MOLECULAR IDENTIFICATION OF GASTROINTESTINAL BACTERIA FROM COPE’S GREY TREEFROG

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ABSTRACT

The objective of this research was to utilize culture-independent methods to identify bacteria in the gastrointestinal tract of adult Cope’s grey treefrogs, *Hyla chrysoscelis*. Five adult *H. chrysoscelis* were collected from a single site in Jacksonville, AL (USA). Gastrointestinal tracts were removed from frogs, and genomic DNA was extracted from these samples. 16S ribosomal RNA genes were amplified using universal eubacterial primers, and PCR products were cloned and sequenced. Sequence analysis and phylogenetic construction were performed using MEGA 4.0 software. After phylogenetic analysis, 98% of the sequences were classified into one of four phyla—Bacteroidetes, Firmicutes, Proteobacteria, and Verrucomicrobia. Relative genera could not be determined phylogenetically, though clones did group into relative families, including Bacteroidaceae (27%), Porphyromonadaceae (23%), Clostridiaceae (22%), Enterobacteriaceae (12%), Lachnospiraceae (8%), Verrucomicrobiaceae (4%), Aeromonadaceae (2%), and unknown Clostridiales (2%).

INTRODUCTION

Cope’s grey treefrogs (*Hyla chrysoscelis*), an anuran species belonging to the Hylidae family, range over much of the eastern United States (Deuchar, E. M. 1966, Pryor, G. et al. 2005, Stebbins, R.C. 1995). During the daytime, individual adults can be found in treetops (often utilizing tree cavities) or on lichen-covered objects above ground. Tadpoles spend their time in ponds and have dramatically different diets from the adults. Tadpoles filter food from the water column and scrape periphyton from submerged surfaces. Adults have been observed feeding on insects, including flies, moths, beetles, roaches, and crickets (Deuchar, E. M. 1966).

The intestinal tract is responsible for food digestion and nutrient assimilation; it also promotes proper immune function (Pryor, G. and K. A. Bjorndal 2005). In the intestines of mammals and birds, the organization of luminal folds includes crypts within the circular folds that further increase surface area, but these crypts are not present in amphibian intestinal tracts. During the anuran larval stage, the intestines are long but lack the complexity of the adult intestinal tract. Only one epithelial fold (consisting of columnar epithelial cells with microvilli), known as the typhlosole, is present, where an abundance of larval connective tissue is located. During postmetamorphosis, the intestinal tract begins to shorten and the amounts of both connective tissue and smooth muscle increase. Larval epithelial cells (primary epithelium) begin apoptosis, and undifferentiated epithelial cells proliferate and differentiate into adult epithelial cells (secondary epithelium), forming circular folds and villi throughout the tract. The microvilli on the secondary epithelium are shorter than those found on primary epithelium. These structural
changes suggest that changes of intestinal physiology may accompany the transformation from being primarily herbivorous tadpole to carnivorous/insectivorous adult frog (Gossling, J. et al. 1982; Tsukinowa, E. et al. 2008). Changes in intestinal microflora may also be associated with these changes.

Attempts to determine bacterial diversity using culture-dependent techniques have notoriously underestimated actual diversity (Akondi and Lakshmi, 2013). Culture-independent techniques include temperature/denaturing gradient gel electrophoresis (TGGE/DGGE), fluorescent in situ hybridization (FISH), dot blot, and 16S rDNA clone libraries (Pryor, G., 2008). The objectives of this research were to use molecular techniques to sequence 16S DNA in an attempt to identify intestinal microflora and establish a baseline microflora from adult *H. chrysoscelis*.

