
ANAEROBIC BACTERIA ISOLATED FROM THE GASTROINTESTINAL TRACTS OF BULLFROG TADPOLES (*RANA CATESBEIANA*)

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Abstract.—The taxonomic identities and biochemical roles of symbiotic microbes living within the guts of amphibians remain very poorly studied to date. This is unfortunate because the normal microbiota has profound significance on the life history of larval amphibians. In this study, I investigated the bacterial flora of the gastrointestinal tracts of wild-caught *Rana catesbeiana* tadpoles under anaerobic conditions. I isolated several strains of bacteria and made attempts to identify them using a variety of culturing methods, biochemical tests, and differential staining techniques. Of the isolated cultures, I identified one as *Edwardsiella tarda* and another as a species of *Clostridium*. There is a paucity of information regarding the bacterial taxa in the guts of healthy larval anurans. I also determined the enzymatic activity of isolated strains for seven different carbohydrates, to elucidate the hydrolytic roles of these bacterial symbionts. Cellulose was the only carbohydrate tested that was not fermented by any isolated bacterial culture. The gastrointestinal microbiology of amphibians has major ecological, evolutionary, and environmental implications, and yet it remains mostly unexplored and warrants further study.

Key Words.—anaerobic; bacteria; *Clostridium*; *Edwardsiella*; fermentation; gastrointestinal tract; *Rana catesbeiana*, tadpoles

INTRODUCTION

Until recently, the possibility of gastrointestinal microbial fermentation in the Amphibia remained uninvestigated (Bjorndal 1997; Pryor and Bjorndal 2005a, 2005b). The first and only account of microbial fermentative digestion within the guts of amphibians occurred in an herbivorous tadpole (*Rana catesbeiana*; Pryor and Bjorndal 2005a, 2005b) and an herbivorous salamander (*Siren lacertina*; Pryor et al. 2006). *Rana catesbeiana* tadpoles make considerable energetic gains from a fermentative digestive strategy, and they share remarkable similarities with other small-bodied herbivores in regards to gut morphology, complex symbiotic microbial communities, digesta passage, and coprophagous behaviors (Pryor and Bjorndal 2005a, 2005b). This has added significantly to our understanding of digestive processing in herbivorous amphibians.

Descriptions of bacteria collected from herbivorous amphibians, such as ranid tadpoles, are extremely rare in the literature and focus on laboratory-reared individuals (e.g., Gossling et al. 1982). This is unfortunate, considering the demonstrated (Pryor and Bjorndal 2005a, 2005b) and purported (Hecnar 1995; Huey and Beitinger 1980) significance of the gut microbiota on the life history and conservation biology of larval amphibians. Most studies investigating the amphibian gastrointestinal microbiota describe only pathogenic or potentially pathogenic taxa isolated from the feces of captive, adult anurans (e.g., as reviewed in Taylor et al. 2001). Furthermore, there is a paucity of information

regarding the enzymatic properties of mutualistic bacteria collected from the amphibian gut.

I investigated the bacterial flora of the gastrointestinal tracts of wild-caught *Rana catesbeiana* tadpoles under anaerobic conditions. I isolated several strains of bacteria and attempted to identify each strain using a variety of culturing methods, biochemical tests, and differential staining techniques. I also determined the enzymatic properties of isolated strains, to elucidate the roles of some of the bacterial symbionts living within the tadpole gut.

MATERIALS AND METHODS

Rana catesbeiana tadpoles were collected by J. Camper on 9 August 2005 from a small, unnamed pond at the Pee Dee Research and Education Center in Darlington County, South Carolina, USA. The GPS coordinates for the pond, in decimal degrees, are: 34.30879N, -79.74963W. In the lab, I maintained the tadpoles (n = 7) in pond water, in a 37 L aquarium kept under cool white fluorescent lighting (12L:12D) at a water temperature of 29°C. They were fed powdered, alfalfa-based rabbit food (Classic Blend Rabbit Food, L/M Animal Farms, Inc., Pleasant Plain, OH) mixed with flaked fish food *ad libitum* (Wardley Premium Goldfish Flakes, Hartz Mountain Corporation, Secaucus, New Jersey, USA) in a ratio of 80:20. Every other day, I transferred the tadpoles to a freshly-prepared aquarium to control water quality without the use of filters and pumps. Small volumes (5 mL) of benthic substrate that I collected from the original pond were added to each

aquarium at the time of each water change to expose tadpoles to the biotic and abiotic materials that would normally be ingested from the environment. The tadpoles used in this study were kept in captivity for 3-7 days before being euthanized for dissection.

