

Research Article

Cytotoxicity of actinomycetes associated with the ascidian *Eudistoma vannamei* (Millar, 1977), endemic of northeastern coast of Brazil

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ABSTRACT. Previous studies demonstrated that the crude extract of the ascidian *Eudistoma vannamei*, endemic from northeastern Brazil, strongly hinders growth of tumor cells *in vitro* by inducing apoptosis due to tryptophan derivatives, which are commonly found in bacteria. This study presents a bioactivity-guided screening among actinomycetes, associated with *E. vannamei*, aiming at recognizing active principles with biological relevance. Twenty strains of actinomycetes, designated as EVA 0101 through 0120, were isolated from colonies of *E. vannamei* among which 11 were selected for cytotoxicity evaluation. The extracts from EVA 0102, 0103, 0106, 0109 and 0113 were the most active, and were further studied for IC₅₀ determination and chemical analysis by ¹H NMR. IC₅₀ values obtained ranged from 3.62 µg mL⁻¹ (for EVA 0109 in leukemia cells) to 84.65 µg/mL (for EVA 0106 in melanoma cells). All active extracts exhibited the same TLC and spectroscopic profiles, suggesting the presence of quinones and other related secondary metabolites. Furthermore, these strains were identified and compared based on their respective 16S rRNA sequences. The results herein identified the five strains as *Micromonospora* spp. while phylogenetic analysis suggests that they are possibly two different *Micromonospora* species producing the cytotoxic compounds.

Keywords: marine biotechnology, *Eudistoma vannamei*, marine microorganisms, *Micromonospora* sp., Brazil.

Citotoxicidad de actinomicetos asociada a la ascidia *Eudistoma vannamei* (Millar, 1977), endémica de la costa noreste de Brasil

RESUMEN. Estudios previos demostraron que el extracto crudo de la ascidia *Eudistoma vannamei*, endémica de la costa noreste de Brasil, obstaculiza fuertemente el crecimiento de células tumorales *in vitro* por inducir apoptosis. El análisis químico del extracto sugirió la presencia de derivados de triptófano, comúnmente encontrados en bacterias. El estudio presenta un screening de citotoxicidad en actinomicetos asociados con *E. vannamei*, para reconocer principios activos biológicamente relevantes. Veinte cepas de actinomicetos, designados como EVA 0101 hasta 0120, fueron aisladas de colonias de *E. vannamei* y 11 fueron seleccionadas para la evaluación de citotoxicidad. Los extractos de EVA 0102, 0103, 0106, 0109 y 0113 resultaron las más activas y fueron estudiadas para determinación de la CL₅₀ y perfiles de CCF y ¹H RMN. Las CL₅₀ obtenidas oscilaron entre 3,62 (EVA 0109 en las células de leucemia) y 84,65 µg mL⁻¹ (EVA 0106 en las células de melanoma). Los extractos activos presentan el mismo perfil de CCF y espectroscópico, lo que sugiere la presencia de quinonas y metabolitos secundarios relacionados. Además, las cinco cepas fueron identificadas y comparadas sobre la base de sus secuencias de 16S ARNr. Las que se identificaron como *Micromonospora* spp. y el análisis filogenético sugiere que sean por lo menos dos especies de *Micromonospora* las que producen los compuestos citotóxicos.

Palabras clave: biotecnología marina, *Eudistoma vannamei*, microorganismos marinos, *Micromonospora* sp., Brasil.

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INTRODUCTION

Colorful soft-bodied sessile marine invertebrates have been a rich source of natural products with biomedical properties for the past 60 years (Baily, 2009; Molinski *et al.*, 2009; Mayer *et al.*, 2010). Among them, the tunicates are one of the most prolific in terms of novel drug prototypes for the anticancer pharmaceutical arsenal (Blunt *et al.*, 2010, 2011). For instance, trabectedin, a tetrahydroisoquinoline alkaloid obtained from *Ecteinascidia turbinata*, was recently approved for the treatment of soft tissue sarcoma, with the commercial name Yondelis[®] (Cuevas & Francesh, 2009; D'Incalci & Galmarini, 2010). Additionally, plitidepsin, a cyclodepsipeptide isolated from *Aplidium albicans* is in advanced trial phase for the therapy of multiple myeloma and other malignant tumors, but has already received orphan drug status for the treatment of multiple myeloma and acute lymphoblastic leukemia (Ocio *et al.*, 2008; Singh *et al.*, 2008).

