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Bacterial diversity and tetrodotoxin analysis in the viscera of the gastropods from Portuguese coast

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ABSTRACT

To trace the pathway of tetrodotoxin (TTX) producing microorganism in the Atlantic coast of Portugal, culture-dependent evaluation of the bacterial isolates from the viscera of the gastropods *Monodonta lineata, Gibbula umbilicalis, Nucella lapillus* and *Patella intermedia*, and from the environmental samples (biofilm and surrounding sea water) was carried out. Samples were collected from eight different coastal locations of Northern Portugal. A total of 311 isolates were identified. The observed bacterial diversity was distributed over five different classes (Gammaproteobacteria, Alphaproteobacteria, Flavobacteria, Bacilli and Actinobacteria) with the greatest number of 16S rRNA gene sequence derived from the Gammaproteobacteria (75%). Phylogenetic analysis based on the 16S rRNA gene showed that bacterial isolates were highly diverse and most of which were found in other marine environment. Among the different species isolated, *Vibrio* was found abundant. Eventhough TTX was not detected (UPLC-MS/MS) in the isolates from this study, PCR screening identified some natural product biosynthesis genes (PKS and NRPS) involved in its assembly. Further PCR screening of the TTX producing two ATCC *Vibrio* sp. reveals that NRPS might be involved in the biosynthesis of TTX through the incorporation of arginine.

1. Introduction

Tetrodotoxin (TTX), commonly known as puffer fish toxin is named after the fish, tetraodontidae (Tetraodon puffer fish). Puffer fish is the most recognizable living organism that contains TTX. TTX is a non-protein small molecule with a low molecular weight (319 $gmol^{-1}$), which blocks the voltage gated sodium channels (VGSCs) in the nerve cell membrane. It is widely distributed in phylogenetically different marine and terrestrial organism from 14 different phyla (Chau et al., 2011; Pratheepa and Vasconcelos, 2013). The wide distribution of TTX among the genetically diverse animals, showing regional, seasonal and individual variability in toxin analogues and the concentrations makes the origin of TTX as one of the controversial topics. This diversified occurrence indicates that it has an exogenous origin and it is proposed that bacteria are responsible for TTX production, with the host accumulating TTX symbiotically or via food chain transmission (Noguchi and Arakawa, 2008). Supporting this hypothesis many TTX producing bacteria have been isolated from the TTX bearing animals. The occurrence of TTX producing bacteria is not restricted to symbiotic life as there are reports of their occurrence in either marine or fresh water sediments (Do et al., 1991; Yasumoto et al., 1986) and marine suspended sinking particles (Hamasaki et al., 1994). At the initial stage, TTX producing organisms was found in Indo–Pacific regions only. Besides this geographical limitation a visible increase of TTX vectors such as *Lagocephalus lagocephalus* (Saoudi et al., 2011), *Lagocephalus sceleratus* (Katikou et al., 2009; Rodríguez et al., 2012), *Charonia lampas lampas* (Rodriguez et al., 2008; Silva et al., 2012) occur in the Mediterranean waters and the Atlantic Ocean. The migration of these species may be due to the increase in global temperature (Lasram and Mouillot, 2008) which influenced the growth rate of the toxic organisms and also toxin production rates (Ashton et al., 2003; Chinain et al., 1999).

TTX-producing bacteria have been isolated from various marine organisms and therefore they have been considered as one of the possible sources for TTX production in many TTX-bearing marine organisms. In 1986, *Vibrio* sp. (isolated from *Atergatis floridus*) and *Pseudomonas* sp. (isolated from red alga, *Jania* sp.) were reported to be the TTX producers. Thereafter, numerous bacterial strains with the capability of producing TTX were isolated and identified. The

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number and types of TTX-producing bacterial strains have been increasing and most of the reported strains belonged to the genus *Vibrio*. Other than the *Vibrio*, considerable bacterial species including *Pseudomonas*, *Bacillus*, *Aeromonas*, *Actinomyces*, *Serratia*, *Microbacterium* and *Shewanella*, were commonly reported to produce TTX. For the past two decades, more than 23 different TTX-producing bacterial strains have been isolated and identified from various organs of TTX vectors. From puffer fish, 14 different TTX producing bacterial genera and 7 from gastropods have been isolated. Because of the presence of TTX in genetically diverse organisms, it is proposed that the accumulation of TTX may be due to the horizontal gene transfer (HGT) through the food chain of TTX producing bacteria.

The total synthesis of TTX has been successfully carried out (Hinman and Du Bois, 2003; Kishi et al., 1972; Ohyabu et al., 2003; Sato et al., 2008). The structure of TTX contains a guanidium moiety attached to the oxygenated carbon backbone. A number of TTX biosynthetic pathways have been proposed (Kotaki and Shimizu, 1993; Shimizu and Kobayashi, 1983; Yasumoto et al., 1988). Chau et al. (Chau et al., 2011) presumed that incorporation of a guanidinium moiety in TTX can be through amidinotransferase (AMT) or non-ribosomal peptide synthetase (NRPS) module incorporating arginine. NRPSs are able to incorporate non-proteogenic amino-acids with biosynthesis enzymes such as PKSs (Cane and Walsh, 1999; Chau et al., 2011), this (PKS-NRPS) enzyme complex are also involved in the biosynthesis of toxins (Bergmann et al., 2007; Schümann and Hertweck, 2007). The genes coding for the TTX biosynthesis enzymes are clustered on the genome of the toxin producers (Chau et al., 2013, 2011).

