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No evidence for an endosymbiotic bacterial origin of tetrodotoxin in the newt *Taricha granulosa*

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Abstract

Tetrodotoxin (TTX) is a potent neurotoxin which is known to occur in numerous taxa, including newts. The origin of TTX is unknown, but production by symbiotic bacteria is suspected for some groups. Using PCR primers that specifically amplify 16S rRNA genes of bacteria, we examined tissues from rough-skin newts, *Taricha granulosa*, for the presence of bacteria which may produce TTX. No amplification of bacterial DNA was seen in samples taken from skin, liver, gonads or oviposited eggs-tissues known to contain TTX. Amplification of bacterial DNA was seen only in samples taken from newt intestines, a tissue with low concentrations of TTX. These results indicate that symbiotic bacteria are unlikely to be the source of TTX in newts.

Keywords: Bacteria; Newt; PCR; Salamandridae; *Taricha granulosa*; Tetrodotoxin

1. Introduction

Tetrodotoxin (TTX) is a low-molecular weight compound that blocks sodium channels and thus inhibits the propagation of action potentials in nerve and muscle cells. This highly potent neurotoxin is found in a diverse array of organisms, including bacteria, dinoflagellates, arthropods, nematodes, mollusks, fish, and amphibians (reviewed by Miyazawa and Noguchi, 2001). TTX has also been identified in sediments from marine (Do et al., 1990; Kogure et al., 1988) and freshwater environments (Do et al., 1993).

Despite the phylogenetic diversity of its occurrence in nature, little is known about the biosynthesis or ultimate origin of TTX. One hypothesis is that TTX is derived from elements of the diet in some taxa (Yasumoto and Yotsu-Yamashita, 1996). In frogs of the genus *Atelopus*, captive-raised individuals do not posses TTX, suggesting a dietary or other environmentally dependent origin of toxicity (Daly et al., 1997). Other types of toxins, primarily alkaloids, found in some amphibians are also sequestered from dietary sources (Daly, 1995; Daly et al., 2002).

Several genera of bacteria have been identified as TTX producers (Simidu et al., 1987; Tiecco et al., 1996; Yasumoto et al., 1986), although these findings are somewhat controversial (Kim and Kim, 2001; Matsumura, 1995; Matsumura, 2001). These potential TTX-producing bacteria are primarily marine (Do et al., 1990; Kogure et al., 1988; Ritchie et al., 2000; Simidu et al., 1987), although a few freshwater species have also been identified (Do et al., 1993). Further, a number of TTX-producing bacteria have been cultured from the digestive tracts of organisms possessing TTX (Cheng et al., 1995; Noguchi et al., 1987; Noguchi et al., 1986), especially puffer fish. Bacteria were presumed to be a source of TTX in some animal species because some intestinal bacteria were found to produce TTX in puffer fish (*Takifugu niphobles*, Matsui et al., 1989), and toxicity could be restored in cultured puffer fish individuals by providing them with a dietary source of TTX-producing bacteria (*Takifugu rubripes* and

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T. niphobles, Matsui et al., 1990). Confounding this apparent exogenous TTX source in puffer fish, increases in TTX concentration through development of artificially fertilized eggs indicates an endogenous origin of TTX in at least one species of puffer fish (*Fugu niphobles*, Matsumura, 1998).

TTX plays an apparent defensive role against predators in many species possessing it, from the expulsion of toxin from specialized skin glands in puffer fish (Kodama et al., 1986) to the potentially deadly bite of the blue-ringed octopus (*Hapalochlaena maculosa*, Flachsenberger, 1986; Sheumack et al., 1978; Williamson, 1987). The antipredator function of TTX has been particularly well studied in the newt *Taricha granulosa* (Brodie, 1968; Brodie et al., 1974; Daly et al., 1987), which is known to be lethal to virtually all potential predators (Brodie, 1968). The only known predators able to survive TTX ingestion are garter snakes of the genus *Thamnophis* (Brodie and Brodie, 1990, 1991), which appear to have evolved resistance to TTX in response to newt toxicity (Brodie et al., 2002, 2004; Geffeney et al., 2002).

Although TTX is a major force in this coevolutionary interaction, its origin in newt tissues is unknown. Determining the source of TTX in newts is critical to interpreting coevolutionary predator–prey dynamics of these two species. If bacteria are capable of producing TTX and do so in newts, the evolutionary implications are much different than if TTX is produced endogenously. Here we show that a symbiotic bacterial origin of TTX in newts is unlikely. Using bacteria-specific PCR primers, bacterial genes coding for 16S rRNA were found only in samples taken from the intestines of newts and not in skin, liver, gonads or eggs.

