Antibacterial and cytotoxic bioactivity of marine Actinobacteria from Loreto Bay National Park, Mexico

Bioactividad antibacterial y citotóxica de actinobacterias marinas del Parque Nacional Bahía de Loreto, México

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ABSTRACT

Production of bioactive compounds is intimately linked to the ecology of the producing organisms. Taking this into account, the objective of this study was to evaluate the bioactive properties of isolated Actinobacteria from sea sediments of a high biodiversity zone; under the hypothesis that the ecological characteristics of this site stimulate the presence of unique and bioactive strains that can be screened for new compounds with antibiotic and anticancer properties. The elected zone was the Loreto Bay National Park in the Gulf of California Mexico, a protected natural area, with high diversity of flora and fauna. The bioactive properties of strains from this area were different to that reported elsewhere. The cytotoxic activity tested by *in vitro* assays was present in 40% of the tested strains and antibacterial activity in 71% of all evaluated strains. This percentage of active strains resulted unusually high when it was compared to similar studies from other regions of the world. This supports the hypothesis of the influence of ecological characteristics of the area of study on the presence of unique and bioactive Actinobacteria. Thereby, the Actinobacteria community found in Loreto Bay, in the Gulf of California, which presented unusual bioactive properties, represents a potential source for obtaining novel compounds with antibacterial and anticancer activity.

Key words*:* Antibiotic activity, Gulf of California, Loreto Bay, Marine Actinobacteria, *Salinispora.*

RESUMEN

La producción de compuestos bioactivos está íntimamente ligada con la ecología de los organismos que los producen. Tomando esto en cuenta, el objetivo de este estudio fue evaluar las propiedades bioactivas de actinobacterias aisladas en el sedimento marino de una región con alta biodiversidad; bajo la hipótesis de que las características ecológicas de este sitio estimulan la presencia de cepas de actinobacterias únicas y bioactivas que pueden ser útiles en la búsqueda de nuevos compuestos con actividad antibiótica y anticancerígena. La zona de estudio elegida fue el Parque Nacional Bahía de Loreto en el Golfo de California. México, un área natural protegida, con alta diversidad de flora y fauna. Las propiedades bioactivas de las cepas procedentes de esta zona fueron diferentes a la reportada en otros sitios. La actividad citotóxica probada mediante ensayos *in vitro* estuvo presente en 40% de las cepas probadas y la actividad antibacterial en el 71% de todas las cepas evaluadas. Este porcentaje de cepas activas resultó inusualmente alto cuando se comparó con estudios realizados en otros sitios del mundo. Esto apoya la hipótesis de la influencia de las características ecológicas de la zona de estudio sobre la presencia de actinobacterias únicas y bioactivas. Por lo tanto, la comunidad de actinobacterias encontrada en Bahía de Loreto, en el Golfo de California, la cual presentó propiedades bioactivas inusuales, representa un recurso potencial para la obtención de nuevos compuestos con actividad antibiótica y anticancerígena.

Palabras clave*:* Actinobacterias marinas, actividad antibiótica, Bahía de Loreto, Golfo de California, *Salinispora.*

INTRODUCTION

Actinobacteria are the main source of bioactive compounds with medical importance known to date. Very important strains of this class, such as *Streptomyces*, have been isolated from terrestrial sources in the past. However, as of three decades ago, several bioactive Actinobacteria have been isolated from the marine environment (Kim *et al.*, 2006; Gontang *et al.*, 2007; Pommier *et al.,* 2007).

The Actinobacteria taxons isolated from the sea are the producers of several bioactive metabolites never seen in terrestrial taxons (Fenical & Jensen, 2006; Bull & Stach, 2007). Therefore, marine Actinobacteria have been recognized, up to now, as the most economically as well as biotechnologically valuable prokaryotes (Dharmaraj, 2010; Manivasagan *et al.*, 2013).

One of the most prolific marine Actinobacteria belongs to the *Salinispora* genus, which is the producer of several new compounds with high cytotoxic activity against HCT-116 cell line such as, arenicolide, salinosporamide A, staurosporine and arenimycin (Gerner & Meyskens, 2004; Williams *et al.*, 2007a, 2007b; Fenical *et al.*, 2009; Asolkar *et al.,* 2010). In fact, the salinosporamide A, is now in early clinical trials as potent anticancer agent (Fenical *et al.*, 2009).

