Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

REVIEW ARTICLE

An overview on the origin and production of tetrodotoxin, a potent neurotoxin

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Keywords
bacteria, environment, exogenous, origin, tetrodotoxin.

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2015/0193: received 29 January 2015, revised 26 May 2015 and accepted 20 June 2015

doi:10.1111/jam.12896

Summary

Tetrodotoxin (TTX) is a deadly neurotoxin which selectively inhibits Na^+ activation mechanism of nerve impulse, without affecting the permeability of K^+ ions. Because of this sodium channel blocking action, it is majorly being studied for biomedical applications. TTX is present in taxonomically diverse groups of animals inhabiting terrestrial, marine, fresh water and brackish water environments, still its origin remains unclear. The extensive study of the toxin has revealed a few possibilities of its origin. This review reports on the aspects of the origin of TTX, where the primary focus is on its exogenous origin. The significance of bacterial, cellular and environmental factors in its biogenesis and accumulation is also discussed. The possible facets for engineering the bacterial genomics to modulate the gene expression for TTX production are also outlined.

Introduction

Tetrodotoxin (TTX) is a naturally occurring deadly neurotoxin that acts by inhibiting the action potential in nerve and muscle cells, in a highly potent and selective manner. Due to this reason, it has emerged as a useful tool for studying excitable cells. A study (Narahashi *et al.* 1960) on lobster giant axon using voltage clamp technique has shown that TTX blocked only the sodium channels, without affecting the resting membrane potential. Based on this characteristic of TTX, the structure of sodium channel was elucidated as a bell-shaped molecule with several cavities and a relative molecular mass of about 300 kD (Sato *et al.* 2001). Since then, TTX has become a popular chemical in the study of neurophysiology (Narahashi 2001). It has also gained importance for its medical potentials in treating migraine, withdrawal symptoms in heroin addicts (Song *et al.* 2011), as an anaesthetic agent (Schwartz *et al.* 1998) and its role in pain sensation (Narahashi 2008). Its future prospective could also be in treating ischaemic neuronal injury (Lysko *et al.* 1994) because of the sodium channel blocking action. Its potential as a pharmaceutical drug was tested by a Canadian company, International Wex technologies in collaboration with a Chinese institute. During their study on the use of TTX for recovery in heroin addicts, serendipitously they discovered its potential to curb pain in cancer patients as well. It also has pharmacological applications as molecular and cellular markers for voltage dependent Na^+ channel, in studying excitable membranes. However, the origin and production of TTX is still not clear. The focus of this review is the influence of external factors on the production of TTX. Also, the significance of bacteria in its *in vitro* culture and mass production is discussed. This review in total summarizes the recent perception on the exogenous origin of TTX.

Diversity of TTX-producing organisms

TTX is produced by a diverse group of animals which are phylogenetically unrelated, such as species from pufferfish, Gobies, Newts, Frogs, Horseshoe Crabs, Xanthid Crabs, Blue-ringed Octopus, Gastropods, Starfish, Flatworms, Ribbon worms, Annelids, Arrow worms, Red calcareous alga, Dinoflagellates, Bacteria, etc. To understand the nature of this potentially emerging compound, TTX bearing animals are exploited extensively. This could be a threat to their number, as large resources can only produce a minute amount of pure toxin, as in the case of puffer fish, tons of puffer fish ovaries are required to produce a gram of pure TTX. If the origin of TTX becomes clear then its mass production, directly from the bacteria would be possible, without affecting the larger phyla. To understand the origin of TTX, it is necessary to study the various hypotheses proposed regarding its origin, which are also discussed in certain detail in this review.

Hypotheses for origin of TTX

To study the origin of TTX, a few probable hypotheses were proposed (Mosher and Fuhrman 1984). Many experiments were performed to exploit the dynamics of the given hypotheses and to find the origin of TTX.

Exogenous

According to this hypothesis, TTX is formed in the environment and is ingested by TTX bearers. This phenomenon could have been occurring for ages, resulting in mutation and evolution making them resistant to TTX. The possibility of exogenous origin of TTX was put forward by Matsui in feeding experiments with artificially bred larvae (Matsui *et al.* 1981), which suggested that TTX bearing organisms were infected by TTX-producing bacteria living symbiotically in their bodies (Matsui *et al.* 1985). A case evidencing exogenous source of TTX is reported from a trumpet shell, *Charonia sauliae* which accumulated TTX on ingesting a toxic starfish (Noguchi *et al.*, 1982). Mechanism for TTX accumulation in puffers through the food chain was proposed in 2006 (Noguchi *et al.* 2006). More than 5000 specimens of pufferfish reared in net cages or land aquaria for a year became nontoxic because of the prevention of invasion of TTX bearing organisms (Noguchi *et al.* 2006). When nontoxic puffers are fed with diet containing TTX, they become toxic (Noguchi 1988).

Endogenous

TTX might be an accidental metabolic product having some physiological functions in the animals that produce it. It may essentially have some survival purpose to fulfil in these animals. Some studies have also favoured this hypothesis. TTX levels in newts increased when they were kept in captivity for a year, suggesting a nonexogenous origin (Hanifin *et al.* 2002). Newts which were kept in captivity for 9 months showed regeneration of TTX in their skin or instead replenished skin TTX from another region of the body (Cardall *et al.* 2004).

Symbiotic micro-organisms

TTX might be produced by the symbiotic association between the animals acquiring it and the micro-organisms that are reported to produce it. The microbial production of TTX in puffers has been studied extensively. It is argued that the micro-organisms produce TTX symbiotically in the body of TTX bearers. Micro-organisms that have been reported to produce TTX till date, are listed in Table 1.

Multiple origins

The origin of TTX might be due to any of the combinations from the above three hypotheses.

Much of the evidence with respect to exogenous, endogenous and symbiotic origin are detailed in this review.

TTX production by symbiotic bacteria in marine organisms

The presence of symbiotic bacteria in TTX bearers were first reported by two research groups in 1986. Noguchi *et al.* (1986) cultured the micro-organisms from the intestine of a Xanthid crab, *Atergatis floridus* collected from Shimoda, Shizuoka Prefecture, Japan. They reported *Vibrio* as the dominant species in PYBG agar culture. Yasumoto *et al.* (1986) also reported the production of TTX by micro-organisms from a red alga, *Jania* sp. They isolated *Pseudomonas* in a medium containing 3% NaCl and 1% polypeptone. TTX-producing bacteria are also reported from other aquatic animals such as Horseshoe Crab, Starfish, Blue-ringed Octopus, Lined Moon Shell, Bivalve Molluscs, Gastropods, several of the pufferfish and also from Marine and Fresh water sediments (Table 1). The origin of TTX in such a diverse group of animals remains unknown. However, bacteria being omnipresent and commonly inhabiting the aquatic system are suspected as the primary source of TTX in the aquatic system.