I collected gut samples from tadpoles that I euthanized by double-pithing (i.e., brain and spinal cord). Because the chemical agents commonly used for amphibian euthanasia, such as tricaine methanesulfonate (i.e., MS-222), might have negative effects on the endemic microbes living within the tadpole gut, I pithed the tadpoles instead of using a chemical anesthetic. Pithing has been approved as an acceptable, effective, and humane means of euthanasia when dealing with amphibians (JAVMA 2001). All procedures involving husbandry and euthanasia of tadpoles were approved by the IACUC at Francis Marion University.

To disinfect the incision site before dissection, I first swabbed the ventral skin with 70% ethanol. I dipped all surgical instruments in ethanol and flame-sterilized them before each use. I measured total length (TL) and snout-to-vent length (SVL) to the nearest 0.1 mm using calipers. I measured mass of the body and gut contents on a wet matter (WM) basis to the nearest 0.01 g.

To estimate bacterial densities in different regions of the gut, I made direct microscopic counts. First, I dissected a tadpole (SVL = 31.4 mm; TL = 77.8 mm; body mass = 4.98 g) and separated the entire gastrointestinal tract into five distinct gut regions following the descriptions provided by Pryor and Bjorndal (2005a). I used a serial dilution technique in which the gut contents were diluted in sterilized physiological buffered saline (PBS). I examined aliquots of the diluted gut contents at 1000X using a Bright Line Counting Chamber (Hausser Scientific, Horsham, Pennsylvania, USA) and phase contrast microscopy. I counted the motile and non-motile bacteria in a volume of each sample, and then made the appropriate calculations involving the various dilution factors and sample volumes to estimate bacterial densities in the original samples. I made these counts in triplicate, and calculated a mean for each sample. I also made bacterial counts for water samples and benthic

substrate/food samples that I collected from the maintenance aquarium.

To isolate bacteria and enumerate bacterial densities under the anaerobic conditions encountered in the gut, I dissected another tadpole (SVL = 31.6 mm; TL = 92.8 mm; body mass = 6.77 g) in an anaerobic chamber that I flushed with CO₂. I quickly removed the gastrointestinal tract, and I collected and diluted the contents from the colon in sterile PBS. I selected this gut region based on its distinct anatomical structure, physiological function, and high microbial fermentation rates (Pryor and Bjorndal 2005a, 2005b). I also used the pour plate technique to inoculate samples into Anaerobic Agar (AA) plates that I then incubated in an anaerobic jar for 48h. After incubation, I counted the colony-forming units (CFUs) on the plates and I calculated the bacterial densities of the original colon contents. I selected and transferred colonies from the AA plates that were morphologically distinct to tubes containing thioglycollate broth (THIO). Of these, six colonies were pure and grew readily in THIO. I maintained these bacterial cultures in THIO, and frequently reinoculated them into new tubes and checked them for purity. The methods I used for collection, isolation, and maintenance of anaerobic bacterial colonies were based on Engelkirk et al. (1992).

For each isolated bacterial culture, I described the growth patterns in THIO broth, as well as colony and cell morphology. Following the procedures of Abramoff et al. (2004), I measured cell sizes with photomicroscopy and image analysis software (ImageJ 1.34s, National Institutes of Health, USA). I inoculated each culture onto MacConkey Agar (MAC) and Eosin Methylene Blue Agar (EMB) to screen for members of the Family Enterobacteriaceae (i.e., the enterics). I conducted carbohydrate fermentations using seven different substrates (i.e., lactose, sucrose, glucose, xylose, starch, chitin, and cellulose) in separate tubes of Phenol Red Broth equipped with inverted Durham fermentation tubes. I noted any acid and/or gas production after 96 hrs. Based on results of the differential MAC and EMB agars, as well as subsequent oxidase and catalase tests, I presumed one culture was an enteric and thus performed a series of additional biochemical tests using the Enterotube II rapid identification system (Becton Dickinson Microbiology Systems, Sparks, Maryland, USA).

TABLE 1. Bacterial densities of samples from the *Rana catesbeiana* tadpole gut, examined by direct microscopic count. Densities are presented as colony-forming units (CFUs)/g digesta (on a wet matter basis, WM) for gut contents, and as CFUs/mL for the aqueous aquarium samples.