**MATERIALS AND METHODS**

**Sample collection, euthanasia, and dissection**

Five adult male *Hyla chrysoscelis* were collected from an ephemeral pool at Henry Farm Park in Jacksonville, Alabama (33.7827, -85.7682). Frogs were anesthetized in a CO₂-rich solution (1 g calcium carbonate and 1 g citric acid in water) and euthanized by freezing at -70°C (AVMA guidelines, 2007). Stomach and intestinal tissues were removed from each individual and weighed. Each tract was slit longitudinally and transferred to a 2 ml screw-cap tube containing 1 ml 1X phosphate-buffered saline (PBS) and 0.5 g sterile ceramic beads (MO BIO Laboratories, Inc., Carlsbad, CA). Tissue was homogenized by vortexing for 5 min at maximum speed, centrifuged at 700 x g for 30 s, and transferred to a fresh 2 ml screw-cap tube. Two hundred fifty microliters (μL) of each suspension was transferred to a single 2.0 ml screw-cap tube. This pooled sample was used for DNA extraction. Remaining tissue homogenate was stored in a -20°C freezer.

**DNA preparation and clone library construction.**

Genomic DNA was extracted from 4-250 μL samples using the PowerSoil DNA Isolation Kit per manufacturer’s protocol (MO BIO Laboratories, Inc., Carlsbad, CA). An additional incubation step of 70°C for 10 min was included for cells that were difficult to lyse. DNA quantities and relative qualities were checked for each sample using GenQuant pro spectrophotometer. 16S rRNA gene sequences were amplified for each of the four samples, as well as a pooled DNA sample, using universal eubacterial 16S rRNA gene primers: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). Twenty-five μl PCR reactions were set up using SmartMix HM PCR beads (Cepheid, Catalog #SMHM1-150N-040). Forty-six μl sterile water and 1 μl of each primer [200 nM] were added to a PCR bead, mixed, and divided into two reactions. One μL of template was added to each reaction. PCR conditions were optimized to include a reduced number of cycles to minimize bias of bacterial species in high abundance in the samples. Conditions used for 16S rDNA amplification included initial denaturation at 95°C for 5 min; 20 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min; and a final extension period at 72°C for 7 min. Amplified products were observed visually on 1% agarose gel in 0.5X TAE buffer.

PCR products were purified using UltraClean PCR Clean-Up Kit (MO BIO Laboratories, Inc., Carlsbad, CA) cloned into pCR2.1-TOPO, and chemically transformed into *Escherichia*
coli DH5α-T1 competent cells using the TOPO TA Cloning Kit (Invitrogen, Catalog #K4520-01). Competent cells were plated on Luria-Bertani (LB) agar containing 50 µg/mL kanamycin and 40 µg/mL X-Gal (5-bromo-4-chloro-3-indoly-β-D-galactoside) and incubated 20 hours at 37°C. Blue/white screening was used for selection of positive transformation clones, and a clone library was constructed by randomly selecting 150 white colonies and replating the colonies on fresh LB plates containing 100 µg/mL ampicillin, 50 µg/mL kanamycin, and 40 µg/mL X-Gal. Each clone was grown in 500 µL LB broth containing 50 µl /mL kanamycin at 37°C for 20 hours. An equal volume of sterile glycerol was added to each clone. Clones were stored at -70°C.

**Sequencing and phylogenetic analysis.**

Each clone was grown in 3 mL LB broth containing 50 µg/mL kanamycin. Plasmids were extracted from each suspension using the E.Z.N.A. Endo-free Plasmid Isolation System (Omega Bio-Tek Inc., Norcross, GA) as per protocol. Plasmid concentrations were determined using GeneQuant pro (Amersham Biosciences Inc.). Reactions were prepared for sequencing according to Beckman Coulter GenomeLab™ Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter Inc., Brea, CA). Ten µL sequencing reactions (half-reactions) consisted of 4 µL DTCS quick start master mix, 1.0 µL respective primer [1.6nM], 115 ng purified plasmid DNA, and sterile water. After sequencing amplification, sterile water was used to bring the reaction volume to 20 µL. Two sequencing reactions were set up per sample with each primer (27F and 1492R) to identify 16S gene sequence data for each clone.