There are mounting evidences that the associated microorganisms are the authentic suppliers for most of the relevant compounds (Zhang *et al.*, 2005; Imhoff *et al.*, 2011). For the ascidians, in particular, there is a compelling amount of information pointing into this direction (Schmidt & Donia, 2010). Despite the oceans are acknowledged as a rich and highly complex microbiological environment, they have only shared their wealth as a natural drug source for the past 10 years. Thus, the study of marine microorganisms as sources of new drugs is still at its youth (Jensen *et al.*, 2005; Fenical & Jensen, 2006; Liu *et al.*, 2010).

This study takes part in a pioneer venture aiming to the exploration of the biotechnological potentials of microorganisms, mainly actinomycetes, from the coast of Ceará. The actinomycetes have proven to be a prolific source of new and bioactive compounds (Fenical & Jensen, 2006). The purpose of the present investigation is to find biologically active strains of actinomycetes and evaluate their respective cytotoxicity, while gathering information on the microbiota associated with *Eudistoma vannamei*. This species is endemic from the northeastern coast of Brazil and is the most abundant ascidian recorded for the state of Ceará. Previous studies have revealed that its crude extract strongly hinders growth of tumor cells *in vitro*,

promoting further apoptosis (Jimenez *et al.*, 2003, 2008).

MATERIALS AND METHODS

Sampling and isolation of bacteria strains

Specimens of *E. vannamei* were hand collected at Taiba Beach (03°30'21.23"S, 038°53'40.16"W), located on the northeastern coast of Brazil. Sampling was carried out with the slightest contamination possible, as the material was gathered and stored with sterile utensils. For isolation of microorganisms, five colonies were homogenized in sterile seawater 1:5 (m/v), and aliquots of 20 L were stroked onto Petri dishes containing different agar based media, which may favor actinomycetes growth (modified from Jensen *et al.*, 1991). The following agar-based media were used herein, all added with cycloheximide 0.1 mg mL⁻¹: SCA (Starch Casein Agar, HIMEDIA, prepared with distilled H₂O), AIA (Actinomycetes Isolation Agar, HIMEDIA, prepared in 75% seawater), SWA (Sea-water Agar, 1.8% agar in 75% seawater) and GYM (glucose 0.4%, yeast extract 0.4%, malt extract 1%, CaCO₃ 0.2%, 1.8% agar in 75% seawater).

Purity of strains were resolved by sequential re-strikes onto brand new agar plates and pure strains were inoculated in liquid A1 medium (10% soluble starch, 4% yeast extract and 2% peptone in 75% seawater) for culture maintenance and up-scaled growth (Jensen *et al.*, 1991). Grown media for each pure strain was sampled into cryovials and supplemented with 25% glycerol for storage at -70°C.

Up-scaled growth and extraction

Initially, pure cultures were inoculated in 500 mL Erlenmeyer flasks filled with 100 mL of liquid A1 medium. Flasks were kept at 28°C and 200 rpm for 5 days. Next, 10 mL of grown media were added to newly assembled Erlenmeyer flasks with 100 mL of fresh media and kept at 28°C and 200 rpm agitation for 10 days.

The resulting media was spun down to separate the bacterial biomass from the broth. Biomass was extracted with MeOH 1:1 (m/v) while broth was extracted in EtOAc 1:1 (v/v). The crude extracts were evaporated under vacuum, washed with MeOH to

remove excessive salt, and fully dried under compressed air, yielding the crude extracts. At this point, extracts were resuspended in DMSO to a final concentration of $10 \mu\text{g mL}^{-1}$ and were ready to be evaluated for their biological activity.

Screening for cytotoxicity-MTT assay

Cytotoxic activity of extracts was evaluated against four human tumor cell lines obtained from the National Cancer Institute (Bethesda, MD, USA): HL-60 (promyelocytic leukemia), MDA-MB-435 (melanoma), SF-295 (glioblastoma) and HCT-8 (colon carcinoma). Cells were grown in RPMI-1640 medium supplemented with 2 mM glutamine, 10% fetal calf serum, $100 \mu\text{g mL}^{-1}$ strep-tomycin and 100 U mL^{-1} penicillin and housed at 37°C with a 5% CO_2 atmosphere. The cell cultures were regularly split to keep them in a logarithmic phase of growth.