2. Materials and methods

2.1. Tissue collection

Tissue samples were obtained from the skin, gonads, liver and intestines of 17 newts recently collected (within 20 days of capture) from Benton County, Oregon, and from the skin and gonads of two long-term captive newts. These tissues were selected because they are known to have high concentrations of TTX (Wakely et al., 1966). Newt eggs, which are also known to have high concentrations of TTX (Hanifin et al., 2003), were collected within 24 h of oviposition from four of the Benton females who spontaneously began laying several days after collection. Newts were collected from Benton County, Oregon, because newts from this area are known to be highly toxic (Hanifin et al., 1999). All tissue samples were surface sterilized by submersion in 99% ethanol to remove bacteria on the skin surface, which could represent contamination from an external source.

2.2. Nucleic acid extraction

Total nucleic acid content of all tissues was extracted using a DNeasy® tissue kit (Qiagen, Inc.), following the protocol for animal tissues.

2.3. PCR

Amplification of bacterial 16S rRNA genes was performed using two bacterium-specific PCR primers (63f and 1387r, Marchesi et al., 1998). These primers selectively amplify a portion of the 16S rRNA gene from bacterial DNA that is present in a sample, but do not amplify mitochondrial genes from other types of DNA (i.e. newt DNA in our case). These primers have been used to successfully amplify sequences from and identify bacteria present in complex environments and samples, including endosymbionts of arthropods (Grindle et al., 2003; Hunter et al., 2003; Stevenson et al., 2003; Wobus et al., 2003). If bacteria are present in a sample, a specific band of approximately 1300 base pairs (the amplicon) will appear when the PCR product is separated on an agarose gel and visualized with ethidium bromide. PCR was performed on 5 μl each of the newt extracts (average amount of DNA per reaction was 410 ng in skin samples, 1905 ng in liver samples, 474 ng in gonad samples and 1483 ng in intestine samples). The PCR was run for 35 cycles of (a) 92 °C for 1 min, (b) 55 °C for 1 min, (c) 72 °C for 2 min, and a final extension at 72 °C for 20 min.

Several types of controls were used with the PCR to help interpret the PCR results. A positive control reaction containing known bacterial DNA was run simultaneously with reactions containing newt tissue extracts to positively identify the appropriate band location on a gel. Similarly, a negative control reaction lacking a DNA template was run simultaneously with reactions containing templates. This blank control reaction enabled identification of contamination in the reactions if a PCR product was present. Additionally, to ensure that no elements of the newt genome interfered with the PCR amplification of bacterial DNA, bacterial DNA was added to newt tissue extracts and these mixed samples were used for PCR (2.5 μl newt, 2.5 μl bacterial DNA). Amplification of bacterial DNA in these mixed reactions would indicate that any bacterial nucleic acids present in the newt samples should amplify. Furthermore, to determine at what amount of bacterial DNA is needed to visualize its amplification, serial dilutions of known bacterial DNA were made until the DNA concentration of two successive solutions was undetectable using a microplate reader (SpectraMax 190, Molecular Devices). These dilutions were then used for PCR, in both bacteria-only and mixed (newt and bacteria) samples. The amount of DNA used in the PCR reaction was then calculated.

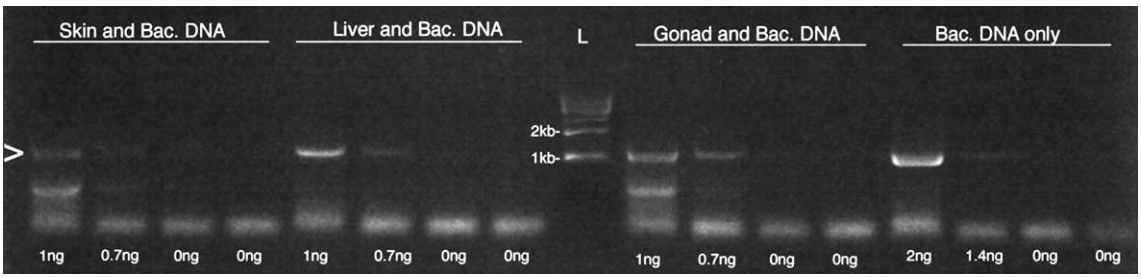

Fig. 1. Product of PCR with bacteria-specific primers using mixed newt tissue extracts and known bacteria samples as templates in each reaction, as visualized on a 1% agarose gel. The amount of bacterial DNA used in the reaction is given below the lane. The lane labeled 'L' contains a 1 kb step ladder for size standards, as indicated. The arrow indicates the amplicon, at 1300 kb, indicative of a bacterial template present in the sample.

