Isolation and Identification of Intestinal Bacteria from Japanese Tree Frog (*Hyla japonica*) with the Special Reference to Anaerobic Bacteria

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ABSTRACT. The bacteria in the large intestines of eight Japanese tree frogs (*Hyla japonica*) were enumerated by using an anaerobic culture system. The microorganisms at approximately 3.1×10^9 bacteria per g (wet weight) of intestinal contents were present in the intestine of all the frogs tested. No difference of the total bacteria in the frog intestine was observed between two different incubation-temperatures (room temperature and 37°C). Eleven genera and 16 species were isolated from the frog intestine. In most frogs, *Bacteroides* (*B.*) *caccae* and *B. vulgatus* were detected as the predominant organisms. *Escherichia coli* was also present in greater numbers in the intestine. Other bacteria isolated at high dilutions were strict anaerobes, including *Fusobacterium* and *Clostridium*. *Enterococcus faecalis* was frequently isolated from the frog intestine. However, four genera of *Bifidobacterium*, *Eubacterium*, *Peptostreptococcus*, and *Lactobacillus* were not isolated from the frog intestine.—**KEY WORDS:** anaerobic bacteria, frog, intestinal flora, tree frog.

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The indigenous microflora of laboratory animals has been controlled as well as genetic relationships and composition of diets. Amphibians are widely used as laboratory animals, especially when systemic tolerance of low or variable temperatures is required [4, 14]. The Japan-USSR space flight program [3, 9] was carried out to research several behaviors of the Japanese tree frogs, *e.g.* visual, feeding, avoiding, and courtship behaviors, onboard “MIR” orbital station. Therefore, it is important to investigate the species and numbers of isolates from frog intestine, skin, and oral cavity for verifying safeness of the living specimens for cosmonauts.

The present study was undertaken to define the composition of intestinal microflora of Japanese tree frogs to the species level.

MATERIALS AND METHODS

Frogs: Eight Japanese tree frogs (*Hyla japonica*; snout-foot length, 4.79 ± 0.73 cm) aged from 1 to 2 years were collected and kept in a plastic tank ($20 \pm 1.5 \times 12.5$ cm³) spread the sponge plate with fresh water at the room temperature. The frogs were given four live meal worms (*Tenebrio obscurus*) per one week as feed.

Bacterial sampling: Each frog was pithed, and its abdominal cavity was opened aseptically. The large intestine, from the ileocolonic constriction to just cranial to the urethral entry, was aseptically removed and weighed. The weight of contents

obtained from the intestine of each frog was from 0.05 to 0.2 g. These procedures were completed less than 10 min for each frog, and the actual samples were exposed to air for less than 10 s.

Culture media and methods: The method for bacteria analysis on the intestinal microflora in this study was essentially the same as the previously described procedures [13]. After thorough mixing, a series of 10-fold dilutions (10^{-1} to 10^{-7}) was prepared in anaerobic diluents [12]. From appropriate dilutions, 0.05 ml aliquots were spread onto three non-selective agar plates and nine selective agar plates. The incubation of each medium was performed by the room temperature (23–25°C) and 37°C from 24 to 48 hrs. Direct bacterial numbers were determined by using microscopic methods [12].

After incubation, each plate was examined for bacterial colonies. The identification of isolates was performed with colonial and cellular morphologies, gram-reaction, spore formation and aerobic growth.

Identification of isolates: All anaerobic isolates were identified by the previously described method [8, 11, 12, 16]. The basal medium for biochemical tests was peptone-yeast extract (PY) broth with 4% (vol/vol) Fildes solution added (designated as PYF broth). Acid production from 25 carbohydrates was determined by using PYF broth containing 0.5% (wt/vol) of the carbohydrate to be tested. The pH was measured directly in the culture tubes by using a combination electrode (Automatic Multipoint pH Measuring System, Lifetech Co., Ltd., Saitama).

The final pH was determined after incubation for 7 days in PYF broth with glucose (designated as PYFG broth).