Firstly, in a qualitative approach, cell lines were exposed to $100 \mu\text{g mL}^{-1}$ of the extracts for 72 h and the effect on cell proliferation was evaluated *in vitro* using the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay, as described by Mosmann (1983). The extracts that reduced over 80% of cell growth, when compared to non-treated cells, were tested again, in a quantitative manner, however, at concentrations varying serially from 0.02 to $100 \mu\text{g mL}^{-1}$ to determine the IC_{50} (the concentration that inhibits growth in 50%), which was calculated, along with the respective 95% CI (confidence interval), by a non-linear regression using the software GraphPad Prism 5.0.

Preliminary chemical analysis

The active extracts (EVA 0102, EVA 0106, EVA 0109 and EVA 0113) were submitted to a preliminary chemical characterization. Initially, these extracts were compared by thin layer chromatography (TLC) on precoated silica gel aluminum sheets (Kieselgel 60 F₂₅₄, 0.20 mm, Merck) using two different solvent systems (CHCl_3 and CHCl_3 : EtOAc 1:1) and the compounds were detected by exposure to UV (312 and 365 nm) light and by spraying with vanillin/perchloric acid/EtOH solution followed by heating at 100°C . The extracts were also analyzed by ^1H NMR spectroscopy. The ^1H NMR spectra were acquired on a Bruker Avance DRX-500 spectrometer equipped with 5 mm inverse detection z-gradient probe. The chemical shifts, given on the δ scale, were referenced to the residual undeuterated CHCl_3 (δ_{H} 7.27) fraction. The methodology was based in a previous work published by Jimenez *et al.* (2008).

Molecular identification: nucleic acid extraction, 16S rRNA gene amplification, and sequencing

The selected microorganisms were characterized using a molecular approach based on the protocol described by Gontang *et al.* (2007) with slight modifications. Genomic DNA was extracted from a fresh, 3 to 5 day grown, bacterial culture following the DNeasy protocol (QIAGEN Inc., USA). Amplification of the 16S rRNA gene was carried out with Ilustra™ PureTaq Ready-To-Go PCR beads (GE Healthcare, UK) using the universal eubacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTACGACTT-3'). PCR products were purified with MiniElute PCR purification kit (QIAGEN Inc., USA).

DNA sequencing was performed by Macrogen Inc. (Seoul, Korea) using the ABI PRISM BigDye™ Terminator Cycle Sequencing kit (Applied Biosystems, USA) and the same primers used for amplification, following the protocols supplied by the manufacturer.

The nucleotide sequences were assembled, analyzed and manually edited using the BioEdit Sequence Alignment Editor (Hall, 1999) and compared to sequences within the NCBI database (<http://www.ncbi.nlm.nih.gov>) using the Basic Local Alignment Search Tool (BLAST).

The identity of the strain was confirmed after an evaluation of the phylogenetic relationships with the most similar sequences obtained from the EzTaxon server (Chun *et al.*, 2007). The sequences were aligned using Muscle ver. 3.6, and the reconstruction was performed using a combined bootstrapping and maximum likelihood approach through RAXML (Stamakis, 2006) on the CIPRES portal (Miller *et al.*, 2010). The tree was produced using Archaeopterix ver. 0.957 (Han & Zmasek, 2009). After the phylogenetic analysis, the sequences were submitted to NCBI GenBank.

RESULTS

Isolation and cytotoxicity screening of bacteria associated with *E. vancouverensis*

Twenty colonies were isolated from *E. vancouverensis* respecting their actinomycete-like distinctiveness. Out of those, 11 were elected for cytotoxicity evaluation on tumor cells. In the single concentration screening, extracts obtained from strains EVA 0102, EVA 0103, EVA 0106, EVA 0109 and EVA 0113 were the most active, reducing proliferation of HCT-8 and SF-295 cells in over 75% (Fig. 1).