A well-known and widely accepted explanation of the high bioactivity and the great difference among marine and terrestrial Actinobacteria is the presence of different environmental conditions between them. It is surmised that marine Actinobacteria, which had to adapt during evolution to more extreme environmental conditions, reflect this evolutionary history in their genetic and metabolic diversity and in the production of different kinds of bioactive compounds. Furthermore, marine Actinobacteria that are geographically isolated from the rest could also present different bioactivity due to the high rates of mutation and horizontal gene transference.

The Gulf of California, Mexico (also known as Sea of Cortez) is a semiclosed, highly productive body of water characterized as having abundant biological resources and a high level of endemism. In the Gulf of California sediment several Actinobacteria related to the genera *Actinomadura*, *Dietzia*, *Gordonia*, *Micromonospora*, *Nocardiopsis*, *Nonomuraea*, *Rhodococcus*, *Saccharomonospora*, *Salinispora*, *Streptomyces*, "*Solwaraspora*" and *Verrucosispora* (Maldonado *et al.*, 2009; Becerril-Espinosa *et al.*, 2013) have been isolated. In addition, several species considered marines autochthones such as *Micromonospora krabiensis* (strain AMS264, HQ877446, 99.06% of similarity), *Saccharomonospora marina* (strain AML899, HQ877432, 99.54% of similarity), *Streptomyces fenhuangensis* (strain AML250, 98.71% similarity), *Verrucosispora maris* (strain AMS604, HQ877435, 99.42% of similarity) and *Verrucosispora sediminis* (strain AMS180, 98.76% of similarity) have been found; moreover, in the Gulf of California, new taxons have been discovered, including two restricted marine taxons related to the genera *Streptomyces* and *Salinispora* (Becerril-Espinosa *et al.*, 2013).

The Gulf of California still possesses ecosystems not yet impacted by anthropogenic activity such as Loreto Bay, which is considered a natural protected area and categorized as National Park (Diario Oficial, 2002). It is feasible to find marine biota in Loreto Bay that is not found in any other place in the world. As such, we test the hypothesis that Actinobacteria strains from Loreto Bay National Park possess different bioactivity properties to the ones isolated from other regions of the world.

MATERIALS AND METHODS

In this research, marine Actinobacteria strains previously isolated and identified according to 16S rRNA in Becerril-Espinosa *et al.* (2013) were used. The strains were isolated from 4 different locations in Loreto Bay: site S1 is named mainland beach (25° 50.2'N, 111° 19.4'W (Juncalito) and 25° 43.3'N, 111° 14.6'W (Ensenada Blanca); site S2 is

