

Marine Actinobacteria from the Gulf of California: diversity, abundance and secondary metabolite biosynthetic potential

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Abstract The Gulf of California is a coastal marine ecosystem characterized as having abundant biological resources and a high level of endemism. In this work we report the isolation and characterization of Actinobacteria from different sites in the western Gulf of California. We collected 126 sediment samples and isolated on average 3.1–38.3 Actinobacterial strains from each sample. Phylogenetic analysis of 136 strains identified them as members of the genera *Actinomadura*, *Micromonospora*, *Nocardiopsis*, *Nonomuraea*, *Saccharomonospora*, *Salinispora*, *Streptomyces* and *Verrucosipora*. These strains were grouped into 26–56 operational taxonomic units (OTUs) based on 16S rRNA gene sequence identities of 98–100 %. At 98 % sequence identity, three OTUs appear to represent new taxa while nine (35 %) have only been reported from marine environments. Sixty-three

strains required seawater for growth. These fell into two OTUs at the 98 % identity level and include one that failed to produce aerial hyphae and was only distantly related (≤ 95.5 % 16S identity) to any previously cultured *Streptomyces* sp. Phylogenetic analyses of ketosynthase domains associated with polyketide synthase genes revealed sequences that ranged from 55 to 99 % nucleotide identity to experimentally characterized biosynthetic pathways suggesting that some may be associated with the production of new secondary metabolites. These results indicate that marine sediments from the Gulf of California harbor diverse Actinobacterial taxa with the potential to produce new secondary metabolites.

Keywords Gulf of California · *Salinispora* · Actinobacteria · *Streptomyces* · Polyketide synthetase (PKS) · Secondary metabolites

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Introduction

In an effort to discover new natural products that can be developed for the treatment of human disease and other biotechnological applications, research has moved towards the study of microorganisms from unexplored habitats such as the ocean. After almost two decades of research in marine microbiology, we now know that marine Actinobacteria are a prolific source of secondary metabolites with antibacterial, immunosuppressive and antitumor activity (Bull and

Stach 2007; Fenical and Jensen 2006; Olano et al. 2009). Marine-derived Actinobacteria include new genera, as demonstrated by the descriptions of *Salinibacterium*, *Salinispora*, *Sciscionella*, *Serinicoccus* and *Marinactinospora* (Han et al. 2003; Maldonado et al. 2005; Tian et al. 2009a; Yi et al. 2004; Tian et al. 2009b), and new species that belong to genera that also occur on land (Helmke and Weyland 1984; Hozzein and Goodfellow 2007; Liu et al. 2010). These discoveries highlight the potential of the marine environment to yield new Actinobacterial taxa and secondary metabolites.

The Gulf of California, also known as the Sea of Cortez, is a marginal sea located between the Mexican mainland and the Baja California peninsula. This 1,100 km long coastal marine ecosystem covers 210,000 km² and includes 37 named islands (Carreño and Helenes 2002). The peninsular coastal zone has little fresh water input due to the sub-desert climate, while the continental shore is characterized by large amounts of fresh water input (Roden and Groves 1959). A past study of marine sediments from the Gulf of California yielded nearly 300 actinomycetes belonging to the genera *Actinomadura*, *Dietzia*, *Gordonia*, *Micromonospora*, *Nonomuraea*, *Rhodococcus*, *Saccharomonospora*, *Saccharopolyspora*, *Salinispora*, *Streptomyces*, “*Solwaraspora*” and *Verrucosisspora* (Maldonado et al. 2009). These genera expand upon those traditionally recovered from marine sediments, such as *Micromonospora*, *Rhodococcus* and *Streptomyces*, and demonstrate the potential of the Sea of Cortez as a source of diverse actinomycete taxa.

The marine actinomycete genus *Salinispora* has been an important source of bioactive secondary metabolites (Fenical and Jensen, 2006). Two species have been formally described, *Salinispora tropica* and *Salinispora arenicola* (Maldonado et al. 2005) while “*S. pacifica*” has been proposed (Jensen and Mafnas 2006). Biogeographical studies have revealed that *S. tropica* is restricted to the Caribbean, “*S. pacifica*” occurs worldwide except for the Caribbean, and *S. arenicola* is broadly distributed and co-occurs with both species (Jensen and Mafnas 2006; Freel et al. 2012). Previous studies have also revealed relationships between *Salinispora* taxonomy and secondary metabolite production (Jensen et al. 2007) and correlations between where the strains were derived and the biosynthetic genes they maintain (Edlund et al. 2011). These results suggest that culturing new *Salinispora*

species or known species from new locations is a potentially productive approach to natural product discovery.

In this study, a culture dependent approach was undertaken to better characterize Actinobacterial diversity in the Gulf of California. The results reveal considerable levels of diversity including the new “*S. pacifica*” 16S rRNA sequence type “K”. In addition, an analysis of polyketide synthase gene sequences suggests that these strains have considerable potential for the production of secondary metabolites not previously reported from the genus.