Bacterial symbiosis, that is host-bacterial association for a prosperous relation is common in marine animals. These bacteria sometimes produce secondary metabolites in the host. Bacterial secondary metabolites have emerged as one of the best resources for innovative therapeutics discovery (Pettit 2011). Having evolved from nature, these metabolites are characterized by their structurally unique heterogeneity which accounts for their potency and selectivity. Industrially important secondary metabolite production is reported from *Vibrio* (Mansson *et al.* 2012), Actinomycetes, myxobacteria (Ichikawa *et al.* 2013), filamentous fungi (Inglis *et al.* 2013) and several

Table 1 Timeline of bacterial origin of TTX

Year	TTX-producing bacteria	Host organism	Reference
1986	*Vibrio* sp.	Intestine of Xanthid crab *Atergatis floridus*	Noguchi *et al.* (1986)
1986	*Pseudomonas* sp.	From an alga, *Jania* sp.	Yasumoto *et al.* (1986)
1987	*Pseudomonas* sp.	Skin of Puffer, *Fugu poecilonotus*	Yotsu *et al.* (1987)
1987	*Vibrio alginolyticus*	Intestine of *Takifugu vermicularis vermicularis*	Noguchi *et al.* (1987)
1987	*V. alginolyticus*, *Vibrio damsela*	Starfish *Astropecten polycanthus*	Narita *et al.* (1987)
1987	*Vibrio fischeri*	Xanthid crab, *Atergatis floridus*	Sugita *et al.* (1987)
1987	*V. alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio anguillarum*, *Photobacterium phosphoreum*, *Aeromonas salmonicida*, *Plesiomonas shigelloides*	Bacterial strains were collected from ATCC & NCMB	Simidu *et al.* (1987)
1988	*V. alginolyticus*	Gastro intestinal tract of Horseshoe crab, *Carcinoscorpius rotundicauda*	Kungsuwan *et al.* (1988)
1989	*V. alginolyticus*	Venom of 4 species of Chaetognatha: *Flussisagitta lyra*, *Parasagitta elegans*, *Zonosagitta nagae*, *Eukrohnia hamata*	Thuesen and Kogure (1989)
1989	*Shewanella putrefaciens*	Puffer, *Takifugu niphobles*	Matsui *et al.* (1989)
1989	*Alteromonas* sp., *Bacillus* sp., *Pseudomonas* sp., *Vibrio* sp.	Posterior salivary gland of *Octopus maculosus*	Hwang *et al.* (1989)
1990	*Listonella pelagia*, *Alteromonas tetraodonis*, *Shewanella alga*	Red alga and Pufferfish	Simidu *et al.* (1990)
1990	*Vibrio* sp., *Bacillus* sp., *Alteromonas* sp., *Aeromonas* sp., *Micrococcus* sp., *Acinetobacter* sp., *Moraxella*	Deep sea sediments	Do *et al.* (1990)
1991	*Actinomycetes*	Marine sediments	Do *et al.* (1991)
1993	*Bacillus*, *Micrococcus*, *Alcaligens*, *Caulobacter*, *Flavobacterium*	Freshwater sediments	Do *et al.* (1993)
1994	*V. alginolyticus*, *Aeromonas* sp.	Lined moon shell, *Natica lineata*	Hwang *et al.* (1994)
1995	*Aeromonas* sp., *Pseudomonas* sp., *Plesiomonas* sp., *V. alginolyticus*, *V. parahaemolyticus*	Gastropod, *Niotha clathrata*	Cheng *et al.* (1995)
2000	Bacteria closely related to *Pseudoalteromonas haloplanktis tetraodonis*	Bacteria was found to be able to cause sudden death in Sea urchin, *Meoma ventricosa*	Ritchie *et al.* (2000)
2000	*Vibrio* sp.	Intestine of *Fugu vermicularis radiatus*	Lee *et al.* (2000)
2003	*Vibrio* sp.	Nemertean worm	Carroll *et al.* (2003)
2004	*Aeromonas molluscorum*	Bivalve mollusc	Galbis *et al.* (2004)
2004	*Microbacterium arabinogalactanolyticum*	Ovary of Puffer, *Takifugu niphobles*	Yu *et al.* (2004)
2004	*Serratia marcescens*	Skin of Puffer, *Chelonodon patoca*	Yu *et al.* (2004)
2004	*V. alginolyticus*	Intestine of Puffer, *Takifugu alboplumbeus*	Yu *et al.* 2004 & Yan *et al.* (2005)
2005	*Bacillus* sp., *Actinomycete* sp.	Ovary, liver, intestine of *Fugu rubripes*	Wu *et al.* (2005a)
2005	*Nocardiopsis dassonvillei*	Ovary of *Fugu rubripes*	Wu *et al.* (2005b)
2007	*Roseobacter*	Copepod *Pseudocaligus fugu* which is present as ectoparasite on the Puffer, *Takifugu pardalis*	Maran *et al.* (2007)
2008	*Vibrio*, *Shewanella*, *Marinomonas*, *Tenacibaculum*, *Aeromonas*	Digestive gland and muscle of marine gastropod, *Nassarius semiplicatus*	Wang *et al.* (2008)
2010	*Aeromonas*	Ovary of Puffer, *Takifugu obscurus*	Yang *et al.* (2010)
2010	*Bacillus*	Puffer, *Fugu obscurus*	Wang and Fan (2010)
2010	*Lysinibacillus fusiformis*	Liver of Pufferfish, *Fugu obscurus*	Wang *et al.* (2010)
2011	*Raoultella terrigena*	Pufferfish, *Takifugu niphobles*	Yu *et al.* (2011)
2011	*Shewanella* sp.	Ovary of Puffer *Takifugu oblongus*	Hien *et al.* (2011)

TTX, tetrodotoxin.

other microbial species. *Vibrio* species produces compounds with biological activities like antibacterial, antiviral and anticancer. In fact, many compounds reported from *Vibrio* sp. have also been isolated from a few other distantly related bacteria indicating the incidence of horizontal gene transfer. An antibiotic andrimid, a secondary metabolite produced by *Vibrio* is an example of such compounds (Mansson *et al.* 2012). Studies carried out on the origin of saxitoxin (STX) (similar in structure to TTX) genes in Cyanobacteria have also shown multiple horizontal gene transfer events in *Anabaena circinalis* (Moustafa *et al.* 2009). Complex biosynthetic pathways of such structurally similar toxins might be helpful in providing insights on the biosynthesis of TTX (Chau *et al.* 2011; Moczydlowski 2013). The synergistic production of secondary metabolites from micro-organisms is also well known (Angell *et al.* 2006). As, TTX has also been reported from diverse bacterial species, so this concept could be possible in case of TTX-producing bacteria.