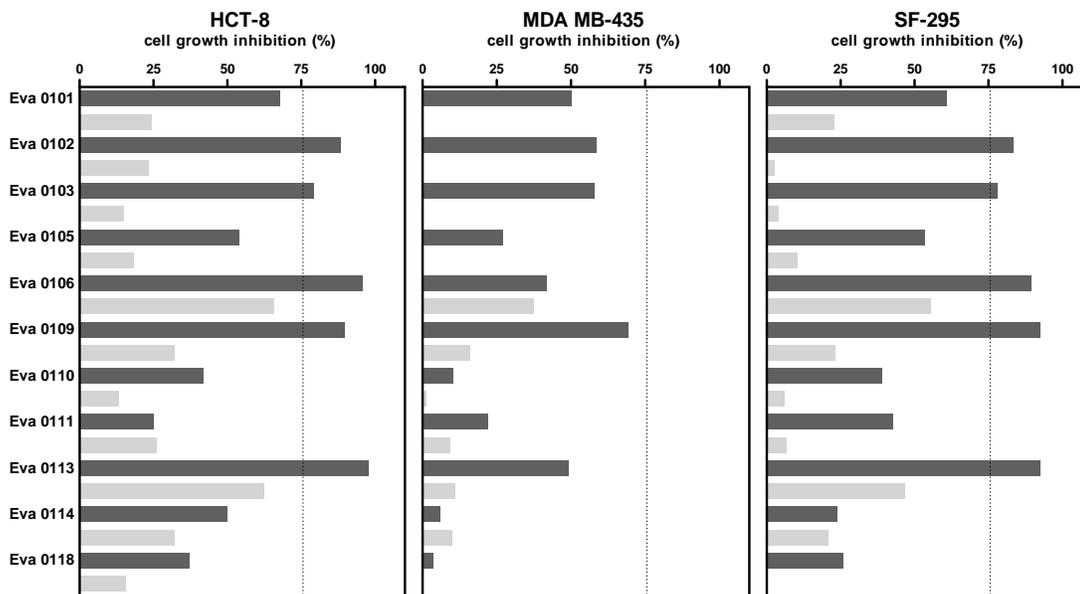


Figure 1. Qualitative cytotoxicity screening of EtOAc extracts from liquid medium (dark gray) and MeOH extracts from biomass (light gray) of bacterial strains isolated from *E. vannamei*. Extracts were tested ($100 \mu\text{g mL}^{-1}$) against HCT-8, MDA-MB-435 and SF-295 cell lines by the MTT assay after 72 h incubation. Data were obtained from two independent experiments and presented as a percentage of growth inhibition relative to untreated cells.

Table 1. Cytotoxic activity of EtOAc extracts obtained from bacterial strains isolated from *Eudistoma vannamei* evaluated by the MTT assay after 72 h incubation. IC_{50} values and their respective 95% confidence intervals were obtained by nonlinear regression on the GraphPad Prism 5.0 software (Intuitive Software for Science, San Diego, CA). N.T.: not tested.

Strain	Isolation media	IC_{50} ($\mu\text{g mL}^{-1}$)			
		HL-60	HCT-8	MDA MB-435	SF-295
EVA 0102	SCA	24.05	16.48	60.41	26.87
		14.81 - 39.06	13.22 - 20.55	49.20 - 74.17	17.77 - 40.65
EVA 0106	AIA	61.92	14.66	84.65	14.06
		32.85 - 116.7	7.831 - 27.44	64.17 - 111.7	8.243 - 23.96
EVA 0109	GYM	3.88	3.62	23.78	5.12
		2.43 - 6.20	0.44 - 0.67	19.86 - 28.48	4.43 - 5.91
EVA 0113	SWA	30.07	10.90	N.T.	9.97
		17.71 - 51.06	6.83 - 17.38		6.92 - 14.38

The IC_{50} was determined for the extracts obtained from EVA 0102, EVA 0106, EVA 0109 and EVA 0113, since the yield of the extract from EVA 0103 was insufficient for further analyses. The tested extracts showed a similar activity profile, with little variation in potency. As shown on Table 1, EVA 0109 yielded the most cytotoxic extract, with IC_{50} values varying from 3.62 to 23.78 $\mu\text{g mL}^{-1}$. EVA 0113

followed, with IC_{50} ranging from 9.97 to 30.07 $\mu\text{g mL}^{-1}$.

Chemical analysis of active extracts

The extracts from EVA 0102, EVA 0106, EVA 0109 and EVA 0113 were similar on TLC and, as expected, showed the same spectroscopic profile by ^1H NMR. For this reason, just one ^1H NMR spectrum was

depicted (Fig. 2). After a detailed analysis, it was clear the presence of chelated hydroxyl groups (δ 15.44–12.29), aromatic and/or olefine hydrogens (δ 8.41–5.92), hydrogens bound to oxygenated carbon (δ 3.64–3.54), as well as methylene, methyne and methyl protons (δ 2.34–0.83) (Silverstein *et al.*, 1991).