Sequences were analyzed using Molecular Evolutionary Genetic Analysis (MEGA) 4.0 software. Select 16S rDNA sequences from the NCBI BLAST Database with percent identities >97% to *Hyla chrysoscelis* adult (HCA) clones were included in alignment and phylogenetic tree analysis. Sequenced clones with percent identities of 97% were considered to represent the same genera found in GenBank, while those with ≥98% identity were considered to represent the same species. HCA sequences and GenBank matches were aligned with ClustalW. After alignment, sequences were trimmed to 530 bp (5’ end) and 420 bp (3’ end) to eliminate bias when calculating percent similarity for rarefaction and phylogenetic analysis. Phylogenetic analysis was performed using bootstrap maximum parsimony (n=1000 replicates). Species diversity and complete coverage were determined using Analytic Rarefaction 1.3 from the University of Georgia-Athens Stratigraphy Lab. Sequences determined from this study were published in the GenBank database.

**RESULTS**

Fifty 16S rDNA clones from the gastrointestinal tracts of *H. chrysoscelis* adults were sequenced with both 27F and 1492R universal primers, resulting in 950 bp partial sequences after alignment. Percent identities observed between *H. chrysoscelis* intestinal sequences and sequences in GenBank ranged from 90 to 100%. Based on sequence homology, 54% of the clones grouped into one of four phyla—Proteobacteria, Firmicutes, Bacteroidetes, and Verrucomicrobia, with the remaining 46% relating to uncultured environmental samples. Organisms within the four phyla were further divided into the following genera: Citrobacter (22%), Enterobacter (19%), Parabacteroides (11%), Bacteroides (15%), Clostridia (7%), Akkermansia (7%), Aeromonas (4%), Robinsoniella (4%), Dorea (4%), and unknown
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Clostridiales (7%) (Fig 1). One Firmicute could not be identified at the genus level, but was reported as a member of the family Clostridiaceae.

Figure 1. A graphical representation of *Hyla chrysoscelis* intestinal sample 16S rDNA sequence results grouped by phyla (A) and genera (B) based on percent identities with GenBank sequences of ≥90% (phyla) and ≥97% (genera). Only sequences grouping into identifiable phyla could be separated by genera.

Percent similarities were determined between each *H. chrysoscelis* intestinal sequence. Sequences with similarities of ≥98% were grouped together, and a representative sequence for each of the twenty-eight groups was used for phylogenetic analysis. GenBank sequences (excluding uncultured bacteria) having percent identities ≥97% to the intestinal samples from *H. chrysoscelis* were included in the analysis. Based on the topology of a consensus bootstrap maximum parsimony tree at 1000 replicates (data not shown), 2% of the clones could not be classified into a specific phylum, but the remaining 98% were among Firmicutes (32%), Proteobacteria (14%), Bacteroidetes (48%), and Verrucomicrobia (4%). Genera could not be predicted, but many organisms within the phyla could be classified into one of seven families: phylum Firmicutes—Clostridiaceae (22%) and Lachnospiraceae (8%), phylum Proteobacteria—Enterobacteriaceae (12%) and Aeromonadaceae (4%), phylum Bacteroidetes—Bacteroidaceae (27%) and Porphyromonadaceae (23%), and phylum Verrucomicrobia—Verrucomicrobiaceae (4%) (Fig. 2). One Firmicute sequence (HCA-1045) was not associated with a particular family and was termed “unknown Clostridiaceae.”

Complete coverage was determined with rarefaction. Rarefaction data were calculated to determine the point where additional sequencing would produce no statistically significant increase in the number of families present in the sample. Eight non-redundant bacterial 16S rDNA sequences were identified in this study and published in the NCBI database (HM060246, HM060247, HM060248, HM060249, HM060250, HM060251, HM060252, and HM060253).
Figure 2. A graphical representation of *Hyla chrysoscelis* intestinal sample 16S rDNA sequence results grouped by phyla (A) and family (B) based on phylogenetic analysis. Sequences within the unknown phyla could not be classified.