Biosynthetic pathways for the synthesis of secondary metabolites are crucial in elucidating the genetic basis of these natural products. These pathways are mainly controlled by transcriptional activities of genes encoding the specific enzyme which is further encoded in the bacterial genome (Ichikawa *et al.* 2013), i.e. genes expressing the biosynthetic pathways for toxins are clustered in the bacterial genome. The understanding of the genomics of these clusters could provide valuable insights into the biosynthesis of secondary metabolites, offering alternatives for discovery of new potential entities. Hence, focusing on the bacterial genome could make it simpler in understanding the origin and production of TTX.

Occurrence of TTX in amphibians

As cited in Table 1, reports for bacterial origin of TTX are mainly from marine biota and a few from freshwater environments. But there are no such reports from the terrestrial TTX bearers. The newt, *Taricha granulosa* which contains TTX, has been highly investigated for the origin of this toxin, due to the presence of glandular skin gland, where TTX is produced. Reports claim that the glandular skin gland is important for the production of TTX in newts (Tsuruda *et al.* 2002; Cardall *et al.* 2004; Hanifin *et al.* 2004). Also, during an investigation on the production of TTX from bacteria in *T. granulosa* no symbiotic bacteria were identified which could be considered responsible for the production of TTX (Lehman *et al.* 2004). Though it is clear that bacteria are not responsible for the production of TTX in newts, involvement of exogenous or endogenous factors is still unknown. TTX levels in newt *T. granulosa* tended to increase when the newt was kept in captivity (Hanifin *et al.* 2002). A study by Hanifin (2010) also favours the endogenous origin of TTX in amphibians (Hanifin 2010). However, it was seen recently that the newts collected from locations in Canada and USA (Yotsu *et al.* 2012) kept for several years (3–6 years) in captivity on nontoxic diet lost their toxicity. Noguchi and Arakawa 2008 have also mentioned the possibility of exogenous origin of TTX in amphibians (Noguchi and Arakawa 2008). The eggs of Japanese newt *Cynops pyrrhogaster* inherit toxicity from the parent that disappears in the larva, but suddenly the juveniles start becoming toxic. When artificially reared, they again lose toxicity (Tsuruda *et al.* 2001; Noguchi and Arakawa 2008). In a similar report by Daly *et al.* 1997 the artificially reared *Atelopus varius* frog produced no TTX. Hence, the exogenous origin of TTX in amphibians cannot be entirely neglected, as these observations focuses on its possibility.

External factors effecting the biosynthesis and origin of TTX

Environmental parameters also play a significant role in the biosynthesis of TTX, which could be a plausible reason for the difference in the biosynthesis and analogue composition in terrestrial and marine biota. Certain analogues of TTX are specific to newts while certain others are specific to pufferfish (Kudo *et al.* 2012). The 6-*epi*TTX, 8-*epi*-type and 1-hydroxy-type analogues of TTX are detected only in newts, while 5, 6, 11-trideoxyTTX is a specific and major analogue in the pufferfish and a few other marine animals. A recent study by Khor *et al.* 2014 reports that the toxin in *Pleurobranchaea maculate* is accumulated through the food chain, but is not the sole reason for their toxicity, suggesting that other factors are also involved in its toxicity.

In marine puffers, toxicity is generally higher in liver and ovary, whereas in brackish and freshwater puffers, toxicity is higher in skin (Asakawa *et al.* 2012). Further, puffers from marine waters are mainly known to contain TTX while pufferfish from freshwater has STX (Ngy *et al.* 2008). But the presence of STX and TTX was documented from the same species in puffer fish *Tetraodon fangi* (Saitanu *et al.* 1991; Sato *et al.* 1997) and *Fugu pardalis* (Jang and Yamashita 2007). In fact, a few reports also suggest STX to be the major component of few marine puffers (Sato *et al.* 2000; Nakashima *et al.* 2004). Such variation in toxicity could be because of the changes in aquatic system caused due to global warming (Arakawa *et al.* 2010; Silva *et al.* 2012) making it more susceptible for aquatic organisms to adapt to different environmental conditions.

TTX production is not only limited to TTX bearers and their symbiotic bacteria, it is also reported from commonly inhabiting bacterial strains. To find the participation of common marine bacterial strains in the production of TTX, Simidu *et al.* (1987) collected several typical strains of marine bacteria from ATCC and NCMB, which were reported to produce AnhydroTTX. TTX was also isolated from aquatic sediments. Kogure *et al.* (1988) have reported the presence of unexpectedly high amount of TTX from marine sediments, indicating that TTX could sustain if the optimum conditions are available, irrespective of their association with TTX bearing animals. Moreover, this complex molecule if produced could bio-accumulate only if the same condition sustains.

Further research was carried out on the presence of TTX in microbial strains from deep sea sediments (Do *et al.* 1990), freshwater environment (Do *et al.* 1993) suggesting, symbiosis is not a necessary factor for the production of TTX. Rather, the necessary stimuli or trigger is required for the production of this secondary metabolite.

Synthesis of natural product represents the chemical interface between the host and surrounding environment. Natural products are produced only in a particular range of culture conditions. The cultivation parameters for their production mainly includes the source for macro and micro nutrients, pressure, temperature, pH, light intensity, enzymes in active state and supply of precursors. The other factors which influence the production are export from the cells for compounds that accumulate outside the cells, interspecific competition, epigenetic factors, environmental stress, predation and interaction of micro-organisms present in natural environment. Therefore, their production under laboratory conditions is a major challenge due to the limitation in *in vitro* culture. Production of secondary metabolites are reported to be affected by environmental factors in others cases like Hawthron species, St. John's wort, etc (Leland *et al.* 2006). Overy *et al.* (2005) have cultured several of necrotrophic *Penicillium* strains by mimicking the fungus' natural habitat that could stimulate the production of corymbiferone.