The aforementioned data were compatible with the presence of quinones or related secondary metabolites.

Identification and phylogenetic analyses

The analysis of the 16S rRNA from the 5 EVA bacterial strains revealed a high degree of similarity among them (Fig. 3). When included in a reconstruction of the phylogeny along with the most similar sequences found on Genbank, the EVA clade came out as the sister group of a clade formed by *Micromonospora echinospora*, *M. marina*, *M. aurantiaca*, *M. halophytica*, *M. humi*, *M. purpureochromogenes*, *M. coxensis*, *M. chalcea* and *M.*

tulbaghiae. The EVA clade was formed with a relatively high support value and EVA 0109, 0113, 0102 and 0106 showed a higher degree of overall similarity. For this clade a single contig was then generated and deposited on NCBI GenBank under accession number JN797618. The sequence from EVA 0103 is slightly different, so it was deposited with a separate accession number (JN797619), despite its 16S rRNA sequence is still more than 99.9% similar to the other EVA strains.

DISCUSSION

The study herein began with a screening for cytotoxicity among actinomycetes isolated from the tunicate *E. vannamei*. The active extracts showed identical ^1H NMR profiles and the respective strains were identified based on a molecular biology approach, where all five isolates were identified within the genus *Micromonospora*. The results pointed

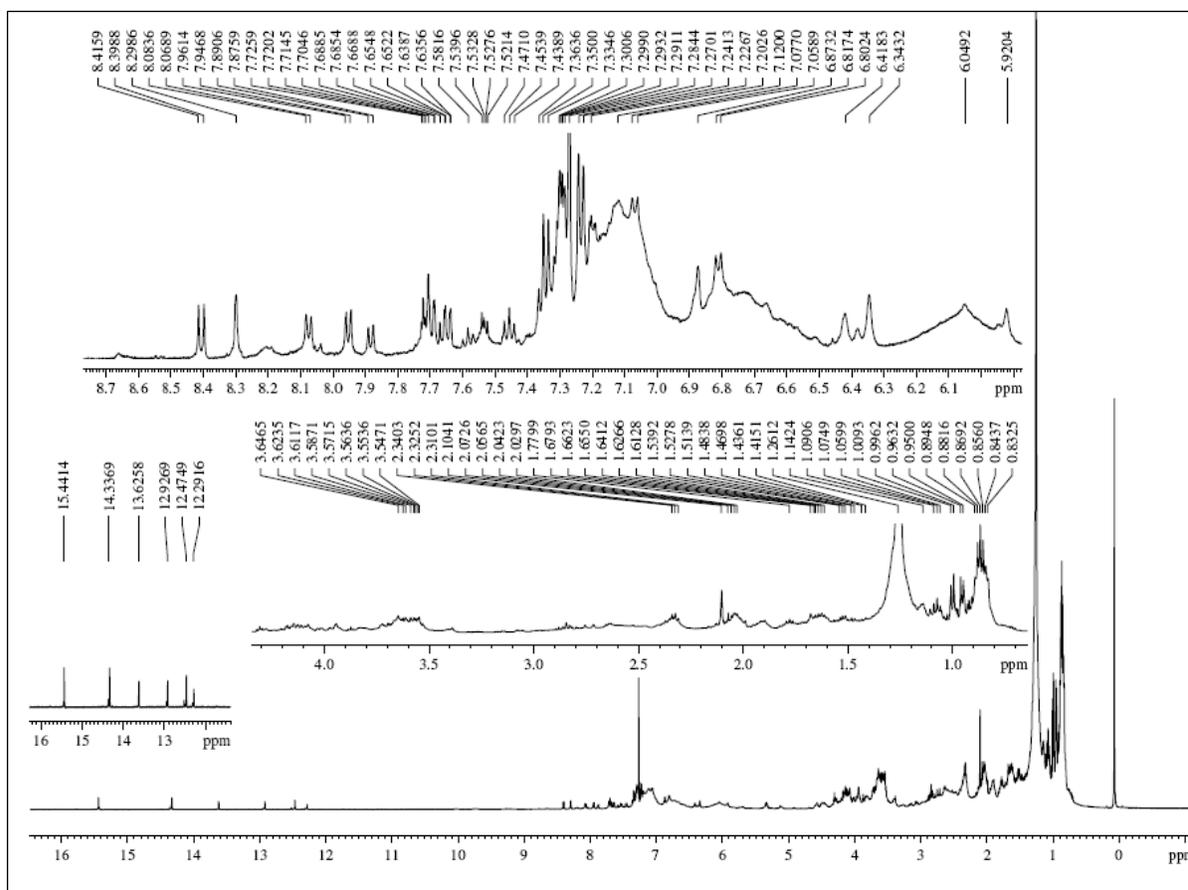


Figure 2. ^1H NMR spectrum (500 MHz, CDCl_3) of the EtOAc extract from EVA 0102.

