glucose, 1 drop of Sorenson buffer [pH 7.2], and 1 drop of 20 mg/100 ml bromcresol purple solution), and the presence of a trypsin-like enzyme (100 μl of 10% BANA solution in pH 8.5 Tris buffer [20]). Two drops of 0.1% tryptophan were added to the remainder of the bacterial suspension to test for the production of indole (15). All suspensions were incubated anaerobically for 48 h at room temperature. Positive reactions were visualized by color changes: yellow for glucose utilization, black for esculin hydrolysis, and purple for nitrate reduction and/or presence of a trypsinlike enzyme. Bacterial characteristics were matched to the generic descriptions described in *Bergey's Manual of Determinative Bacteriology* (3).

Hibernation induction. Two attempts were made to induce hibernation in a group of frogs. In the first attempt approximately 30 frogs were kept within two Michigan Environmental Enclosures for Small Animals in a refrigerator. The temperature was gradually decreased from approximately 20 to 9°C over a 2-week period. Light was decreased from 10 to 8 h of exposure per 24 h. The temperature was allowed to hover around 8 to 9°C for a couple days, since this is the critical temperature at which frogs stop feeding. Frogs were fed crickets twice a week until this point. Enough water was added to the Michigan Environmental Enclosures for Small Animals to allow the frogs to submerge themselves. The water was changed every day. In week 3, the temperature was reduced to 2°C and the surviving frogs were moved to permanent housing in plastic containers in the cold room (1 to 4°C). During the induction process the refrigerator door was opened each day to allow fresh oxygen.

The second attempt was modified slightly. The temperature and light were decreased to 8 to 9°C and 8 h of exposure per 24 h during week 1. The temperature was allowed to hover around 8°C for 3 days, and the next week it was brought down to 2°C. At this point the frogs were removed to the cold room.

RESULTS

Significant qualitative and quantitative differences were observed between the large intestinal flora in a nonhibernator and the large intestinal flora in a hibernator (Table 1). A logarithmic decrease of approximately one of bacteria per milligram of tissue was observed in the intestinal contents or associated with the intestinal lining of hibernating frogs compared with that of nonhibernating frogs. The physiology of these bacteria differed as well; the flora in hibernators was composed of significantly higher proportions of facultative anaerobes and facultative psychrophilic bacteria (bacteria which grew at both 4 and 25°C) relative to the flora of nonhibernators. In absolute quantities the hibernators contained 1/10 the total number of bacteria, 1/3 to 1/5 the number of facultative anaerobes, and approximately the same number of psychrophilic bacteria as nonhibernators.

Of the frogs examined, 9 to 50% had bacteria in a detectable quantity (greater than 10 bacteria per mg of tissue) in the liver or muscle (Table 2). Bacteria were isolated at a significantly greater frequency from the thigh muscles of nonhibernators than from those of hibernators but were isolated slightly more frequently from the livers of hibernators than from those of nonhibernators. Often, bacteria were isolated from one or the other tissue in a particular frog. The average concentration of the bacteria in these tissues was greater in the nonhibernators than in the hibernators.

The hibernators used were obtained in the naturally hibernating state and were kept under hibernating conditions in the laboratory. The data presented in Tables 1 and 2 represent the means of results from all nonhibernators and hiber-

nators that were studied over a 4-year period and represent different shipments of frogs from the same supplier. One might expect that there would be variation in the flora from year to year. However, the proportions of facultative anaerobes in these hibernators remained significantly greater than proportions in nonhibernators from year to year as determined by comparison of the ratio of median percentage of facultative anaerobes in nonhibernators to the median percentage in hibernators.