The production of secondary metabolite in an artificial mode may cause silencing of some genetic pathways (Wang *et al.* 2013). The genes for their biosynthesis get activated under specific conditions and are known as cryptic genes. The *in vitro* production of secondary metabolite is dependent on optimizing the right conditions in which these cryptic genes are expressed. An example of such metabolite production is seen in rhizoxin (Shwab and Keller 2008). The biosynthetic pathways of such genes could be triggered through external cues, co-cultivation and genomic approaches (Scherlach and Hertweck 2009). Further, the induction of such orphan genetic loci into heterologous host has been successful in expression of such genes.

Analytical studies have reported that the quantity of TTX produced by micro-organisms is much lower than the TTX bearing animals, as the optimum conditions required to stimulate the amount of TTX in *in vitro* cultures is still not known. Gallacher and Birkbeck (1993) reported the concentration of phosphate to affect the TTX production in *Alteromonas tetraodonis*. They also suggested that TTX was produced during the stationary phase of the cell cycle. Hashimoto *et al.* 1990 reported that no toxicity in *Vibrio alginolyticus* was observed in a 24 and 48 h medium containing 1% NaCl–1% Phytone peptone at 25°C, but a sudden increase in toxicity up to 213 MU was noted in a 72 h's culture. While Yu *et al.* 2011 have reported that, in the bacterial culture isolated from marine puffer fish *Takifugu niphobles* the toxicity in 24 h (log phase) was two-fold higher than in the 48 h (stationary phase) culture. A study by Cheng *et al.* 1995 reports that the toxicity of TTX-producing bacteria in gastropod varied from one location to other. Toxicity of TTX-producing bacteria in lined moon shell *Natica lineata* showed no relationship with the viable count of bacteria suggesting that bacteria alone do not cause toxicity in this species (Hwang *et al.* 1994). Its seasonal variation in toxicity also signifies the involvement of an external stimulus. Moreover, as reported by Saito *et al.* 1985; when the skin of Puffer is lightly wiped with gauze, TTX is released, which might be due to the stimulus. These observations emphasize the importance of optimal conditions that trigger the production of signalling molecules for TTX biosynthesis. Such small signalling molecules are important in regulating the metabolite productions in several other bacteria as well (Horinouchi 2002). These conditions are well provided in the body of TTX bearers and in the sediments where TTX is present. But, the production of such conditions *in vitro* is still not successful.

Significance of bacteria in the production of TTX

Substantial studies have been carried out on the bacterial origin of TTX. Spectral analysis by HPLC (Do *et al.* 1990), ESI-MS (Wang *et al.* 2010), GC-MS (Cheng *et al.* 1995), etc. makes it clear that TTX was extracted from the above reported micro-organisms, which were present within the TTX-producing animals.

Bacteria are not the sole producers of TTX as the biosynthesis of TTX is considered to be different in marine animals and amphibians (Kudo *et al.*, 2012). However, the biosynthetic pathway of TTX could be simplified if bacteria are analysed for its origins. Thus, we have focused on the bacterial group reported to produce TTX.

Table 2 Phylogenic order of tetrodotoxin producing bacteria

Kingdom	Phylum	Class	Order	Family	Genus
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	*Actinomycetes* sp.
				Microbacteriaceae	*Microbacterium* (*Mic. arabinogalactanolyticum*)
				Micrococcaceae	*Micrococcus* sp.
	Bacteroides	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Flavobacterium* sp.
					Tenacibaculum sp.
	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus* sp.
					Lysinibacillus (*L. fusiformis*)
	Proteobacteria	Alphaproteobacteria	Caulobacteriales	Caulobacteriaceae	*Caulobacter* sp.
			Rhodobacteriales	Rhodobacteriaceae	*Roseobacter* sp.
		Betaproteobacteria	Burkhobacteriales	Alcaligenaceae	*Alcaligenes* sp.
		Gammaproteobacteria	Aeromonadales	Aeromonadaceae	*Aeromonas* (*Aer. salmonicida* & *Aer. molluscorum*)
			Alteromonadales	Alteromonadaceae	*Alteromonas* (*Aer. tetraodonis*)
				Shewanellaceae	*Shewanella* (*S. putrefaciens* & *S. alga*)
			Enterobacteriales	Enterobacteriaceae	*Raoultella* (*R. terrigena*)
					Serratia (*Ser. marcescens*)
				Plesiomonaceae	*Plesiomonas* (*Ple. shigelloides*)
			Oceanospirillales	Oceanospirillaceae	*Marinomonas* sp.
			Pseudomonadales	Pseudomonadaceae	*Pesudomonas* (*Ps. haloplanktis tetrodonis*)
				Moraxellaceae	*Moraxella*
					Acinetobacter
			Vibrionales	Vibrionaceae	*Listonella* (*L. pelagia*)
					Photobacterium (*P. phosphoreum*)
					Vibrio (*V. alginolyticus* & 4 other species)

From the data presented in Table 1, we can ascertain that TTX and its analogues are present in diverse range of related microbes and animals. We have found that around 25 genera of bacteria are involved in the production of TTX (Table 2). The major TTX-producing microbes belong to the genus *Vibrio*, *Aeromonas*, *Pseudomonas*, *Bacillus*, *Shewanella* and *Alteromonas*. Of these microbes, *Vibrio*, *Aeromonas*, *Pseudomonas*, *Shewanella* and *Alteromonas* belong to *Gammaproteobacteria*. Hence, mostly bacteria are reported from the class *Gammaproteobacteria* belonging to *Proteobacteria* phylum. Other phyla that contribute to TTX production are *Actinobacteria*, *Bacteroides*, *Firmicutes* (Fig. 1). Majority of the findings to date have reported *V. alginolyticus* (belonging to *Gammaproteobacteria*) as the typical TTX-producing bacterium. We can see in Table 2 that the bacteria identified to be TTX producers are in diverse phyla symbolizing different characteristics. This leads to the speculation that the bacteria possibly could produce TTX when certain physiological parameters are present. Understanding the parameters required for production of TTX could help in the mass production of TTX from bacterial cultures.