3. Results

When PCR was performed with both newt tissue extracts and bacterial DNA as templates, the amplicon was visible on an agarose gel (Fig. 1). This occurred with all tissue types. The amplicon was visible when reactions contained less than 1 ng of bacterial DNA (Fig. 1). DNA extracted from the skin and gonads of both long-term captive and recently collected newts did not show amplification (Fig. 2), nor did DNA from liver tissue or eggs of recently collected newts (Fig. 3). The amplicon was only seen in extract of newt intestinal tissue (Fig. 4).

4. Discussion

These results indicate that bacteria are not present in *T. granulosa* skin, liver, gonads or eggs—tissues that are

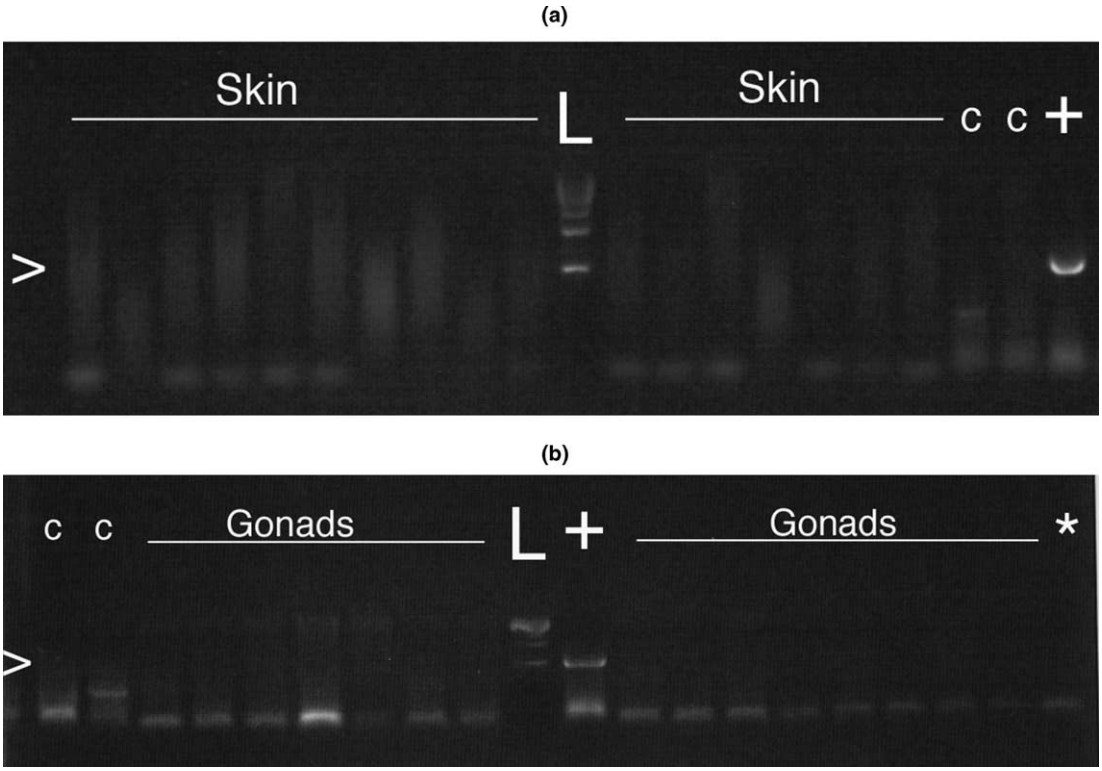

Fig. 2. Product of PCR with bacteria-specific primers using extracts from (a) newt skin and (b) newt gonads, as visualized on a 1% agarose gel. The lanes labeled 'L' contain 1 kb step ladder, lanes labeled with ' + ' contain only bacteria template, and lanes labeled '*' contain no known template. Lanes labeled 'c' in (a) contain skin samples from captive newts. The arrow indicates the approximate location of the amplicon. This band is only seen in lanes containing PCR product from reactions known to contain bacteria, indicating that no bacteria are present in skin or gonadal tissues in newts.