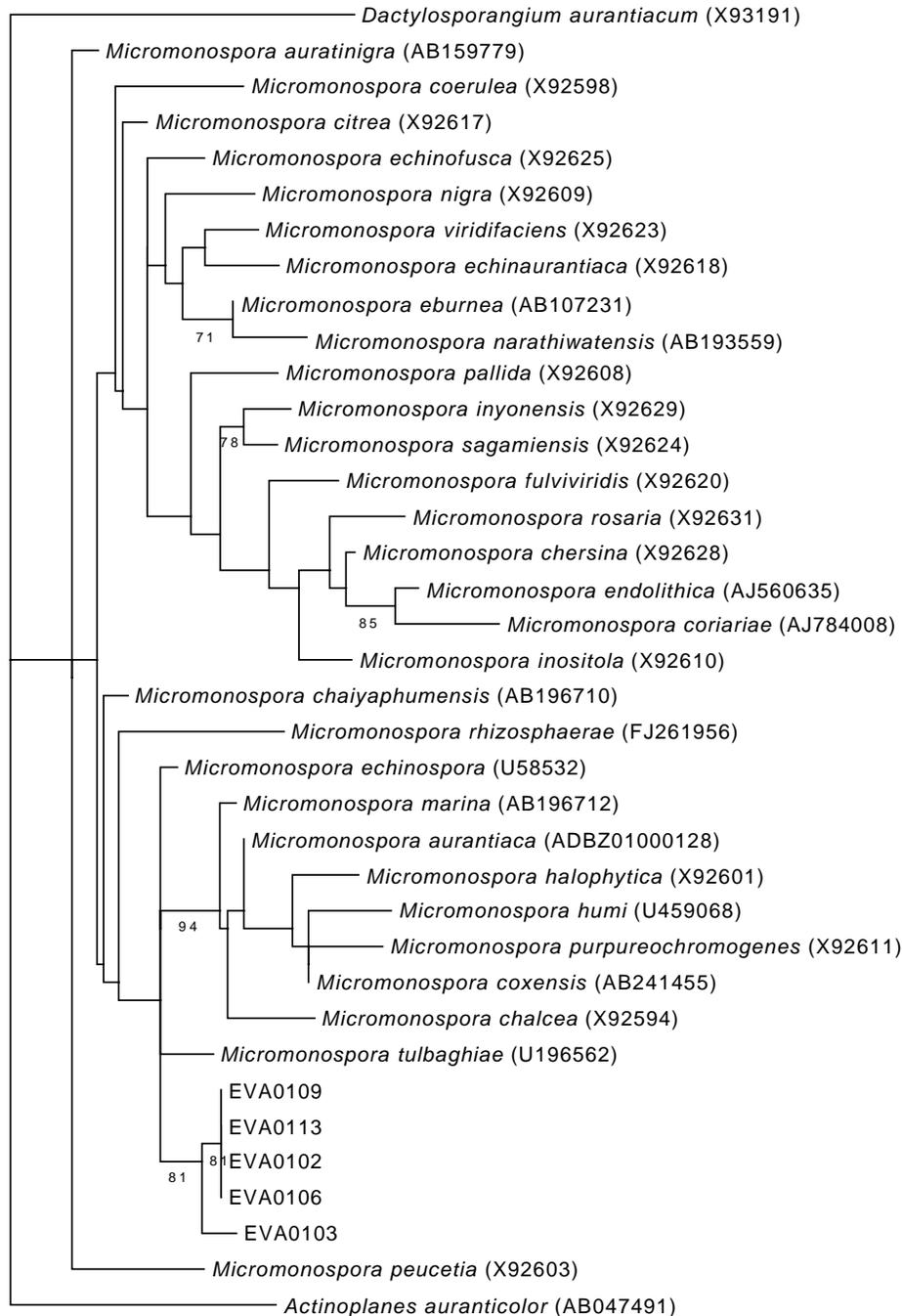


Figure 3. Maximum likelihood tree of the *Micromonospora* lineages isolated from *E. vannamei* and most similar species. *Dactylosporangium* and *Actinoplanes* were used as outgroups.