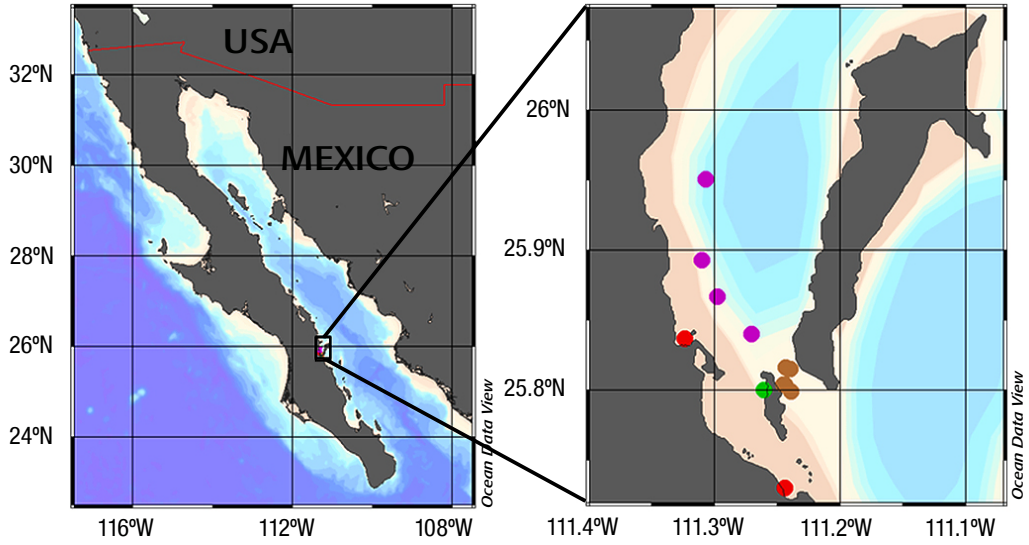


Figure 1. Map of the Gulf of California in the left. Enlargement of Loreto Bay area in the right. Sampling sites as follow: red circles indicates sites of the area 1(S1), green circles indicate sites of the area 2 (S2), purple circles indicates sites of the area 3 (S3) and brown circles indicate sites of the area 4 (S4).

named island beach (25° 48'N, 111° 15.35'W); site S3 is named Danzante Island Northwest zone (25° 57.0'N, 111° 18.37'W to 25° 48'N 111° 15.42'W) and site S4 was named Danzante Island East zone (25° 48.91'N, 111° 14.36'W to 25° 47.95'N, 111° 14.32'W). Sites S1 and S2 are located near the shoreline at very shallow depths (0 -15 m) while sites S3 and S4 were located in deeper sites of the national park (200-300 m) (Fig. 1). The isolation was performed using the plate stamping technique (Mincer *et al.,* 2002). The 16S rRNA sequences were taken from GenBank (http://www.ncb.nlm.nih.gov/genbank/index.html) under the accession numbers HQ873926-HQ873952 and HQ877423-HQ877448.

A total of 216 Actinobateria were selected for this study. According to Becerril-Espinosa *et al.* (2013) these strains were grouped as follows: Group I (29 strains) bacteria with mycelium substrate morphology and with no requirement of seawater for growth (*Micromonospora*), Group II (127 strains) strains with mycelium substrate morphology and with a requirement of seawater for growth (*Salinispora*) and Group III (60 strains) strains with aerial mycelium and no requirement of seawater for growth (*Streptomyces*).

Phylogenetic analysis for bioactive Actinobacteria. 16S rRNA gene sequences of bioactive strains (1300 bp) were assembled and analyzed using BLAST (Basic Local Alignment Search Tools) (Altschul *et al*., 1990) and aligned using the ClustalX program (Staley & Konopka, 1985) and then imported into the Bioedit program (Hall & Brown, 2001) with manual alignment. The phylogenetic tree was constructed with the neighbor-joining algorithm with 10,000 replications using the MEGA4 program (Tamura *et al*., 2007) and a 1300 bp segment.

Operational taxonomic units (OTUs). 16S rRNA gene sequences (1300 bp) of bioactive strains were aligned and grouped into OTUs based on 98% of the identity sequences using the Cluster program (http://www.bugaco.com/mioritic/clusterer_jlp.php).

Preparation of organic extracts. Before the bioactivity test, Actinobacteria strains preserved in glycerol were grown in A1 media (18 g agar, 10 g starch, 2 g peptone, 4 g yeast extract, 1l natural sea water) (Mincer *et al.* 2002; Gontang *et al.* 2007), then a well-defined young colony was transferred to 10 ml of liquid A1 media and growth was allowed for 3 to 5 days at 25 °C with continuous stirring at 215 rpm to induce higher biomass. After 3-5 days the 10 ml culture was transferred into 100 ml of A1 media at same temperature and agitation for 8 days. After this period, Amberlite XAD7 was added at a concentration of 20 g/l of media allowing agitation for 2 h at 100 rpm. Amberlite was then filtered and washed with 100 ml of acetone for 2 h at 100 rpm. Finally, the extract was filtered, concentrated and kept at 15 °C, ready to use in the bioactivity tests.