Sample	Mass of Contents (WM)	Bacterial Densities
manicotto (foregut)	0.024	3.0 X 10 ⁹
anterior small intestine	0.160	9.4 X 10 ⁹
posterior small intestine	0.124	1.1 X 10 ¹⁰
colon	0.068	7.7 X 10 ⁹
rectum	0.013	9.4 X 10 ⁹
aquarium water	n/a	1.7 X 10 ⁶
aquarium benthic substrate	n/a	1.6 X 10 ⁷

RESULTS

I found the greatest numbers of bacteria in the posterior small intestine and the least in the manicotto (i.e., foregut) (Table 1). The numbers of bacteria in the various gut regions were higher than in the aquarium water and a benthic substrate sample collected from the bottom of the aquarium.

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The anaerobic bacteria densities of the colon, as determined by the pour plate technique, was 1.2×10^8 CFU/g WM digesta. This estimate is lower than that obtained from direct microscopic count (7.7×10^9 CFU/g WM), suggesting that not all microbes in this gut region are obligate or facultative anaerobes, that many cells observed in the direct count were dead, or that some species are noncultivable using Anaerobic Agar. Of the six pure cultures that I isolated, one closely matched morphological descriptions for the genus *Clostridium* (i.e., anaerobic, Gram-positive, thin, regular, bacilli with terminal endospores; Holt et al. 1994). Considering these morphological characteristics, and the fact that this culture was catalase-negative, it was likely not a species of *Bacillus* (the only other genus of endospore-forming, Gram-positive rods). Due to concerns about maintaining a potentially pathogenic strain such as *C. tetani* without proper laboratory facilities and security measures, I destroyed the culture via autoclaving, without further analyses.

Cell morphologies, AA growth patterns, and THIO growth patterns of bacteria collected from the tadpole colon are summarized in Table 2. The cell sizes of these bacteria are shown in Table 3. Whereas two of the cultures (Cultures 1 and 2) grew well on MAC and EMB agar plates, none of the other cultures grew on these media. I presumed that Culture 1 was an enteric (i.e., Family Enterobacteriaceae) because of the appropriate color changes I observed in the media near the colonies. In subsequent tests, Culture 1 was oxidase-negative and catalase-positive, further suggesting an enteric. In a series of additional biochemical tests, glucose was fermented with acid and gas production; lysine decarboxylase and ornithine decarboxylase enzymes were produced; and hydrogen sulfide and indole production was evident. Adonitol, lactose, arabinose, sorbitol, and dulcitol were not fermented; phenylalanine deaminase and urease enzymes were not produced; citrate was not metabolized; and the Voges-Proskauer test was negative. Based on these characteristics, I identified Culture 1 as *Edwardsiella tarda* (i.e., after the morphological and biochemical test results described by Holt et al. 1994).

Carbohydrate fermentation test results involving pure cultures of bacteria collected from the tadpole colon are

TABLE 2. Cell morphology, Anaerobic Agar (AA) growth patterns, and Thioglycollate (THIO) broth growth patterns of bacteria collected from the *Rana catesbeiana* tadpole colon.

	Culture ID				
	1	2	3	4	5
Cell morphology ¹	G- CB Sm	G- CB Med	G-/Gv Sq/Vac Lg	G-/Gv Sq/Vac Lg	G- R Var
AA growth ²	Lg Sp CW Tr Ro	Lg Sp CW Tr S	Lg Sp Tr	Sm CW Op	Sm/Med Sp Tr
THIO growth ³	FA G	FA NG	FA G	FA G	FA G

¹ Cell morphology codes: G- (Gram-negative), Gv (Gram variable), CB (coccobacilli), Sq (squared ends), Vac (vacuolated or irregular stain retention), R (rounded ends), Sm (small cells, around 1 µm), Med (medium cells, 2-6 µm), Lg (large cells, generally >6 µm), Var (variable cell size; some short, some very long and coiled/twisted)

² AA growth pattern codes: Sm (small colony diameter, <3mm), Med (medium colony, 3-10mm), Lg (large colony, >10mm), Sp (spreading), CW (creamy white color), Tr (translucent), Op (opaque), Ro (rough texture), S (smooth texture)

³ THIO growth pattern codes: FA (facultative anaerobe), G (gas production), NG (no gas)

summarized in Table 4. Cellulose was the only carbohydrate tested that was not fermented by any isolated bacterial culture.