A total of 879 strains isolated from three nonhibernators and six hibernators were partially characterized. Table 3 lists the percentage of bacteria isolated at 25°C that could be classified in the various genera that were identified. Those genera that are grouped together indicate that tests to identify the bacteria were not sufficient to differentiate among those genera. The 25°C isolates from nonhibernators were predominantly anaerobic species. The gram-positive anaerobic strains had characteristics consistent with *Clostridium*, *Eubacterium*, or *Bifidobacterium* spp. Spore tests on random isolates indicated that most of these organisms probably belonged to the genus *Clostridium*. The gram-negative anaerobes could usually be assigned to the genera *Bacteroides* or *Fusobacterium*. The majority of the facultative isolates were coliform or gram-positive enterococci, but isolates typical of *Bacillus*, *Corynebacterium*, *Pseudomonas*, and *Flavobacterium* or *Azotobacter* were also represented.

The anaerobes isolated at 25°C from hibernators were similar to the anaerobes isolated from nonhibernators. Differences were observed in the facultative flora, since the hibernators had a significantly higher proportion of *Pseudomonas* species both in the intestinal contents and associated with the intestinal lining and higher proportions of *Bacillus* species and coliforms than were observed in the nonhibernators.

At 4°C, the bacterial profile changed considerably for the nonhibernators (Table 3). Fewer anaerobic species were isolated, and the majority of facultatives were coliformlike. High proportions of streptococci and, to a lesser extent, of *Bacillus* and *Flavobacterium* or *Azotobacter* spp. were seen. In the hibernators, more than half of the 4°C isolates were *Pseudomonas* species, and the levels and proportions found differed significantly from those found in the nonhibernators. Coliform and *Bacillus* bacteria were also well represented among the facultative bacteria in the hibernators.

Analysis of the distribution of isolates using the percent coverage formula developed by Good (8) indicated that the identification of additional bacteria would not have significantly altered the patterns shown in Table 3. By this analysis it was unlikely that significant numbers of bacteria would belong to any genus not represented in the table, since in all cases over 95% of the flora was estimated to belong to the listed genera.

An effort was made to determine whether the differences between nonhibernators and hibernators, in terms of absolute numbers of bacteria present in the large intestine, were related to the length of time the animal was in hibernation. Scatter plots were generated by plotting the total bacterial population, the number of facultative organisms, and the number of psychrophiles per milligram of intestinal tissue or intestinal contents versus the length of time in hibernation. Regression analysis was used to generate a best-fit line through the points, assuming a linear relationship. It appeared that the total number of bacteria, the number of facultative anaerobes, and the number of psychrophiles all increased with the length of time frogs spent in hibernation.

TABLE 1. Large intestine bacteriology

Group	Total bacteria[a] in intestinal:		% Facultative and anaerobic bacteria[b] in intestinal:				% Psychrophilic bacteria[b] in intestinal:	
			Lining		Contents			
	Lining	Contents	Anaer.[c]	Fac.[c]	Anaer.	Fac.	Lining	Contents
Nonhibernators	5.5 ± 0.1 (23)[d]	6.7 ± 0.1 (19)	86.4 ± 4.6 (23)	13.6 ± 4.6 (23)	85.0 ± 6.3 (19)	15.0 ± 6.3 (19)	3.6 ± 2.2 (16)	4.6 ± 2.9 (12)
Hibernators	4.4 ± 0.2 (15)	5.6 ± 0.3 (11)	62.2 ± 9.2 (15)	37.8 ± 9.2 (15)	49.0 ± 9.8 (11)	51.0 ± 9.8 (11)	31.1 ± 10.2 (15)	60.7 ± 24.0 (11)

[a] Log_{10} bacteria per milligram of tissue.
[b] Percentage of total count at 25°C.
[c] Anaer., Anaerobic; Fac., facultative.
[d] Value plus or minus the standard error of the mean, with number tested in parentheses. Values for the nonhibernators are significantly different from values for the hibernators in each column as determined by a Kruskal-Wallis test, $P < 0.05$.

TABLE 2. Bacterial concentrations in liver and muscle of nonhibernating and hibernating frogs

Group	Thigh muscle bacteria			Liver bacteria		
	Isolation frequency[a]	Isolation %	Mean concn[a]	Isolation frequency	Isolation %	Mean Concn
Nonhibernators	8/16[b]	50	2.0 ± 0.3	4/16	25	1.8 ± 0.5[b]
Hibernators	1/11[b]	9	1.3	4/11	36	1.4 ± 0.3[b]

[a] Frequencies in this column are significantly different as determined by a chi-square analysis, $P < 0.05$.
[b] Log_{10} of bacteria per milligram of tissue plus or minus the standard error of the mean, determined at the time of isolation.