Antibiotic activity. For antibiotic activity, the sensitivity or resistance of the Gram negative pathogens *Pseudomonas aeruginosa* (ATCC27853) and *Proteus vulgaris* (ATCC13315) and the Gram positive pathogen methicillin resistant *Staphylococcus aureus* (MRSA), were tested using paper discs impregnated with the organic extract. Each pathogen was cultured at a final concentration of 10^5 colony formed units (UFC)/ml, and an aliquot of 1 ml was massively inoculated into nutritive agar. All the assays were performed by triplicate. At the end of the inoculation, the paper discs impregnated with each organic extract at a concentration of 10µg/ml were positioned into the Petri dish. Bacterial growth was allowed for 24-48 h at 37 °C before the reading of the inhibition zone. The Gram negative pathogens were selected due to their resistance to rifampicin, which is actively secreted by most of the Actinobacteria strains of group II (*Salinispora*; Jensen *et al*., 2007). The Gram positive bacteria were included to see a wide range of activity in the analyzed strains, as well as the high activity in *Salinispora arenicola* (Asolkar *et al*., 2010) and *Streptomyces* strains (Torres-Beltrán *et al*., 2012). Therefore, the positive antimicrobial activity evaluated here is due to different and potentially new antibiotic compounds.

Cytotoxic activity. Each organic extract was tested against human colon carcinoma cells HCT-116, human lung cancer cells H460 and human cervical cancer cells HeLa. Bioassays of HCT-116 cells were performed at a concentration of 10 mg/ml of the dry organic extract in dimethyl sulfoxide (DMSO) and the medium inhibitory concentration IC_{50} (µg/ml) was calculated from the activity assay according to the method described in Becerril-Espinosa *et al.* (2012); bioassays of H460 lung cancer cells and HeLa breast cancer cells were performed at a concentration of 20 mg/ml of dry organic extract in dimethyl sulfoxide (DMSO), from which the survival rate of less than 50% was obtained under conditions described by Torres-Beltrán *et al.* (2012); all the assays were realized by triplicate.

RESULTS

Antibiotic activity. 71.0% (154 strains) of Actinobacteria organic extracts (217 extracts) showed antibiotic activity against at least one of the pathogens tested. From the bioactive strains, 43.0% (66) showed activity against *Pseudomona aeruginosa*, 69.0% (47 strains) against *Proteus vulgaris* and 30.5% (47 strains) against MRSA. In addition, from the bioactive strains, only 19 showed an inhibition zone smaller than 10 mm in diameter, while the rest (135) of the strains showed an inhibition zone between 10 and 30 mm in diameter, which is considered a strong inhibition.

Of the 29 strains from group I, 38.0% (11 strains) showed activity against *P. aeruginosa*, 45.0% (13 strains) showed activity against *P. vulgaris* and 17.0% (5 strains) were active against MRSA. Of the 127 strains from group II, 27.0% (34 strains) showed activity against *P. aeruginosa*, 46.4% (59 strains) showed activity against *P. vulgaris* and 13.0% (17 strains) showed activity against MRSA. Of the 60 strains from Group III, 35.0% (21 strains) showed activity against *P. aeruginosa*, 58.0% (35 strains) showed activity against *P. vulgaris* and 42.0% (25 strains) were active against MRSA (Fig. 2).

The selected strains were grouped into 16 OTUs based on 98% similitude to the 16S rRNA sequence, of which two OTUs showed activity only against *P. aeuroginosa*, two OTUs where bioactive only against *P. vulgaris* and one OTU showed activity against MRSA, seven OTUs showed activity against both pathogens and four OTUs showed activity against three pathogens (Table 1).

Cytotoxic activity. From the 217 organic extracts prepared, only 89 strains belonging of the *Micromonosporaceae* family, specifically the *Micromonospora* and *Salinispora* genus, were the most important according to these and previous investigations for this geographic zone (Maldonado *et al*., 2009; Becerril-Espinosa *et al*., 2013). Only 24 strains of Group I (*Micromonospora*) and 65 strains of group II (*Salinispora*) where assayed for cytotoxic activity. From the 89 Actinobacteria tested, 36 strains (40.0 %) showed cytotoxic activity, from which 20 strains

Table 1. Antibiotic activity of Actinobacteria against *Proteus vulgaris ATCC33152*, *Pseudomona aeruginosa ATCC27853* and Methicillin Resistant *Staphylococcus aureus* (MRSA).