DISCUSSION

The bacterial densities of the tadpole gut described here fall within the range of densities reported for other animals. For example, Stevens and Hume (1997) report densities ranging from 10^6 - 10^{12} bacteria/g wet mass (g WM) of intestinal contents in mammals and birds, 10^{10} bacteria/g WM in two reptiles (i.e., Green Sea Turtle, *Chelonia mydas*, and Green Iguana, *Iguana iguana*), and 10^9 bacteria/g WM in an amphibian (i.e., Leopard Frog, *Rana pipiens*). From wild-caught, herbivorous *I. iguana*, McBee and McBee (1982) made colony counts of 3.3×10^9 to 23.5×10^9 CFUs/g WM, and direct microscopic counts of 23.5×10^9 cells/g WM. Pinn et al. (1997) described bacterial densities of 4.4×10^9 CFUs/g WM in the intestines of the plant- and detritus-feeding marine thalassinidean mud shrimp, *Upogebia stellata*. In the large intestine of humans -- arguably the best-studied

TABLE 3. Means, standard deviations, and ranges of cell sizes (µm) of bacteria collected from the *Rana catesbeiana* tadpole colon. For each culture, 10 cells were measured.

	Culture ID				
	1	2	3	4	5
Length: mean ± SD	1.3 ± 0.3	1.6 ± 0.4	3.9 ± 1.1	3.9 ± 1.1	12.7 ± 10.9
Length: range	0.8 - 1.7	1.0 - 2.1	2.8 - 5.3	2.3 - 5.9	3.7 - 41.5
Width: mean ± SD	0.6 ± 0.2	0.7 ± 0.1	1.1 ± 0.2	1.0 ± 0.1	1.9 ± 0.5
Width: range	0.4 - 0.8	0.6 - 0.8	0.8 - 1.4	0.9 - 1.2	1.1 - 2.5

TABLE 4. Carbohydrate fermentation test results involving bacteria collected from the *Rana catesbeiana* tadpole colon. Codes: acid and gas production (A / G); acid but no gas production (A / -); no acid or gas production (- / -).

Carbohydrate	Culture ID				
	1	2	3	4	5
Lactose	- / -	A / -	- / -	- / -	- / -
Sucrose	- / -	A / -	- / -	- / -	- / -
Glucose	A / G	A / -	A / G	- / -	- / -
Xylose	A / -	- / -	A / -	A / -	A / G
Starch	A / -	A / -	- / -	- / -	- / -
Chitin	A / -	- / -	- / -	A / -	- / -
Cellulose	- / -	- / -	- / -	- / -	- / -

gastrointestinal tract in the animal kingdom -- there are 10^8 facultative anaerobes/g WM and 10^{11} obligate anaerobes/g WM (Wilson 2005). Using direct microscopic counts from laboratory-reared *R. pipiens* tadpoles, Gossling et al. (1982) reported 7.9×10^7 to 6.3×10^9 bacteria/g WM of midgut contents.

My observation of *Edwardsiella tarda* and a *Clostridium* species within the guts of *R. catesbeiana* tadpoles is a new account, but it is not surprising. *E. tarda* is a bacterium in the Family Enterobacteriaceae that is commonly found in the intestines of freshwater fishes (Holt et al. 1994). It is an opportunistic pathogen in some species, including humans (Janda et al. 1991; Van Damme and Vandepitte 1980; Vandepitte et al. 1983). Apparently *E. tarda* has been isolated “from the intestinal contents of healthy amphibians” (Taylor et al. 2001), although the authors provided no reference to the literature for this statement, and the amphibian host species were not described.

Similarly, various species of *Clostridium* are commonly isolated from the gastrointestinal tracts of healthy individuals, yet they can be pathogenic when they produce extracellular toxins and enterotoxins (Wilson 2005). An unidentified species of *Clostridium* has been described as a dominant member of the gut microbiota of *I. iguana* (McBee and McBee 1982), and at least eight species of *Clostridium* are routinely collected from the human gastrointestinal tract (Wilson 2005). Although the nonpathogenic roles of *E. tarda* and *Clostridium* in the gut remain enigmatic, they are apparently part of the normal bacterial flora of healthy tadpoles.

None of the other bacterial strains that I isolated could be positively identified to a generic or specific taxonomic level with the biochemical tests I used in this study. It is possible that some of these strains are the same as the ten species of Enterobacteriaceae isolated from laboratory-reared *R. pipiens* tadpole guts by Hird et al. (1983), but this remains unconfirmed because those researchers employed different sampling and isolation techniques (involving different culture media and enrichment procedures), and a different rapid

identification test system for the identification of enterics (i.e., the API 20E system).