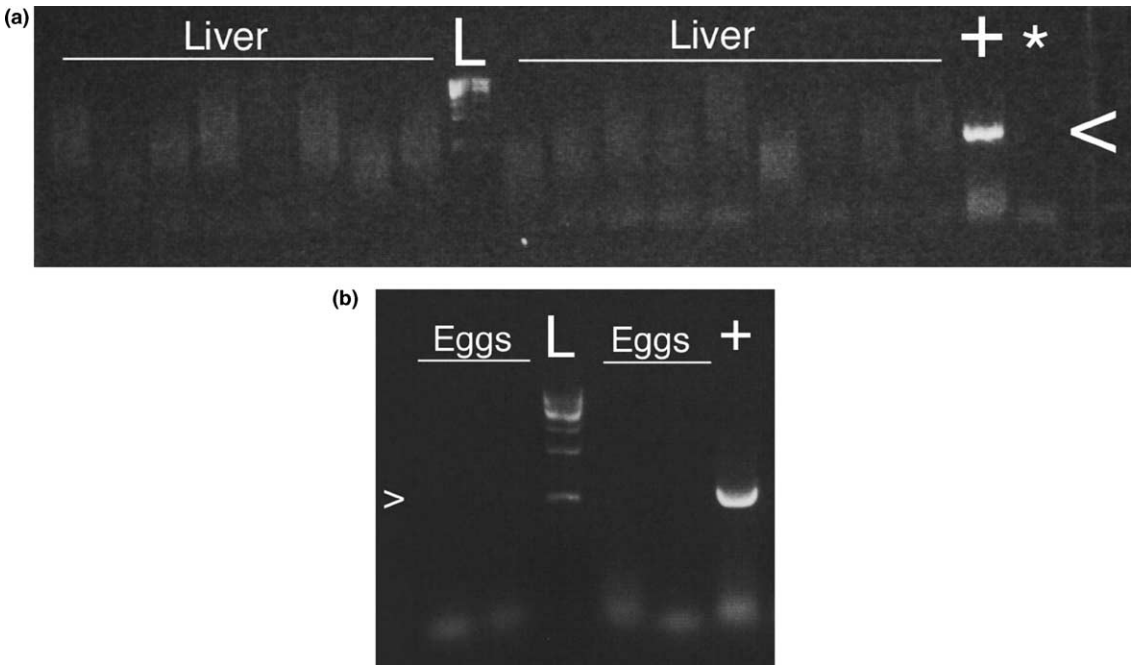

Fig. 3. Product of PCR with bacteria-specific primers using extracts from (a) newt liver and (b) newt eggs, as visualized on a 1% agarose gel. The lanes labeled 'L' contain 1 kb step ladder, lanes labeled with ' + ' contain only bacteria template, and lanes labeled '*' contain no known template. The arrow indicates the approximate location of the amplicon. This band is only seen in lanes containing PCR product from reactions known to contain bacteria, indicating that no bacteria are present in liver tissue or laid eggs in newts.

known to contain high concentrations of TTX (Wakely et al., 1966). If bacteria are responsible for the production of TTX in newts, they are not harbored directly in the TTX containing tissues within the detection limits of this method. In fact, the only newt tissue in which bacterial DNA was detected is the intestine. Finding evidence of bacteria in the intestine is not a surprising result, given that bacteria inhabit the digestive tracts of most animals. Concentrations of TTX in viscera of newts are estimated to be 200 times lower than those found in skin and ovaries (Wakely et al., 1966). If intestinal bacteria are the source of TTX in newts, a small number of bacterial cells would have to produce vast quantities of TTX. Further, newts would have to possess a TTX transfer system that moves the vast majority of a secondarily produced toxin out of the intestines and into other tissues, as well as mechanisms to concentrate the TTX in other tissues. The lack of bacteria in tissues containing high concentrations of TTX indicates that symbiotic bacteria are unlikely to be the ultimate source of TTX in *T. granulosa*.

Given that the bacterial amplicon was visible with very low amounts of template DNA, it seems that this technique is sensitive enough to detect any bacteria present in newt tissues. In addition to our findings, several other lines of