Family	Genera	Group	# OTU	Bioactivity		
				P. vulgaris	*P. aeruginosa*	MRSA
Micromonosporaceae	*Micromonospora*	GI	1	+	-	-
	Micromonospora	GI	2	-	+	-
Micromonosporaceae	*Salinispora*	GII	3	+	-	+
Streptomycetaceae	*Streptomyces*	GII	4	-	-	+
	Streptomyces	GIII	5	-	+	-
	Streptomyces	GIII	8	-	+	+
	Streptomyces	GIII	7	+	+	+
	Streptomyces	GIII	8	+	+	-
	Streptomyces	GIII	9	+	+	-
	Streptomyces	GIII	10	+	-	+
	Streptomyces	GIII	11	+	+	+
	Streptomyces	GIII	12	+	-	+
	Streptomyces	GIII	13	+	+	+
	Streptomyces	GIII	14	+	-	-
	Streptomyces	GIII	15	+	+	-
	Streptomyces	GIII	16	+	+	+

Symbols utilized: + (activity), - (no activity. OTUs with 98% of identity

(55.5%) were active against HCT-116 cell line, 11 strains (30.5%) were active against HeLa cell line, and 9 strains (25%) were active against H460 cell line (Fig.2). From all the bioactive strains, only four strains presented activity against two cell lines and one strain presented activity against the three cell lines.

Of the 24 strains related to the *Micromonospora* ssp. genera (group I) only 6 extracts (25%) had cytotoxic activity; for strains related to the *Salinispora* genus (group II) cytotoxic activity was present in 30 (46.15%) of 65 assayed strains (Fig. 2).

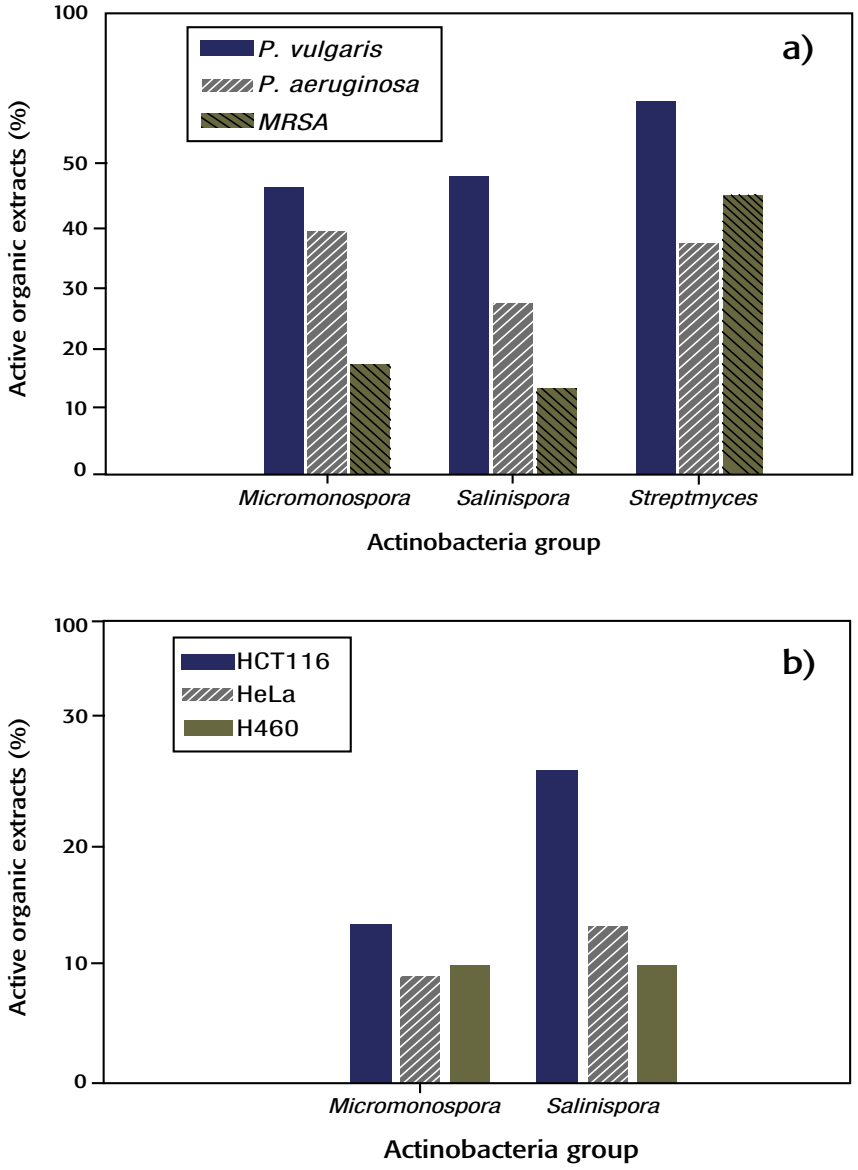
Molecular classification. A few bacteria from Loreto Bay were related, according to the 16S rRNA, to bioactive strains isolated in other regions of the world. For example, the strain AMS515 (HQ873952) is related to the *S. arenicola* (CP000850, 99.27% similarity) producer of several bioactive compounds (Fenical & Jensen, 2006), and the strains related to the genus *Streptomyces* such as the strain AML852 (HQ873944) of group III was related to the type of *Streptomyces hygroscopicus subsp. hygroscopicus* (AB184428, 97.36% similarity), the marine strains of *Streptomyces* sp. (EU214940, 100% similarity, and TFS59-23 (HM001271, 97.65% similarity), which has antibacterial and antifungal activity (Engelhardt *et al*., 2010) (Fig. 3).