to at least two species of *Micromonospora* producing cytotoxic compounds associated with *E. vannamei*. *Micromonospora* are a well-known group of Gram-positive, spore forming microbes, and are considered the most abundant actinobacteria, along with *Rhodococcus* and *Streptomyces* in marine environments (Maldonado *et al.*, 2005). Additionally, they are

long accepted as an important source of antibiotics and other substances with commercial value. For instance, some of the best-known antibiotics, such as the aminoglycosides amikacin, gentamicin and netamicin, were initially isolated from these microorganisms (Bérdy, 2005). Furthermore, *Micromonospora* species with marine occurrence have

yielded many compounds with biomedical relevance. Thiocoraline is produced by a strain of *M. marina* isolated from a soft coral collected near the coast of Mozambique (Romero *et al.*, 1997). This cyclic thiopeptide showed strong cytotoxic activity against various tumor cell lines, with IC₅₀ around 2nM. Moreover, thiocoraline induced cell cycle perturbations due to inhibition of DNA-polymerase activity (Pérez-Baz *et al.*, 1997; Romero *et al.*, 1997; Erba *et al.*, 1999). Other example is diazepinomicin, a dibenzodiazepine alkaloid with antimicrobial properties isolated from a *Micromonospora* strain recovered from *Didemnum proliferum*, a tunicate collected off the Japanese coast (Charan *et al.*, 2004).

In fact, associations of *Micromonospora* and other actinomycetes with tunicates have been documented elsewhere (Menezes *et al.*, 2010). The Fijian tunicate *Polysyncraton lithostrotum* is the host to the then unidentified species *M. lomaivitiensis*, the producer of the potent anticancer compounds lomaivitins A and B (He *et al.*, 2001). These molecules also have antimicrobial activity, which is thought to scare off other bacteria (He *et al.*, 2001).

The results presented here, showing the cytotoxicity of a *Micromonospora* strain and describing its association with the Brazilian endemic ascidian *E. vannahmei*, highlight their potential as producers of relevant molecules. The chemical profile of *Micromonospora* extracts was different from the one observed by Jimenez *et al.* (2008) for *E. vannahmei* extracts. While the active compounds presented in ascidian extracts correspond to tryptophan derivatives, spectra data generated for the bacterial extracts herein hinted on the presence of quinoid-like compounds, such as anthracyclines or anthracyclinones (Pretsch *et al.*, 2000; Laatsch & Fotso, 2008). This data suggested that the isolated *Micromonospora* strain is not responsible for the production of ascidian bioactive compounds. Nonetheless, the activity was similar for both ascidian extract (IC₅₀ ranging from below 2.00 to 23.80 µg mL⁻¹, Jimenez *et al.*, 2008) and bacterial ones (IC₅₀ ranging from 3.62 to 84.65 µg mL⁻¹).

The occurrence of anthracyclines or related compounds withholding some sort of bioactivity is rather common within the *Micromonospora* genus. *M. lupini* yielded the anti-cell invasion anthraquinones lupinacidins A, B and C (Igarashi *et al.*, 2007, 2011). The anthracyclines micromonomycin (Yang *et al.*, 2004) and spartamicins A and B (Nair *et al.*, 1992), produced by different strains of *Micromonospora*, have antimicrobial and antifungal properties, respectively. Moreover, the previously mentioned lomaivitins are hybrid molecules flanked with naphthoquinone moieties (He *et al.*, 2001).

Finally, it can be concluded that actinomycetes isolated from the Brazilian endemic ascidian *E. vannahmei* are a promising source of bioactive compounds. Therefore, additional studies are in progress to resolve the active principles and to characterize the means by which they may exert cytotoxicity.

ACKNOWLEDGMENTS

This work has been supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP) and International Foundation for Science (IFS). The authors also wish to thank Silvana França, for technical assistance with the cell cultures, and M.Sc. José Gustavo de A. Lima for generating and editing the ¹H NMR spectra.

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Received: 16 May 2011; Accepted: 22 October 2012