The formation of gas was detected by the appearance of crevices in PYFG broth containing 1.5% (wt/vol) Bacto-Agar (Difco Laboratories, Detroit, Mich., U.S.A.). Indole production and nitrate reduction in indole-nitrate medium (BBL Microbiology System, Cockeysville, Md., U.S.A.) were determined. The following tests were carried out with the media and methods previously described [8]: gelatin liquefaction, starch and esculin hydrolysis, growth stimulation or inhibition by 20% bile, and production of H_2S .

For the analysis of fermentation products, strains were grown for 7 days in PYFG broth. Volatile fatty acids, and nonvolatile acids were analyzed by the methods previously described [10].

Enterobacteriaceae and *Enterococcus* were classified by using two identification kits (EB-20, Nisui, Tokyo for enterobacteria and API Strep, Analytab Products, Painview, N.Y. for streptococci, respectively).

Microbial counting: For the bacterial species identified, the bacterial count per gram of wet intestinal content was calculated and converted into a logarithmic equivalent. The total viable count was calculated from the sum of the counts of each bacterial groups.

RESULTS AND DISCUSSION

Facultative bacteria from the intestine of frogs have been investigated as a source of the septicemia, often associated with chilling and hibernation [2, 4], which occasionally kills large numbers of frogs in the laboratory and in the wild [4].

Table 1 shows the counts and composition of certain bacterial groups isolated from the large intestine of eight tree frogs. When the bacterial numbers from frog intestinal specimens were determined by the microscopic method, the microorganisms at approximately 3.1×10^9 bacteria per g (wet weight) of intestinal contents was present in the intestine of all the frogs tested. No significant changes in total viable counts or the types of bacteria incubated at the different temperatures (room temperature and 37°C) were observed. On the other hands, Gossling *et al.* [6] demonstrated that the lowering of temperature rather than the absence of food is the important factor in the

Table 1. Counts and composition of certain bacterial types isolated from large intestines of eight tree frogs

Method of counting and bacterial type	Bacterial counts	
Method of counting		
Microscopic	9.6±0.4 ^{a)}	(8/8) ^{b)}
Anaerobic culture at room temperature	9.5±0.4	(8/8)
Anaerobic culture at 37°C	9.5±0.4	(8/8)
Bacterial types		
Total bacteria	9.5±0.4	(8/8)
Anaerobic bacteria		
Gram negative nonsporeforming rods	9.4±0.6	(8/8)
Curved rods	8.2	(1/8)
Sporeforming rods	6.6±0.5	(3/8)
Aerobic bacteria		
Enterobacteria	9.2±0.5	(8/8)
Enterococci	6.9±1.3	(8/8)
Micrococci	3.6	(1/8)
Molds	3.3	(1/8)

a) Bacterial counts expressed as mean±S.D. of $\log_{10}$ /gram of wet intestinal contents.

b) Frequency of occurrence expressed as numerator=No. of subjects detected; denominator=No. of subjects examined.

reduction of bacterial flora seen in hibernating frogs. Carr *et al.* [2] also indicated that composition of intestinal aerobes from bullfrogs was differed by the maintenance at low temperatures.

Obligate anaerobic bacteria in the frogs consisted of anaerobic gram-negative nonsporeforming rods, curved rods, and sporeforming rods. Facultative anaerobes and aerobes comprised enterobacteria, enterococci, micrococci, and molds. The predominant organisms in the frog intestine were anaerobic gram-negative nonsporeforming rods and enterobacteria. Four anaerobic bacteria of lactobacilli, bifidobacteria, eubacteria, and peptostreptococci were not isolated from the tree frog intestine in the present study. The intestinal flora of meal worms and the bacterial flora of environmental water were not investigated, but they may influence the composition of the frog intestinal flora.