One of the isolated strains (Culture 5) in this study exhibited highly variable cell morphology; some cells were shorter than $4\mu\text{m}$ whereas others were longer than $41\mu\text{m}$, and some were straight whereas others were coiled and twisted (Tables 2 and 3). This pattern held true, even after I made special attention to ensure the culture was pure. Highly variable cell shape, including relatively long bacilli, is observed in a variety of Gram negative, facultative anaerobes such as *Flexibacter* and *Pectinatus* (Holt et al. 1994). The former is a common bacterium in freshwater ecosystems, and the latter is often isolated from samples collected from breweries and wastewater treatment facilities. Narrowing down the identification of these bacteria might seem straightforward based on their unusual cell morphology. However, definitive identification would necessitate a variety of specialized tests, including genetic analyses (Holt et al. 1994).

Regardless of taxonomic designation of bacterial strains isolated in this study, the enzymatic properties of these isolates are nonetheless insightful. Of the carbohydrates I tested in fermentation assays, only cellulose could not be digested by any of the isolated strains. Cellulose is a structural carbohydrate in the plant matter ingested by herbivorous tadpoles, and thus, an inability to digest cellulose would seem disadvantageous. However, other strains of bacteria in the tadpole gut might hydrolyze cellulose and other refractory structural polysaccharides in plants. Alternatively, tadpoles might benefit from the activity of cellulolytic microbes in the environment (e.g., *Cellulomonas*), which act upon the cellulose in decaying plant matter before ingestion. Indeed, most of the discernable plant matter in the anterior gut regions of wild-caught *R. catesbeiana* appears partially disintegrated and detrital, and in captivity these tadpoles do not ingest significant quantities of live plants such as *Elodea*, *Lemna*, or *Najas* (pers. obs.). Evidently, the possibility of cellulose digestion by microbes in the guts of herbivorous tadpoles merits further investigation.

I also isolated chitin-digesting bacteria from the tadpoles, which seems advantageous because the foreguts of wild-caught tadpoles regularly contain the chitinous exoskeletons of aquatic invertebrates (pers. obs.). Furthermore, because adult anurans are highly insectivorous, any chitinolytic bacteria retained through metamorphosis would confer obvious digestive advantages to the frogs. The ability of tadpole gut bacteria to hydrolyze the sugars and starch in the fermentation tubes is also understandable, considering the ease with which most animals digest these carbohydrates (Stevens and Hume 1997).

The roles of the gastrointestinal microbiota in amphibians have profound ecological, evolutionary, and

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environmental implications and yet they remain, for the most part, unexplored. For example, Pryor and Bjorndal (2005a, 2005b) demonstrated important energetic gains made by tadpoles from a fermentative microbial digestion within their guts. These gains, in turn, are expected to be directly proportional to metamorphic size and/or timing. In the context of conservation biology, Huey and Beitinger (1980) and Hecnar (1995) speculated that the ailments they observed in nitrate-exposed tadpoles were related to, or caused by, a disturbance in the normal gut microbiota. Nitrate pollution of the aquatic environment is extensive and pervasive, and may represent a major threat to amphibian populations (Rouse et al. 1999; Edwards et al. 2006; Burgett et al. 2007). More research is needed on the potential effects of aquatic contaminants on the gastrointestinal microbiota, and thus, the digestive function of amphibians.

In conclusion, our understanding of normal and abnormal conditions of the gastrointestinal microbiota in amphibians, and what factors affect the microbiota, remains vague at best. Clearly, these topics warrant further microbiological study and promise to change the way we think about amphibian health in captivity and in the natural environment.

Acknowledgments.—I thank Everard Armstrong and Jeff Camper for their assistance with tadpole collection and care, and Tamatha Barbeau and Larry McCumber for reviewing early drafts of this manuscript. All experiments were conducted with approval by the Francis Marion University IACUC (Protocol #010, 2005).

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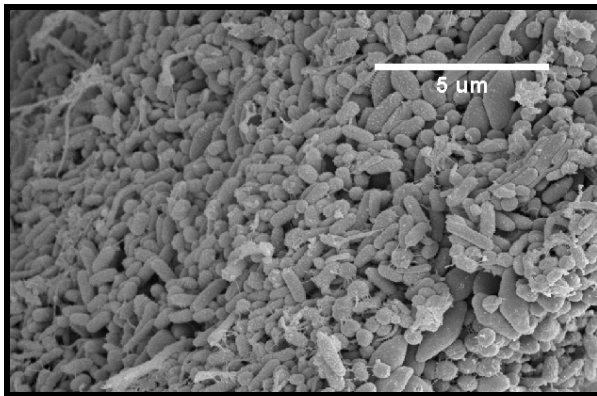
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ABOVE: Bacteria in the Bullfrog (*Rana catesbeiana*) tadpole gut. SEM by Greg Pryor.



ABOVE: Bullfrog tadpole (*Rana catesbeiana*). Photographed by Greg Pryor.