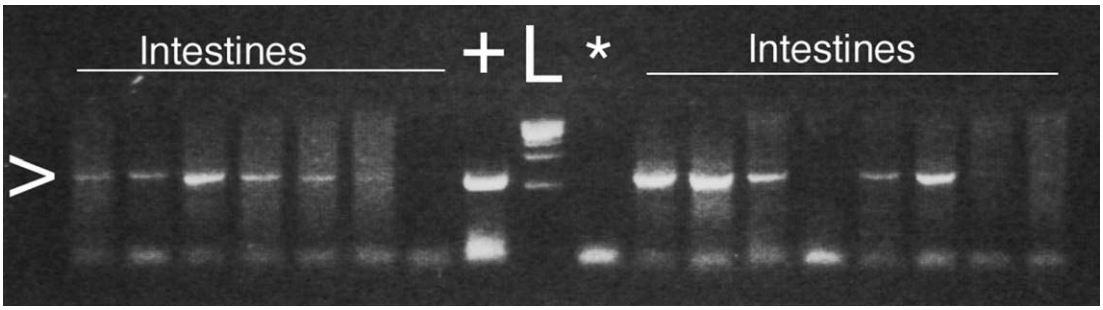

Fig. 4. Product of PCR with bacteria-specific primers using extracts from newt intestines, as visualized on a 1% agarose gel. The lanes labeled 'L' contain 1 kb step ladder, lanes labeled with ' + ' contain only bacteria template, and lanes labeled '*' contain no known template. The arrow indicates the approximate location of the amplicon. This band is seen in lanes containing PCR product from reactions known to contain bacteria, as well as lanes containing product from reactions using extracts from newt intestines. This result indicates that bacteria are present in the intestines.

evidence support the hypothesis of an endogenous origin of TTX in *T. granulosa* as an alternative to a bacterial origin of TTX. Because the only bacteria found in newt tissues were present in the digestive tract, it is possible that TTX in newts may come from a dietary source of TTX-producing bacteria. However, newts kept in captivity for over 1 year maintain or increase their TTX levels (Hanifin et al., 2002) despite not consuming their natural diet. If TTX-producing bacteria are present in the newts' normal habitat and are consumed during feeding, toxicity levels should decrease (or at least not increase) in captivity when the dietary source of TTX-producing bacteria becomes unavailable, in contrast to the observed maintenance of toxicity. Although it is possible that TTX-producing intestinal bacteria are maintained in captivity, other lines of evidence suggest that this is not the case.

Microscopic examination of skin sections of *T. granulosa* does not reveal any bacterial cells in granular glands in the skin (A. Gayda and B. Williams, unpublished data), consistent with the lack of amplification of bacterial DNA observed here. Immunohistological study of *Cynops pyrrhogaster,* another newt species possessing TTX, also does not reveal bacterial cells in the TTX-storing granular glands of the skin (Tsuruda et al., 2002). Given the relative sizes of granular glands and bacterial cells, bacteria should be easily visualized by these methods, particularly if they are synthesizing and contain TTX.

Furthermore, TTX is found in only three of the four life stages of newts; larvae lack the toxin soon after the yolk has been absorbed (C. Hanifin, personal communication). Early work indicated a rapid decline in toxicity as larvae developed post-hatching and suggested that TTX is present in the yolk (Twitty, 1937), as has now been confirmed (Hanifin et al., 2003). Larval newts are presumably non-toxic because the skin has not yet developed granular glands to store TTX (Tsuruda et al., 2002). If bacteria are responsible for TTX production in terrestrial juvenile and adult newts, and the bacteria are not from a dietary origin, then bacteria must be present in the larvae but not actively synthesizing TTX. Additionally, eggs are toxic (Hanifin et al., 2003) but do not harbor bacteria (Fig. 3), ruling out vertical transmission of a TTX producing bacteria. Any bacterial source of TTX in terrestrial juveniles and adults would have to be acquired after the larval stage, and probably not through the diet (see above).

TTX is also found in the blood of adult newts, although there is a discrepancy between studies in the relative toxicity of blood from males and females (Twitty, 1937; Wakely et al., 1966). Twitty (1937) proposed that differences in the toxicity of blood from females is related to their reproductive status, with levels potentially being highest during the deposition of yolk in developing eggs. Given that the toxicity of eggs, embryos and newly-hatched larvae is due to TTX in yolk, and that there is a strong correlation between a female's toxicity and that of her eggs (Hanifin et al., 2003), it is likely that females deposit TTX in the yolk of their eggs and that this may be under hormonal control.

Based on the results presented here and previous knowledge of toxicity patterns in *T. granulosa*, it appears most likely that TTX in *T. granulosa* is of endogenous origin. If bacteria are capable of producing TTX, there is no evidence of a role for them in the toxicity of newts, although these findings cannot be extrapolated to all taxa possessing TTX. An endogenous source of TTX implies that toxicity may have a genetic basis, although this has yet to be confirmed. Establishing a genetic basis to a trait is an important step in studying its evolution, as evolution cannot occur without heritable variation in a trait. Further investigation into the genetics and heritability of toxicity in *Taricha* may shed light on the origins and biosynthesis of TTX in this and other species, and aid interpretation of the predator–prey coevolutionary interaction between newts and snakes.

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