When log_{10} values were used for the bacterial concentrations (to minimize the influence of outliers) there was still the increase in the bacterial concentrations with length of hibernation, but only the increase in the concentration of bacteria within the intestinal contents approached significance. The population of frogs used (year purchased) did not seem to make a difference.

Attempts to induce hibernation and thereby maintain a year-round supply of hibernators were unsuccessful. In the first attempt many frogs began dying during week 3 of the induction process. Only about one-third of the frogs survived long enough to be placed in the cold room at 4°C, and most of these died shortly thereafter. The second attempt resulted in 25 of 35 surviving the induction process. However, these frogs lived only about 1 month at 4°C as opposed to the 4 or 5 months that natural hibernators survived. Some of the artificial hibernators that survived the induction process and displayed no external symptoms of disease were sacrificed, and specimens were taken for culture. These artificial hibernators had greater proportions of facultative anaerobes than the average natural hibernator in both the intestinal lining (75 versus 38%) and the intestinal contents (76 versus 51%). The difference between these median facultative organism percentages was significant for the bacteria associated with the intestinal lining. The artificial hibernators also had a lower concentration of bacteria in the intestines, but these differences were not significant.

DISCUSSION

The results documented the substantial differences that exist in the large intestinal flora between nonhibernators and hibernators with regard to bacterial numbers and proportions of facultative anaerobes and facultative psychrophiles. The main change was a significant increase in both the proportion and absolute number of pseudomonads in the hibernators. The remaining flora was predominantly anaerobic and, on the whole, quite complex, with many different species isolated for each genus represented. Thus, hibernation and the conditions associated with it did not result in a flora simplified to the point that it was predominated by only a few species. In this way, frogs (which do not eat during periods of hibernation) differ from rodents, in which there is an increase in enterobacteria under starvation conditions (14, 22) or the selection of one or two predominant bacterial species after maintenance on a sterile diet (13).

The results of this investigation are in partial agreement with those obtained by Gossling et al. (9), who concluded that a complex intestinal flora could be maintained at temperatures close to freezing. In both studies the concentra-

TABLE 3. Characterization of bacterial isolates from large intestine lining and contents

Organism type and genus	% of 25°C isolates from:				% of 4°C isolates from:			
	Nonhibernators		Hibernators		Nonhibernators		Hibernators	
	Lining (84)[a]	Contents (82)	Lining (138)	Contents (147)	Lining (74)	Contents (57)	Lining (140)	Contents (157)
Anaerobic								
Clostridium, *Eubacterium*, *Bifidobacterium*	51.1	40.2	25.3	24.4	29.5	12.2	8.0	7.8
Propionibacterium	3.5	2.2	0.0	0.0	0.0	0.0	0.0	0.0
Bacteroides, *Fusobacterium*	25.2	22.4	16.3	10.8	1.1	0.0	6.5	3.0
Peptococcus, *Peptostreptococcus*, *Ruminococcus*	3.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wolinella	2.4	0.0	5.7	1.1	0.0	0.0	0.0	0.0
Facultative								
Bacillus	0.0	1.4	2.0	7.9	2.2	11.8	7.8	6.9
Streptococcus, *Enterococcus*	4.8	10.7	0.0	0.0	25.1	19.4	0.0	0.0
Corynebacterium	2.6	0.0	0.0	1.1	0.0	1.1	0.0	0.0
Coliform-*Vibrio* groups								
Indole (+), esculin (+)	3.6	8.3	13.9	15.6	22.9	34.6	12.9	17.3
Indole (+), esculin (−)	1.3	0.0	1.3	3.3	7.7	15.9	6.3	6.2
Indole (−), esculin (+)	1.3	7.7	0.7	1.2	4.3	3.6	0.6	0.0
Indole (−), esculin (−)	0.0	2.4	0.7	3.8	0.0	1.1	1.2	3.7
Pseudomonas[b]	1.3	0.0	31.6	27.7	1.1	0.0	55.6	52.6
Flavobacterium, *Azotobacter*	0.0	4.8	0.7	1.7	7.2	0.0	0.0	1.7
Lactobacillus	0.0	0.0	2.0	1.1	0.0	0.0	0.0	0.6