The knowledge of the factors determining the TTX yield is imperative due to its biomedical importance. To fit the market requirements optimizing the culture conditions for higher yield of TTX is essential, for which, microbes could be exploited. As mentioned earlier, several factors when rightly coupled contribute to the production of natural products. The insufficient understanding of regulation of natural product biosynthesis has resulted in the failure of *in vitro* production. These parameters could be optimized by focusing on the TTX-producing bacterial strains. Thus, *in vitro* culturing of bacteria could increase the scope to identify the necessary optimal conditions for the production of TTX. Further, bacteria can be more easily cultured compared to the farming of eukaryotic organisms. It is even less laborious and time saving to

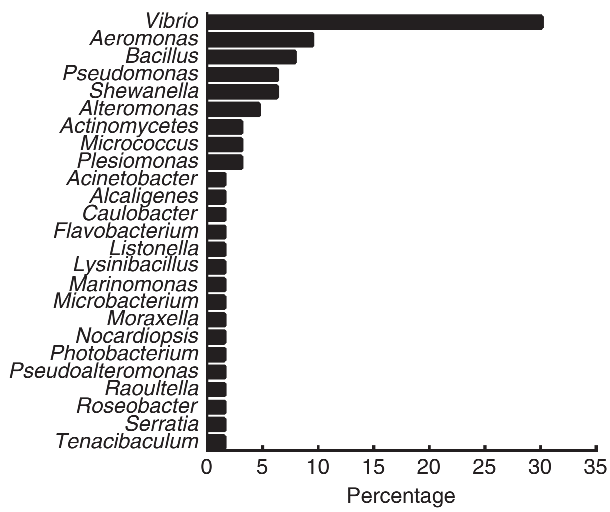

Figure 1 Frequency of bacteria reported to produce tetrodotoxin.

grow bacteria. Hence, bacteria could be an attractive alternate model system by which the study of the biosynthesis of this natural product can be up-regulated to ultimately increase its yield. As the prokaryotic genomic data are easily available and the genome size would be comparatively smaller, further investigations involving TTX-producing bacteria rather than larger animals can be studied to understand the biogenesis of TTX. This reflects that TTX-producing bacteria plays a significant role, not only in understanding the biosynthetic pathway and origin of TTX, but also in its mass production.

Conclusion and future perspectives

Based on the analysis of various experiments conducted on the inception of TTX, it is possible that the occurrence of TTX in such diverse group of animals might be exogenous. The biosynthetic pathway of TTX is too complex and physiological conditions are found to play an enormous role. Symbiotic association of bacteria for the origin of TTX in aquatic environment may not possibly be necessary as TTX was reported in common strains of marine inhabiting bacteria collected from ATCC and NCMB (Simidu *et al.* 1987) and also in deep sea sediments (Do *et al.* 1990), freshwater sediments (Do *et al.* 1993). Instead, the environmental parameters and the physiological conditions could have considerable significance in TTX production.

Understanding the optimal conditions for TTX production can give valuable insights into the origin and mechanism of TTX production. To improve secondary metabolite production, it is pivotal to optimize the conditions for their production in *in vitro* cultures. Once the *in vitro* conditions are established, the development of transformation system can expedite genetic enhancement for greater yield. Therefore, focusing on the gene clusters from bacterial genome might help in a better understanding of the production and accumulation of TTX. The chemical activation for gene expression for TTX production could be attributed to their ecological response. We envisage that it is important to study the cellular and environmental factors that affect the synthesis of this natural product.

Conflict of Interest

The authors report no conflict of interest.

References

Angell, S., Bench, B.J. and Williams, H., Watanabe, C.M. (2006) Pyocyanin isolated from a marine microbial population: synergistic production between two distinct bacterial species and mode of action. *Chem Biol* **13**, 1349.

Arakawa, O., Hwang, D.F., Taniyama, S. and Takatani, T. (2010) Toxins of pufferfish that cause human intoxications. In *Coastal Environmental and Ecosystem Issues of the East China Sea* ed. Ishimatsu, A. and Lie, H.J. pp. 227–244. Nagasaki City, Japan: Terrapub and Nagasaki University.

Asakawa, M., Shida, Y., Miyazawa, K. and Noguchi, T. (2012) Instrumental analysis of tetrodotoxin. In *Chromatography — The Most Versatile Method of Chemical Analysis* ed. de Azevedo Calderon, L. pp. 245–270. Rijeka, Croatia, Europe: InTech publication.

Cardall, B.L., Brodie, E.D. Jr, Brodie, E.D. and Hanifin, C.T. (2004) Secretion and regeneration of tetrodotoxin in the rough-skin newt (*Taricha granulosa*). *Toxicon* **44**, 933–938.

Carroll, S., McEvoy, E. and Gibson, R. (2003) The production of tetrodotoxin-like substances by nemertean worms in conjunction with bacteria. *J Exp Mar Biol Ecol* **288**, 51–63.

Chau, R., Kalaitzis, J.A. and Neilan, B.A. (2011) On the origins and biosynthesis of tetrodotoxin. *Aquat Toxicol* **104**, 61–72.

Cheng, C.A., Hwang, D.F., Tsai, Y.H., Chen, H.C., Jeng, S.S., Noguchi, T., Ohwada, K. and Hasimoto, K. (1995) Microflora and tetrodotoxin-producing bacteria in a gastropod (*Niotha clathrata*). *Food Chem Toxicol* **33**, 929–934.

Daly, J.W., Padgett, W.L., Saunders, R.L. and Cover, J.F. Jr (1997) Absence of tetrodotoxin in a captive raised riparian frog, *Atelopus varius*. *Toxicon* **35**, 705–709.

Do, H.K., Kogure, K. and Simidu, U. (1990) Identification of deep-sea-sediment bacteria which produce tetrodotoxin. *Appl Environ Microbiol* **56**, 1162–1163.

Do, H.K., Kogure, K., Imada, C., Noguchi, T., Ohwada, K. and Simidu, U. (1991) Tetrodotoxin production of actinomycetes isolated from marine sediment. *J Appl Microbiol* **70**, 464–468.

Do, H.K., Hamasaki, K., Ohwada, K., Simidu, U., Noguchi, T., Shida, Y. and Kogure, K. (1993) Presence of tetrodotoxin and tetrodotoxin-producing bacteria in freshwater sediments. *Appl Environ Microbiol* **59**, 3934–3937.

Galbis, D.M., Farfan, M., Fuste, M.C. and Loren, J.G. (2004) *Aeromonas molluscorum* sp. nov., isolated from bivalve molluscs. *Int J Syst Evol Microbiol* **54**, 2073–2078.

Gallacher, S. and Birkbeck, T.H. (1993) Effect of phosphate concentration on production of tetrodotoxin by *Alteromonas tetraodonis*. *Appl Environ Microbiol* **59**, 3981–3983.