**DISCUSSION**

Previous studies of intestinal microflora in anurans have been performed using electron microscopy for bacterial morphology and culture-dependent techniques (e.g., selective media). This study has produced results similar to many previous studies in that the genera Bacteroides and Clostridium were two of the most common intestinal isolates of leopard frogs (Banas, J. et al. 1988) and are members of the two major groups of organisms detected in vertebrate digestive systems using 16S rDNA sequencing—the Cytophaga-Flavobacter-Bacteroides (CFB) and Firmicutes groups (Pryor, G. 2008). Similar 16S rDNA gene studies of the digestive tract of dugongs (*Dugong dugong*) also showed similar results, with primary phyla including Firmicutes, Bacteroidetes, Actinobacteria, Lentisphaerae, Proteobacteria, and Verrucomicrobia (Tsukinowa, E. et al. 2008). Forty-eight percent of the sequences from this study grouped phylogenetically within the phylum Bacteroidetes and represented the largest portion of the adult *H. chrysoscelis* intestinal components. Several *H. chrysoscelis* adult intestinal sequences showed homology with organisms within the Bacteroidaceae family, including *Bacteroides fragilis*, *B. ovatus*, *B. intestinalis*, and *B. clarus* at homologies of 97%, 99%, 99%, and 97%, respectively. Several genes have been discovered in *B. fragilis* that are involved in the acquisition of complex carbohydrates (Choat, J.H. and K.D. Clements, 1998), indicating that homologous organisms in *H. chrysoscelis* digestive tract may have the ability to metabolize chitin—the primary component of insect exoskeletons. This species also has multiple mechanisms to change cell surface markers and enhance colonization of the intestinal tract due to surface fucosylation; both functions may be involved in preventing the immune system from attacking the bacteria (Pryor, G. 2008).

The second largest group of taxa were from the phylum Firmicutes and represented 32% of the sequences from *H. chrysoscelis* intestinal samples. *Clostridium ramosum* and
**Robinsoniella peoriensis** were 99% and 97% (respectively) similar to three sequences. One sequence (HCA-1056) was 97% homologous to a *Dorea spp.* Organisms within Firmicutes are obligate anaerobes and include numerous butyrate producers, whose presence is thought to improve health since colonocytes use butyrate as a primary energy source. Some organisms within this phylum have the ability to utilize N-acetylglucosaminoglycans, a product of chitin metabolism which enhances growth of *Bacteroides spp.* and Firmicute organisms (Choat, J.H. and K.D. Clements, 1998).

Proteobacteria and Verrucomicrobia are often observed as minor groups within vertebrate intestinal tracts, along with members of Bifidobacteria, lactic acid bacteria, Fusobacteria, Actinobacteria, Spirochetes, Deinococcus, and Cyanobacteria (Pryor, G. 2008). Proteobacteria represented 14% of cloned 16S rRNA genes produced from the *H. chrysoscelis* samples, and 4% represented Verrucomicrobia. Many Proteobacteria are known to metabolize chitin; one specific organism of interest is *Aeromonas hydrophilia*, which showed 99% homology to one sequence from the reported *H. chrysoscelis* samples. *A. hydrophilia* uses chitin as a source of carbon and nitrogen and produces all enzymes necessary for chitin degradation (Cline, G.R. 2005). Both sequences within the phylum Verrucomicrobia were 99% similar to *Akkermansia muciniphila*, a common mucin-degrading colonic bacterium. *A. muciniphila* is an obligate anaerobe, and mucin is its primary source of carbon and nitrogen (Forney, L. J. et al. 2004).

Minor intestinal groups of bacteria previously described by Manson, et al. primarily utilized culture-dependent techniques, which favor identification of aerobic organisms over anaerobes (Pryor, G. 2008). Using molecular techniques allow for identification of both anaerobic and aerobic organisms, which may shift the proportions of minor bacterial groupings described. Previously mentioned genera may not have been identified within the *H. chrysoscelis* intestinal tract because populations are smaller than expected for some bacteria and larger for other minor groups—such as the Proteobacteria and Verrucomicrobia.