In the present investigation, we have used gastropods (*Monodonta lineata*, *Gibbula umbilicalis*, *Nucella lapillus* and *Patella intermedia*) collected from the Atlantic coastal area of Portugal for the isolation of the bacteria present in their viscera and to screen the ability of the bacteria to produce TTX. Among the four gastropods *M. lineata* and *G. umbilicalis* (family Trochidae) are reported to have TTX. Their habitat ranges from high intertidal to deep sea and in shallow waters, and they are generally herbivores or detritivores (Williams et al., 2010). *P. intermedia* belong to the family Patellidae. The common limpet *Patella* sp. occur in intertidal rocks of North-western Europe and are grazers attached to the macroalgae by feeding on their mature thalli (Lorenzen, 2007). *N. lapillus*, belongs to the family Muricidae and feed on mussels, barnacles, bivalves, limpets, top purple shell *Gibulla* (Gosselin and Chia, 1994).

This study describes the isolation of bacteria from the gastropods and its surrounding environment (biofilm and seawater). The bacterial isolates were also screened for the biosynthesis genes (PKS and NRPS) and for the TTX production through UPLC -MS/MS. Two bacterial samples *Vibrio alginolyticus* (ATCC 17749) and *Vibrio parahaemolyticus* (ATCC 17802) previously reported to produce TTX (Simidu et al., 1987) was used as reference standard for the analysis of biosynthesis genes (PKS, NRPS and AMT) and the quantification of TTX through UPLC-MS/MS.

2. Materials and methods

2.1. Sample collection and processing

Samples were collected from distinct geographical locations of the Atlantic rocky shore of North-western coast of Portugal from February 2012 to January 2013. The sampling locations were chosen due to their large extension of rocky intertidal substrate which affords an ideal habitat for the marine gastropods. Sampling was carried out on monthly intervals at the referred beaches on their corresponding low tide hours.

Specimens of live gastropod species such as *M. lineata, G. umbilicalis, N. lapillus* and *P. intermedia* were identified by field guides and collected in the sterile plastic bags. Along with gastropods, sea water and the biofilm surrounding the gastropods were also collected. Biofilm samples were obtained by scraping the rocky film with a sterile knife and collected in a sterile container.

From each gastropod species viscera ($n = 10$ from each sampling sites) was dissected by using the sterilized equipment (70% EtOH and flamed) and pooled together, and triturated in a sterile pestle. The obtained homogenate was 10 fold serially diluted (up to 10^{-3} dilution) in sterile marine water (filtered using 0.45 μm membrane filter). To procure desired bacterial growth, 100 μl of serially diluted homogenate was spread plated in petri dishes containing marine agar medium (Difco 2216). The inoculated bacteria were incubated for 4–5 days at 25 °C. Bacterial isolation was then achieved through macroscopical observation of differing colonial morphologic characteristics (form, elevation, margin, opacity, chromogenic and surface). Each unique colony was further sub cultured for four times to ensure that it was a pure culture before using for bacterial identification. The individual colonies were incubated in Difco marine broth for 3 days at 25 °C. The single colonies were stored at −20 °C for further use.

2.2. 16S rRNA gene sequence – based identification of active isolates

2.2.1. DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted using the kit PureLinkTM Genomic DNA Mini Kit (INVITROGEN), following the instructions of the manufacturer. The extracted genomic DNA was used for the amplification of bacterial 16S ribosomal RNA (rRNA). The PCR amplification of the 16S rRNA gene was carried out with the primer pair 8F and 1492R(I) (Turner et al., 1999). Reactions were performed in 50 μl volume, with 5 μl of 5X reaction buffer (promega), 5 μl of 2.5 mM DNTPs, (CITOMED), 2.5 μl of 25 mM $MgCl_2$ (promega), 2.5 μl of 10 mM each primer, 0.1 μl of 5 U/ml TAQ DNA polymerase (promega) and 2.5 μl of genomic DNA. Amplification reaction was performed on the thermocycler (Biometra Professional multigradient thermocycler) under the following conditions: initial denaturation at 94 °C for 10 min, followed by 30 cycles of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 2 min, and a final extension step at 72 °C for 10 min. The amplified products (ca.1400 bp) were confirmed by gel electrophoresis (1% agarose gel in 1X TAE buffer). Amplified products were directly purified with PCR Purification Combo Kit (INVITROGEN) and sent to Macrogen for sequencing in both directions using the same PCR primers. The sequences were submitted in the Genbank with the accession numbers KF009746–KF009883, KT185283–KT185450.

2.2.2. PCR screening of bacterial isolates for biosynthesis genes

The isolated bacterial samples were screened for the biosynthesis genes. PKS and NRPS genes were amplified by using the degenerate primers DKF/DKR (Moffitt and Neilan, 2001) and MTF/MTR (Neilan et al., 1999) respectively. AMT genes were amplified using the degenerate primers ATF/ATR (Kellmann, 2005). Amplifications were carried out at the following condition: initial denaturation at 92 °C for 2 min, 35 cycles of 92 °C for 10 s, annealing temperature as given in (Table 2) for 30 s, 72 °C for 1 min, followed by a final extension of 72 °C for 7 min. The anticipated sizes for the PCR amplified product were 700 bp for PKS and AMT; 1000 bp for NRPS products. The amplified products were gel purified (Macherey-Nagel) and ligated into the pGEM®-T Easy Vector