A total of 79 anaerobic isolates from eight tree frogs were identified to 14 bacterial groups by biochemical and physiological tests as shown in Table 2. From the analysis of fermentation production, groups A to H were classified as *Bacteroides* and groups I and J were identified as *Fusobacterium*. Group K was classified as *Selenomonas* by cell morphology and propionate production from PYFG broth. The bacteria in the large intestines of northern leopard frogs (*Rana pipiens*) were enumer-

Table 2. Identification of anaerobic bacteria isolated from large intestines of eight tree frogs^{a)}

Characteristics	A	B	C	D	E	F	G	H	I	J	K	L	M	N
No. of isolates	37	22	1	1	1	1	1	1	3	2	2	1	2	1
Gram reaction	—	—	—	—	—	—	—	—	—	—	—	V	+	—
Spore formation	—	—	—	—	—	—	—	—	—	—	—	+	+	+
Cell morphology														
Acid produced from:														
Arabinose	A	A	—	A	A	A	—	A	—	—	—	—	A	—
Xylose	A	A	A	A	A	A	A	A	—	—	—	—	A	—
Rhamnose	A	W	W	A	A	—	A	W	—	—	—	A	A	—
Ribose	W	—	—	A	A	—	—	A	—	—	—	—	A	—
Glucose	A	A	A	A	A	A	A	A	A	A	A	A	A	—
Mannose	A	A	A	A	A	A	A	A	A	A	A	A	A	—
Fructose	A	A	A	A	A	A	A	A	A	A	—	A	A	—
Galactose	A	A	A	A	A	A	A	A	A	W	A	A	A	—
Sucrose	A	A	A	A	A	A	A	—	A	—	—	A	A	—
Maltose	A	A	A	A	A	A	A	W	A	—	—	A	A	—
Cellobiose	A	A	—	A	A	A	—	—	A	—	A	A	A	—
Lactose	A	A	A	A	A	A	A	A	A	—	A	A	A	—
Trehalose	A	—	—	A	A	—	—	—	A	—	—	A	A	—
Melibiose	A	A	A	A	A	A	—	W	A	—	—	W	A	—
Raffinose	A	A	A	A	A	A	—	—	W	—	—	W	A	—
Melezitose	A	A	—	A	A	—	—	—	A	—	—	A	A	—
Starch	—	W	A	A	A	A	A	—	—	—	—	—	A	—
Glycogen	—	W	A	A	A	A	A	—	—	—	—	—	—	—
Mannitol	—	—	—	—	—	—	—	—	—	—	—	A	—	—
Sorbitol	—	—	—	—	—	—	—	—	—	—	—	—	W	—
Inositol	—	—	—	—	—	—	—	—	—	—	—	—	W	—
Esculin	A	A	—	A	A	A	A	—	A	—	A	A	W	—
Salicin	A	A	—	A	—	A	—	—	A	—	—	A	W	—
Amygdalin	A	A	—	A	A	A	—	—	A	—	A	A	W	—
Starch hydrolyzed	—	—	—	+	+	+	—	—	—	—	—	—	+	—
Esculin hydrolyzed	+	+	+	+	+	+	+	W	+	—	+	+	+	—
Gas formation	—	—	—	—	—	—	—	—	—	—	—	—	+	+
Growth in 20% bile	S	S	S	S	S	S	S	S	—	—	—	—	—	—
Indole produced	—	—	—	+	+	+	+	—	—	—	—	—	—	W
H ₂ S produced	+	+	—	—	—	—	—	W	—	—	—	—	—	W
Products from PYG	SA piv	SA piv	SA piv	SA piv	SA piv	SA piv	SA piv	SA piv	B ap	B lap	PA	A fl	AS	A

a) None of all strains produced acid from glycerol, reduced nitrite, and liquefied gelatin. A, all strains produced acid from the carbohydrates; +, positive reaction; —, negative reaction; W, weak reaction; V, variable. S, Enhancement of the growth by 20% bile. Products from 1% peptone-1% yeast extract-1% glucose (PYG) broth cultures: Capital letters indicate an average (from multiple cultures) of >1 meq of acid/100 ml broth; small letters, <1 meq/100 ml. A, acetate, B, butyrate, f, formate, iv, iso-valerate, L, lactate, P, propionate, and S, succinate.

ated and partly characterized by Gossling *et al.* [5] who stated that *Bacteroides* spp. were the dominant organisms in most frogs. Their finding shows that the intestinal flora of frogs is similar to that of mammals and birds [1, 12, 15]. The results in this study, however, did not agree with their report [5].