Studies of tadpole and adult frog microflora have shown more anaerobic organisms growing in culture than aerobic organisms (Halliday, T. 2005). One study reported finding much higher percentages of anaerobic bacteria (64.8-85.7%) in the intestinal lining and contents of nonhibernating leopard frogs than aerobic bacteria (14.9-35.3%) when incubated at 25°C, but when incubated at 4°C, the populations shifted toward aerobic bacteria (*Bacillus*, *Streptococcus*, *Enterococcus*, *Vibrio* groups, etc.) were predominant with anaerobic *Clostridium*, *Eubacterium*, and *Bifidobacterium* making up the majority (12.2-29.5%) of the anaerobic population (Banas, J.A. et al. 1988). The bacteria identified in the intestinal flora of *H. chrysoscelis* are comparable to these studies, with the suggestion that more anaerobic organisms were found than aerobic organisms. Many of the identified phyla, including Bacteroidetes, Firmicutes, and Verrucomicrobia, are primarily obligate anaerobes. Proteobacteria contain both facultative and obligate anaerobes. Since many of the *H. chrysoscelis* intestinal sequences could not be classified to the species level, ratios of anaerobic to aerobic bacterial populations could not be accurately assessed, but it is probable that there is a higher ratio of anaerobic to aerobic bacteria. Hird, D.W. et al. (1983) studied the occurrence of members of the family Enterobacteriaceae and *Aeromonas hydrophilia* in the intestinal tract of leopard frogs (*Rana pipiens*) by using a series of biochemical tests. He found that the most common intestinal isolates in adult frogs were *Citrobacter freundii* and *A. hydrophilia* (both found in 46% of 222 adult frogs) (Manson, J.M. et al., 2008). Six clones showed 100% similarity to an uncultured *Citrobacter spp.*, and one sequence (HCA-1033) showed 99% identity to *A. hydrophilia*, one of several causative agents of red-leg disease in many amphibians and reptiles (Mason, K.L. et al. 2008). *A. hydrophilia* is a
normal inhabitant of adult frog intestinal flora but is an opportunistic pathogen, causing disease when not within the intestinal tract (Manson, J.M. et al. 2008). Some authors have suggested that viral or fungal infections may expose amphibians to secondary infections by opportunistic pathogens with devastating effect (Holland, S. 2008; Viertel, B. and S. Richter, 1999). It is possible those opportunistic pathogens in the intestinal tract, such as *A. hydrophilia* and *B. fragilis*, cause secondary infections, resulting in decreased health and increased morbidity of infected frogs.

Results from this study support previous studies which reported large populations of Bacteroidetes (including the families Bacteroidaceae and Porphyromonadaceae) and Firmicutes (including the families Clostridiaceae and Lachnospiraceae) present within the digestive tract, as well as organisms from the phyla Proteobacteria (including the families Enterobacteriaceae and Aeromonadaceae) and Verrucomicrobia. Since no information has been documented on the physiological effects of intestinal bacteria on nutrient assimilation in adult *H. chrysoscelis*, identification of the bacteria will provide insight into these physiological processes. The intestinal tract is one of the more complex transformations during metamorphosis and diets change pre- and post-metamorphosis; therefore, it would be interesting to compare the results of adult intestinal flora to the intestinal flora of *H. chrysoscelis* tadpoles. Recent studies have displayed that enteric microbiota play important roles in dimorphic sex differences in hormone metabolism in humans, particularly as it relates to estrogen metabolism (Flores et al. 2012). Therefore, comparisons of additional adult *H. chrysoscelis*, including female specimens and organisms from different collection sites, would give a better insight on the overall intestinal flora of this species.

**LITERATURE CITED**


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