Hanifin, C.T. (2010) The chemical and evolutionary ecology of tetrodotoxin (TTX) toxicity in terrestrial vertebrates. *Mar Drugs* **8**, 577–593.

Hanifin, C.T., Brodie, E.D. III and Brodie, E.D. Jr (2002) Tetrodotoxin levels of the rough-skin newt, *Taricha granulosa*, increase in long-term captivity. *Toxicon* **40**, 1149–1153.

Hanifin, C.T., Brodie, E.D. and Brodie, E.D. Jr (2004) A predictive model to estimate total skin tetrodotoxin in the newt *Taricha granulosa*. *Toxicon* **43**, 243–249.

Hashimoto, K., Noguchi, T., Watabe, S. (1990) New aspects of tetrodotoxin. In *Microbial Toxins in Foods and Feeds* ed. Poland, A.E. *et al.* pp. 575–588. New York, NY: Plenum.

Hien, B.T.T., Long, P.Q. and Thanh, K.H. (2011) *Isolation and Identification of Tetrodotoxin-Producing Bacteria from Ovary of the Vietnamese Puffer Fish Takifugu Oblongus*. Vladivostok, Russia: The Ist Asia Pacific Meeting on Animal, Plant And Microbial Toxins.

Horinouchi, S. (2002) A microbial hormone, A-factor, as a master switch for morphological differentiation and secondary metabolism in *Streptomyces griseus*. *Front Biosci* **7**, 45–57.

Hwang, D.F., Arakawa, O., Saito, T., Noguchi, T., Simidu, U., Tsukamoto, K., Shida, Y. and Hashimoto, K. (1989) Tetrodotoxin-producing bacteria from the blue-ringed octopus *Octopus maculosus*. *Mar Biol* **100**, 327–332.

Hwang, D.F., Cheng, C.A., Chen, H.C., Jeng, S.S., Noguchi, T., Ohwada, K. and Hashimoto, K. (1994) Microflora and tetrodotoxin-producing bacteria in the lined moon shell (*Natica lineata*). *Fish Sci* **60**, 567–571.

Ichikawa, N., Sasagawa, M., Yamamoto, M., Komaki, H., Yoshida, Y., Yamazaki, S. and Fujita, N. (2013) DoBISCUIT: a database of secondary metabolite biosynthetic gene clusters. *Nucleic Acids Res* **41**, 408–414.

Inglis, D.O., Binkley, J., Skrzypek, M.S., Arnaud, M.B., Cerqueira, G.C., Shah, P., Wymore, F., Wortman, J.R. *et al.* (2013) Comprehensive annotation of secondary metabolite biosynthetic genes and gene clusters of *Aspergillus nidulans*, *A. fumigatus*, *A. niger* and *A. oryzae*. *BMC Microbiol* **13**, 91.

Jang, J.H. and Yamashita, Y. (2007) 6, 11-Dideoxytetrodotoxin from the puffer fish, *Fugu pardalis*. *Toxicon* **50**, 947–951.

Khor, S., Wood, S.A., Salvitti, L., Taylor, D.I., Adamson, J., McNabb, P. and Cary, S.C. (2014) Investigating diet as the source of tetrodotoxin in *Pleurobranchaea maculata*. *Mar Drugs* **12**, 1–16.

Kogure, K., Do, H.K., Thuesen, E.V., Nanba, K., Ohwase, K. and Simidu, U. (1988) Accumulation of tetrodotoxin in marine sediments. *Mar Ecol Prog Ser* **45**, 303–305.

Kudo, Y., Yasumoto, T., Konoki, K., Cho, Y. and Yotsu, M. (2012) Isolation and structural determination of the first 8-*epi*-type tetrodotoxin analogs from the newt, *Cynops ensicauda popei,* and comparison of tetrodotoxin analogs profiles of this newt and the puffer fish, *Fugu poecilonotus*. *Mar Drugs* **10**, 655–667.

Kungsuwan, A., Noguchi, T., Arakawa, O., Simidu, U., Tsukamoto, K., Shida, Y. and Hashimoto, K. (1988) Tetrodotoxin-producing Bacteria from Horseshoe Crab *Carcinoscorpius rotundicauda*. *Nippon Suisan Gakkaishi* **54**, 1799–1802.

Lee, M.J., Jeong, D.Y., Kim, W.S., Kim, H.D., Kim, C.H., Park, W.W., Park, Y.H., Kim, K.S. *et al.* (2000) A tetrodotoxin-producing *Vibrio* strain, LM-1, from the puffer fish (*Fugu vermicularis radiates*). *Appl Environ Microbiol* **66**, 1698–1701.

Lehman, E.M., Brodie, E.D. Jr and Brodie, E.D.I.I.I. (2004) No evidence for an endosymbiotic origin of tetrodotoxin in the newt *Taricha granulosa*. *Toxicon* **44**, 243–249.

Leland, J., Kirakosyan, C.A., Kaufman, P.B., Warber, S. and Duke, J.A., Brielmann, H.L. (eds) (2006) Plant Biotechnology for the production of natural products. In: *Natural Products from Plants*, 2nd edn. pp. 237. Boca Raton, FL: CRC Press, Taylor and Francis group.

Lysko, P.G., Webb, C.L., Yue, T.L., Gu, J.L. and Feuerstein, G. (1994) Neuroprotective effects of tetrodotoxin as a Na+ channel modulator and glutamate release inhibitor in cultured rat cerebellar neurons and in gerbil global brain ischemia. *Stroke* **25**, 2476–2482.

Mansson, M., Gram, L. and Larsen, T.O. (2012) Production of bioactive secondary metabolites by marine Vibrionaceae. *Mar Drugs* **9**, 1440–1468.

Maran, B.A.V., Iwamoto, E., Okuda, J., Matsuda, S., Taniyama, S., Shida, Y., Asakawa, M., Ohtsuka, S. *et al.* (2007) Isolation and characterization of bacteria from the copepod *Pseudocaligus fugu* ectoparasitic on the panther puffer *Takifugu pardalis* with the emphasis on TTX. *Toxicon* **50**, 779–790.

Matsui, T., Hamada, S. and Konosu, S. (1981) Difference in accumulation of puffer fish toxin and crystalline tetrodotoxin in the puffer fish, *Fugu rubripes rubripes*. *Bull Jpn Soc Sci Fish* **47**, 535–537.