[a] Percentages of total numbers of isolates are given in parentheses.
[b] Values for pseudomonads in nonhibernators and hibernators are significantly different as determined by a Kruskal-Wallis test, $P < 0.05$.

tions of bacteria in the intestines of nonhibernators were comparable. Gossling et al. reported only slightly fewer bacteria in four hibernating frogs, whereas the results of this investigation showed significantly fewer bacteria (1 log less) in hibernators (Table 1). The discrepancy may be due to the low number of frogs in the Gossling experiment and the length of time for which the animals were in hibernation. In the present study, in which strict anaerobic procedures were used, approximately 3 logs more bacteria per g of intestinal tissue from hibernators were recovered than in an earlier study in which only aerobic procedures were used to culture the flora of bullfrogs maintained at low temperatues (4).

The significantly greater proportion of psychrophiles isolated from hibernators was expected, since the low temperatures of hibernation should operate in the selection of bacteria that could best maintain themselves at these temperatures. Reasons for the larger proportion of facultative anaerobes are unknown. Perhaps the obligate anaerobic portion of the flora was more susceptible to the low temperatures or to the lack of nutrient intake by the host. Alternatively, it is conceivable that bacterial metabolism on the whole was greatly decreased for the same reasons, and so the low E_h at which anaerobes can survive was not maintained in the intestinal tract.

Characterization of the bacteria isolated from the large intestines of nonhibernators and hibernators revealed that the flora in hibernators was not merely a surviving subset of the flora in the nonhibernating state. *Pseudomonas* species increased not only in proportion but also in absolute number in hibernators relative to nonhibernators. Pseudomonads represented approximately one-third of the flora in hibernators but were rarely isolated at all from nonhibernators. Suggestive evidence that the bacteria in a hibernator were actively growing and multiplying was obtained by plotting the number of bacteria isolated from the hibernating frogs versus the length of time the frogs had been in hibernation. It might be expected that since the frogs do not eat during hibernation there would be a decline in the bacterial concentration with increasing time in hibernation, since portions of the flora would periodically be excreted or die of starvation. Contrary to these expectations, there appeared to be an increase, though not statistically significant, in the bacterial concentrations with the length of hibernation.

It was not unusual to isolate bacteria from portions of the liver and thigh muscle. The types of bacteria found were similar in most instances to the types isolated from the large intestine. Whether the large intestinal flora was a direct source of this systemic contamination is not known with certainty. This is of particular interest, because many species of *Pseudomonas* are considered pathogenic for frogs (7) and these organisms were predominant in the intestines of hibernators. Thus, if these intestinal pseudomonads can gain entry into the underlying host tissues, they could contribute to, or be responsible for, the septicemias that are sometimes associated with hibernation (16).

The results presented in this report are an accumulation of data from different populations of frogs over different years. The higher proportion of facultative anaerobes in hibernators was maintained from year to year, and thus the differences between nonhibernators and hibernators could not be attributed to differences in frog shipments. At the same time, artificial hibernators were shown to have greater proportions of intestinal facultative anaerobes than natural hibernators. It is unlikely that the differences were due to different lengths of hibernation between the two groups. It is possible that the shorter induction time in the laboratory and a different water environment resulted in a different flora in the artificial hibernators than would have occurred in nature with the more gradual induction into hibernation in the pond or stream ecosystem. This indicates that frogs artificially induced into hibernation have an intestinal flora that is not typical of either true hibernators or nonhibernators. This compromises the ability to compare nonhibernators and hibernators under rigid experimental conditions.

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