DISCUSSION

Antibiotic activity. The three Actinobacteria groups isolated from Loreto Bay and assayed for antibiotic activity: *Micromonospora* (GI), *Salinispora* (GII) and *Streptomyces* (GIII) where highly bioactive against the pathogens tested. The antibiotic activity showed by the strains of group I and II was very similar, due probably to the fact that they belong to the same family (Micromonosporaceae). This behavior was different for the group III strain. The highest proportion of Actinobacteria with antibiotic activity belonged to this group (*Streptomyces*). Moreover several of these strains where highly active against the gram positive pathogen MRSA. This finding is very important nowadays, because of the increasing number of resistant pathogen bacteria to many of the known antibiotics. In this regard, the evaluation of the antibiotic activity investigated in this study contributes to the effort to isolate and elucidate novel antibiotic compounds capable to fight resistant pathogens.

Citotoxic Activity. Nowadays, the *Salinispora* genus is considered the most prolific in terms of production of new compounds (Ziemert *et al.* 2014). This information was important in the selection of strains for this study due to the high cost of the cytotoxic tests. Therefore and due to the fact that they belong to the same family (Micromonosporaceae) only the extracts belonging to the *Salinispora* and *Micromonospora* genus (group II and III), were used to evaluate cytotoxicity. As expected, several *Salinispora* strains and a few of *Micromonospora* were active against different cell lines. It is an expected result due to the unique diversity of strains related to *Salinispora* genus previously found in the Gulf of California. In this region new phylotypes of *S. pacifica* "k" and *S. arenicola* "A" and "B" has been discovered. No formal description or metabolic characterization has been presented for these bacteria. However, it was reported that new phylotypes present ketosynthase domains encoding the synthesis of new compounds (Edlund *et al*., 2011; Becerril-Espinosa *et al*., 2013). Therefore a high possibility of finding new compounds able to fight cancer in any of the 30 different strains of *Salinispora* that resulted cytotoxic in this study exists.

A particular bioactivity behaviour found in actinobacteria strains of Loreto Bay. The capacity of Actinobacteria to produce secondary metabolites is widely accepted. However is quite interesting to



Figures 2a-b. a) Percentage of active organic extracts from Actinobacteria of group I (*Micromonospora*), II (*Salinispora*) and III (*Streptomyces*) against the pathogen bacteria: *Proteus vulgaris*, *Pseudomona aeruginosa* and Methicillin Resistant *Staphylococus aerus* (MRSA). b) Percentage of active organic extracts from Actinobacteria of group I, II and III against human colon carcinoma cells HCT-116, human lung cancer cells H460 and human cervical cancer cells HeLa.

note that the recuperation percentage of bacteria with antibiotic activity found in this work is quite different to other regions. For example, Ellaiah *et al.* (2004) reported 18 to 30% antibiotic activity of Actinobacteria isolated in a bay of the Bengal Ocean near the Kakinada coast of Andhra Pradesh, India, and Valli *et al.* (2012) reported 9.5% and 23% antibiotic activity against *P. aeruginosa* and *P. vulgaris* respectively; which are at least two times less than the 71% of recuperation percentage of Actinobacteria with antibiotic activity found in this work. It is probable that the Biosphere reserve of Bahía de Loreto, which represents a non-impacted and geographically isolated ecosystem, presents such particular conditions as to induce this variability on bioactivity. It is known that isolated strains from different places and at different conditions have evolved in their own way. Factors that influence on the presence or dominance of bioactive over no-active Actinobacteria has not been investigated formally and could be very interesting in ecological terms and beneficial for planning future sampling expeditions for biotechnological purposes. From the actual study, it is possible to conclude that the Actinobacteria community found in Loreto Bay, in the Gulf of California, which possesses unusual bioactive properties represents a potential source of novel compounds with antibacterial bioactivity.