Matsui, T., Yamamori, K., Chinone, M., Takatsuka, S., Sugiyama, S., Sato, H. and Shimizu, C. (1985) Development of toxicity in cultured puffer fish kept with wild puffer fish-I. Development of toxicity in cultured Kusahugu (*Fugu niphobles*). In *Proceedings of the Annual Conference of the Japanese Society of Scientific Fisheries*. pp. 156. Japan: Japanese Society of Scientific Fisheries.

Matsui, T., Taketsugu, S., Kodama, K., Ishii, A., Yamamori, K. and Shimizu, C. (1989) Production of tetrodotoxin by the intestinal bacteria of a puffer fish (*Takifugu niphobles*). *Nippon Suisan Gakkaishi* **55**, 2199–2203.

Moczydlowski, E.G. (2013) The molecular mystique of tetrodotoxin. *Toxicon* **63**, 165–183.

Mosher, H.S. and Fuhrman, F.A. (1984) Occurrence and origin of tetrodotoxin. In *Seafood Toxins* ed. Ragelis, E.P. pp. 333–344. Washington, DC: American Chemical Society.

Moustafa, A., Loram, J.E., Hackett, J.D., Anderson, D.M., Plumley, F.G. and Bhattacharya, D. (2009) Origin of saxitoxin biosynthetic genes in cyanobacteria. *PLoS ONE* **4**, e5758.

Nakashima, K., Arakawa, O., Taniyama, S., Nonaka, M., Takatani, T., Yamamori, K., Fuchi, Y. and Noguchi, T. (2004) Occurrence of saxitoxins as a major toxin in the ovary of a marine puffer *Arothron firmamentum*. *Toxicon* **43**, 207–212.

Narahashi, T. (2001) Pharmacology of tetrodotoxin. *J Toxicol – Tox Rev* **20**, 67–84.
Narahashi, T. (2008) Tetrodotoxin: a brief history. *Proc Jpn Acad Ser B Phys Biol Sci* **84**, 147–154.
Narahashi, T., Deguchi, T., Urakawa, N. and Ohkubo, Y. (1960) Stabilization and rectification of muscle fiber membrane by tetrodotoxin. *Am J Physiol* **198**, 934–938.
Narita, H., Matsubara, S., Miwa, N., Akahane, S., Marukami, M., Goto, T., Nara, M., Noguchi, T. *et al.* (1987) *Vibrio alginolyticus*, a TTX-producing bacterium isolated from the Starfish (*Astropecten polycanthus*). *Nippon Suisan Gakkaishi* **53**, 617–621.
Ngy, L., Tada, K., Yu, C.F., Takatani, T. and Arakawa, O. (2008) Occurrence of paralytic shellfish toxins in Cambodian Mekong pufferfish *Tetraodon turgidus*: selective toxin accumulation in the skin. *Toxicon* **51**, 280–288.
Noguchi, T. (1988) Food chain-associated toxification of tetrodotoxin-bearing animals. In *Recent Advances in Tetrodotoxin Research* ed. Hashimoto, K. pp. 85–93. Tokyo: Koseisha-Koseikaku.
Noguchi, T. and Arakawa, O. (2008) Tetrodotoxin - distribution and accumulation in aquatic organisms, and cases of human intoxication. *Mar Drugs* **6**, 220–242.
Noguchi, T., Narita, H., Maruyama, J. and Hashimoto, K. (1982) Tetrodotoxin in the starfish *Astropecten polyacanthus*, in association with toxification of a trumpet shell, "boshubora", *Charonia sauliae*. *Bull Jpn Soc Sci Fish* **48**, 1173–1177.
Noguchi, T., Jeon, J.K., Arakawa, O., Sugita, H., Deguchi, Y., Shida, Y. and Hashimoto, K. (1986) Occurrence of tetrodotoxin and anhydrotetrodotoxin in *Vibrio* sp. isolated from the intestines of a xanthid crab (*Atergatis floridus*). *J Biochem* **99**, 311–314.
Noguchi, T., Hwang, D.F., Arakawa, O., Sugita, H., Deguchi, Y., Shida, Y. and Hashimoto, K. (1987) *Vibrio alginolyticus*, a tetrodotoxin-producing bacterium, in the intestines of the fish (*Fugu vermicularis vermicularis*). *Mar Biol* **94**, 625–630.
Noguchi, T., Arakawa, O. and Takatani, T. (2006) Toxicity of pufferfish *Takifugu rubripes* cultured in netcages at sea or aquaria on land. *Comp Biochem Physiol Part D Genomics Proteomics* **1**, 153–157.
Overy, D.P., Zidorn, C., Petersen, B.O., Duus, J., Dalsgaard, P.W., Larsen, T.O. and Phipps, R.K. (2005) Medium dependant production of corymbiferone a novel product from *Penicillium hordei* cultured on plant tissue agar. *Tetrahedron Lett* **46**, 3225–3228.
Pettit, R.K. (2011) Culturability and secondary metabolite diversity of extreme microbes: expanding contribution of deep sea and deep-sea vent microbes to natural product discovery. *Mar Biotechnol* **13**, 1–11.
Ritchie, K.B., Nagelkerken, I., James, S. and Smith, G.W. (2000) Environmental microbiology: a tetrodotoxin-producing marine pathogen. *Nature* **404**, 354.
Saitanu, K., Laobhripatr, S., Limpakanjanarat, K., Sangwanloy, O., Sudhasaneya, S., Anuchatvorakul, B. and Leelasitorn, S. (1991) Toxicity of the freshwater puffer fish *Tetraodon fangi* and *T. palembangensis* from Thailand. *Toxicon* **29**, 895–897.
Saito, T., Noguchi, T., Harada, T., Murata, O. and Hashimoto, K. (1985) Tetrodotoxin as a biological defense agent for puffers. *Nippon Suisan Gakkaishi* **51**, 1175–1180.
Sato, S., Kodama, M., Ogata, T., Saitanu, K., Furuya, M., Hirayama, K. and Kakinuma, K. (1997) Saxitoxin as toxic principle of a freshwater puffer, *Tetraodon fangi*, in Thailand. *Toxicon* **35**, 137–140.
Sato, S., Ogata, T., Borja, V., Gonzales, C., Fukuyo, Y. and Kodama, M. (2000) Frequent occurrence of paralytic shellfish poisoning toxins as dominant toxins in marine puffer from tropical water. *Toxicon* **38**, 1101–1109.
Sato, C., Ueno, Y., Asai, K., Takahashi, K., Sato, M., Engel, A. and Fujiyoshi, Y. (2001) The voltage-sensitive sodium channel is a bell-shaped molecule with several cavities. *Nature* **409**, 1045–1051.
Scherlach, K. and Hertweck, C. (2009) Triggering cryptic natural product biosynthesis in microorganisms. *Org Biomol Chem* **7**, 1753–1760.
Schwartz, D.M., Duncan, K.G. and Duncan, J.L. (1998) Experimental use of tetrodotoxin for corneal pain after excimer lazer keratectomy. *Cornea* **17**, 196–199.
Shwab, E.K. and Keller, N.P. (2008) Regulation of secondary metabolite production in filamentous actiomycetes. *Mycol Res* **112**, 225–230.
Silva, M., Azevedo, J., Rodriguez, P., Alfonso, A., Botana, L.M. and Vasconcelos, V. (2012) New gastropod vectors and tetrodotoxin potential expansion in temperate waters of the atlantic ocean. *Mar Drugs* **4**, 712–726.
Simidu, U., Noguchi, T., Hwang, D.F., Shida, Y. and Hashimoto, K. (1987) Marine bacteria which produce tetrodotoxin. *Appl Environ Microbiol* **53**, 1714–1715.
Simidu, U., Tsukamoto, K.K., Yasumoto, T. and Yotsu, M. (1990) Taxonomy of four marine bacterial strains that produce tetrodotoxin. *Int J Syst Bacteriol* **40**, 331–336.
Song, H., Li, J., Lu, C.L., Kang, L., Xie, L., Zhang, Y.Y., Zhou, X.B. and Zhong, S. (2011) Tetrodotoxin alleviates acute heroin withdrawal syndrome: a multicentre, randomized, double-blind, placebo-controlled study. *Clin Exp Pharmacol Physiol* **38**, 510–514.
Sugita, H., Ueda, R., Noguchi, T., Arakawa, O., Hashimoto, K. and Deguchi, Y. (1987) Identification of a tetrodotoxin-producing bacterium isolated from the xanthid crab (*Atergatis floridus*). *Nippon Suisan Gakkaishi* **53**, 1693.
Thuesen, E.V. and Kogure, K. (1989) Bacterial production of tetrodotoxin in four species of chaetognatha. *Biol Bull* **176**, 191–194.
Tsuruda, K., Arakawa, O. and Noguchi, T. (2001) Toxicity and toxin profiles of the newt, *Cynops pyrrhogaster* from western Japan. *J Nat Toxins* **10**, 79–89.