A total of 37 strains in group A was identified as *B. caccae*. Group B (22 strains) was classified as *B. vulgatus*. Groups C, D, E, F, and G were also identified as *B. fragilis*, *B. ovatus*, *B. thetaiotaomicron*, *B. uniformis*, and *B. stercoris*, respectively. Group J could not be identified to species level by using currently accepted identification protocols. The higher populations of *B. caccae* and *B. vulgatus* were observed in the tree frog intestinal flora (Table

3).

The anaerobic sporeforming rods (genus *Clostridium*) divided into three groups (L to N) were identified as *C. ramosum* for group L, *C. coccoides* for group M, and unidentified *Clostridium* sp. for group N, respectively. Gossling *et al.* [5] indicated that the species of the genus *Clostridium* isolated from the frog intestine were *C. cocleatum* and *C. spiroforme*. In this study, those *Clostridium* species were not detected from the tree frog intestine.

The facultative anaerobes and aerobes in the frog intestines belonged to seven genera and five species (Table 3). The most prevalent facultative anaerobes were *Escherichia coli* and *Enterococcus faecalis*. Hird *et al.* [7] indicated that *Aeromonas hydrophila* was

Table 3. Bacterial species isolated from large intestines of eight tree frogs

Species	Bacterial counts	
<i>Bacteroides</i>	8.6–10.1 ^{a)}	(8/8) ^{b)}
<i>B. caccae</i>	8.0–10.1	(8/8)
<i>B. vulgatus</i>	8.6–10.0	(8/8)
<i>B. fragilis</i>	7.3	(1/8)
<i>B. ovatus</i>	8.3	(2/8)
<i>B. thetaiotaomicron</i>	6.1	(1/8)
<i>B. uniformis</i>	7.4–8.4	(2/8)
<i>B. stercoris</i>	7.9–8.3	(2/8)
<i>Bacteroides</i> sp.	8.2–9.4	(2/8)
<i>Fusobacterium</i>	8.4–9.8	(2/8)
<i>F. mortiferum</i>	8.4–9.8	(2/8)
<i>F. varium</i>	7.3	(1/8)
<i>Selenomonas</i> sp.	8.2	(1/8)
<i>Clostridium</i>	6.1–7.1	(4/8)
<i>C. ramosum</i>	6.1–7.1	(3/8)
<i>C. coccooides</i>	5.1	(1/8)
<i>Clostridium</i> sp.	6.8	(1/8)
<i>Escherichia coli</i>	8.3–9.9	(8/8)
<i>Citrobacter freundii</i>	9.9	(1/8)
<i>Klebsiella pneumoniae</i>	7.3–9.3	(2/8)
<i>Haffnia alvei</i>	9.9	(1/8)
<i>Enterococcus faecalis</i>	5.3–8.9	(8/8)
<i>Micrococcus</i> sp.	3.6	(1/8)
Molds	3.3	(1/8)

a) Bacterial counts expressed as the extent of log₁₀/gram of wet intestinal contents obtained from each frog.

b) Frequency of occurrence expressed as numerator=No. of subjects detected; denominator=No. of subjects examined.

frequently isolated from frog intestine and the prevalence of members of the family *Enterobacteriaceae* was the lowest in frogs captured in early spring and the highest in frogs captured in late summer. Therefore, it is of interest to determine the effects, if any, of the different temperature of breeding environment on the frog intestinal flora.

These results in the present study show that the intestinal microflora in the tree frogs consists of obligately anaerobic bacteria and facultatives, whereas lactobacilli and bifidobacteria, which have the beneficial effects in animals and birds, were not detected from these tree frog intestine.

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