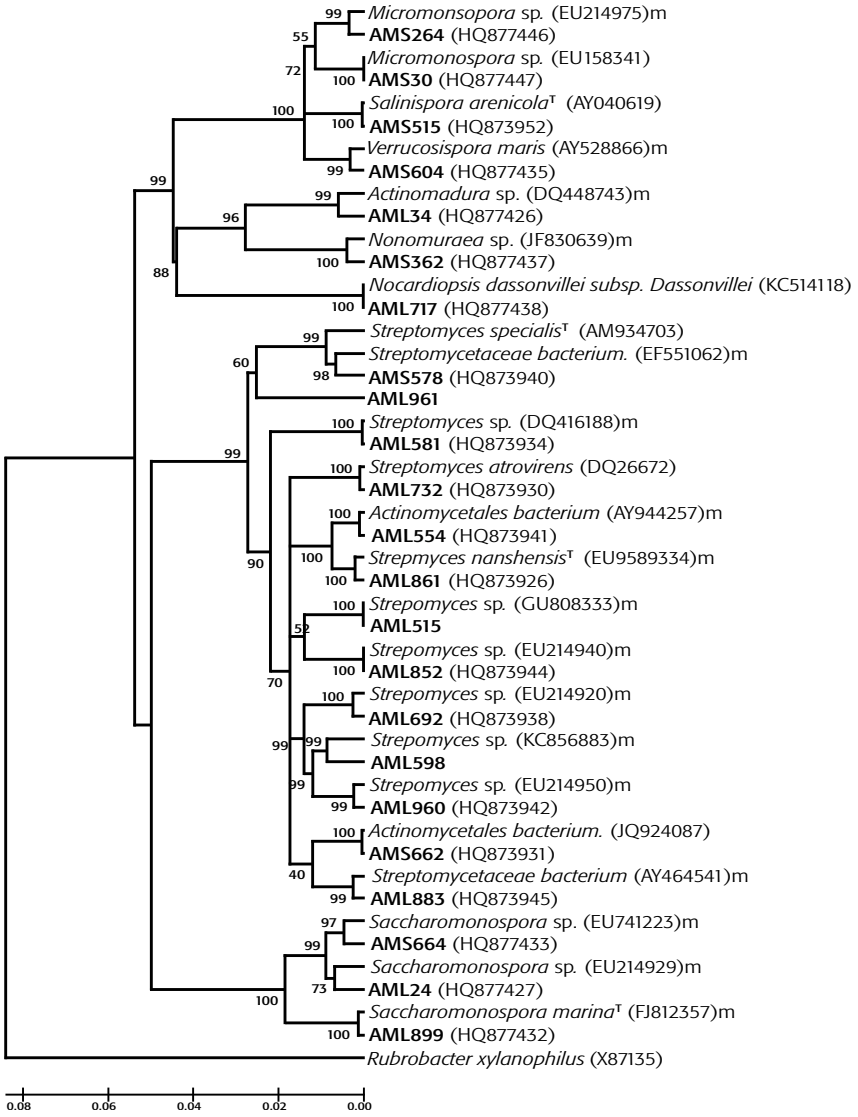


Figure 3. A Phylogenetic tree based on 16S RRNA gene sequences from 16 *Actinomycetales* OTUs (calculated using a sequence identity value of ≥ 98%) observed in this study (AMS numbers) and the nearest strains. Letters after the species name designate 16S "sequences types". Those without letters represent the "standard" sequence type (i.e., the first sequence type observed for the species). The tree was constructed using the neighbor-joining method and the program MEGA4 (1000 botstrap replicates). Accession numbers (in parenthesis). m= marine source.

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