Tsuruda, K., Arakawa, O., Kawatsu, K., Hamano, Y., Takatani, T. and Noguchi, T. (2002) Secretory glands of tetrodotoxin in the skin of the Japanese newt *Cynops pyrrhogaster*. *Toxicon* **40**, 131–136.

Wang, J. and Fan, Y. (2010) Isolation and characterization of a *Bacillus* species capable of producing tetrodotoxin from the puffer fish *Fugu obscurus*. *World J Microbiol Biotechnol* **26**, 1755–1760.

Wang, X.J., Yu, R.C., Luo, X., Zhou, M.J. and Lin, X.T. (2008) Toxin-screening and identification of bacteria isolated from highly toxic marine gastropod, *Nassarius semiplicatus*. *Toxicon* **52**, 55–61.

Wang, J., Fan, Y. and Yao, Z. (2010) Isolation of a *Lysinibacillus fusiformis* strain with tetrodotoxin-producing ability from puffer fish *Fugu obscurus* and the characterization of this strain. *Toxicon* **56**, 640–643.

Wang, M.H., Li, X.M., Li, C.S., Ji, N.Y. and Wang, B.G. (2013) Secondary metabolites from *Penicillium pinophilum* SD-272, a marine sediment-derived fungus. *Mar Drugs* **11**, 2230–2238.

Wu, Z., Xie, L., Xia, G., Zhang, J., Nie, Y., Hu, J., Wang, S. and Zhanga, R. (2005a) A new tetrodotoxin-producing actinomycete, (*Nocardiopsis dassonvillei*), isolated from the ovaries of puffer fish (*Fugu rubripes*). *Toxicon* **45**, 851–859.

Wu, Z., Yang, Y., Xie, L., Xia, G., Hu, J., Wang, S. and Zhang, R. (2005b) Toxicity and distribution of tetrodotoxin-producing bacteria in puffer fish (*Fugu rubripes*) collected from the Bohai Sea of China. *Toxicon* **46**, 471–476.

Yan, Q., Yu, P. and Li, H. (2005) Detection of tetrodotoxin and bacterial production by *Serratia marcescens*. *World J Microbiol Biotechnol* **21**, 1255–1258.

Yang, G., Xu, J., Liang, S., Ren, D., Yan, X. and Bao, B. (2010) A novel TTX-producing Aeromonas isolated from the ovary of *Takifugu obscurus*. *Toxicon* **56**, 324–329.

Yasumoto, T., Yasumura, D., Yotsu, M., Michishita, T., Endo, A. and Kotaki, Y. (1986) Bacterial production of tetrodotoxin and anhydrotetrodotoxin. *Agric Biol Chem* **50**, 793–795.

Yotsu, M., Yamazaki, T., Meguro, Y., Endo, A., Murata, M., Naoki, H. and Yasumoto, T. (1987) Production of tetrodotoxin and its derivatives by *Pseudomonas* sp. isolated from the skin of a pufferfish. *Toxicon* **25**, 225–228.

Yotsu, M., Gilhen, J., Russell, R.W., Krysko, K.L., Melaun, C., Kurz, A., Kauferstein, S., Kordis, D. *et al.* (2012) Variability of tetrodotoxin and of its analogues in the red-spotted newt, *Notophthalmus viridescens* (Amphibia: Urodela: Salamandridae). *Toxicon* **59**, 257–264.

Yu, C.F., Yu, P.H.F., Chan, P.L., Yan, Q. and Wong, P.K. (2004) Two novel species of tetrodotoxin-producing bacteria isolated from toxic marine puffer fishes. *Toxicon* **44**, 641–647.

Yu, V.C.H., Yu, P.H.F., Ho, K.C. and Lee, F.W.F. (2011) Isolation and identification of a new tetrodotoxin-producing bacterial species, *Raoultella terrigena*, from Hong Kong marine puffer fish *Takifugu niphobles*. *Mar Drugs* **9**, 2384–2396.