Methods

Sediment collection

A total of 126 marine sediment samples were collected during two expeditions. The first took place in November 2007 off San Felipe, B.C. The second took place in April 2008 and targeted three sites along the west coast of the Gulf of California (Fig. 1) under collection permit DGOPA.06472.110909.3745 (marine protected areas from Los Ángeles Bay to Loreto Bay). Sediments were obtained from depths of 20–300 m using a modified Kahlisco grab sampler (model # 214WA110). Samples from 0 to 15 m were collected by hand. The specific sampling sites were S1: San Felipe (31°62'N, 114°48.95'W to 31°59.74'N, 114°49.04'W); S2: Los Ángeles Bay (28°58.93'N, 113°29.54'W to 28°57.19'N, 113°25.85'W); S3: Concepción Bay (26°34.17'N, 111°47.11' W to 26°45.73'N, 111°53.50'W). The Loreto Bay sampling sites were S4: mainland Beach (25°50.2'N, 111°19.4'W and 25°43.3'N, 111°14.6'W); S5: island beach (25°48'N, 111°15.35'W); S6: around Danzante Island (25°57.0'N, 111°18.37'W to 25°48'N 111°15.42'W) and S7: around Danzante Island (25°48.91'N, 111°14.36'W to 25°47.95'N, 111°14.32'W). Each sample was transferred to a previously labeled sterile bag (Nasco whirl-pack) and stored at 4 °C for transport to the Universidad Autónoma de Baja California (UABC) Ensenada, Mexico for processing.

Actinobacterial isolation

All sediment samples were dried in a laminar flow hood for 24 h. Once dried, they were ground and inoculated using the plate stamping technique (Mincer

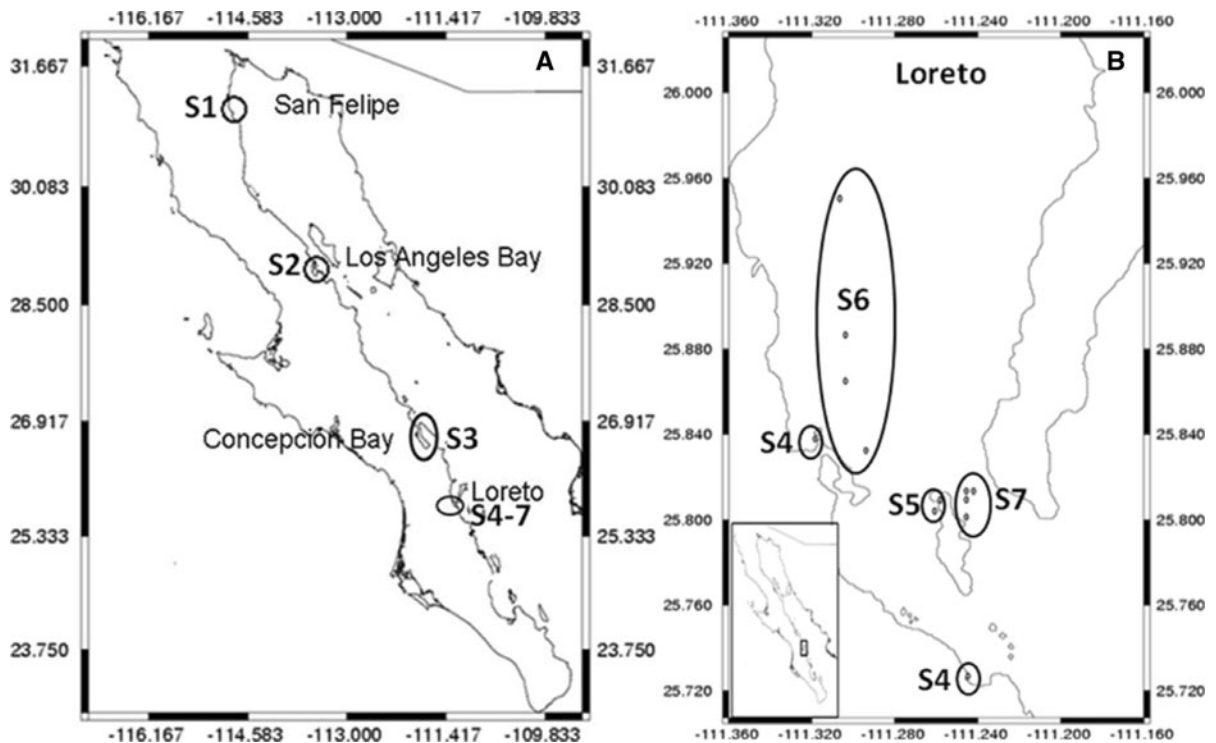


Fig. 1 a Map of the Gulf of California. b Map of Loreto Bay. Circles indicate sampling areas

et al. 2002) on 6 different culture media: M1 (18 g agar, 1 l natural sea water), M2 (18 g agar, 0.5 g mannitol, 0.1 g peptone, 1 l natural sea water), M3 (18 g agar, 1.0 g starch, 0.2 g peptone, 0.4 g yeast extract, 1 l natural sea water), M4 (18 g agar, 2.5 g mannitol, 1 g peptone, 1 l natural sea water), M5 (18 g agar, 0.5 g mannitol, 0.1 g casamino acids, 1 l natural sea water), and M6 (10 g agar, 0.6 g tryptone, 1.0 g casitone, 0.8 g glucose, 1 l natural sea water). Cyclohexamide ($100 \mu\text{g ml}^{-1}$ final concentration) was added to all media to reduce fungal contamination and the antibiotic rifamycin or gentamicin ($5 \mu\text{g ml}^{-1}$ final concentration) was added to select for Actinobacteria. In total, 12 different media and antibiotic combinations were used for the isolation of Actinobacteria.

After 2–12 weeks of incubation at 25–28 °C, all well separated Actinobacterial colonies observed by eye or using a stereomicroscope were removed from the original isolation plate and repeatedly sub-cultured on medium A1 (18 g agar, 10 g starch, 2 g peptone, 4 g yeast extract, 1 l natural sea water) until pure cultures were obtained as judged by uniform colony morphology. Colony characteristics were observed using a stereomicroscope and all pure isolates were

tested for Gram reaction following the non-staining KOH method (Powers 1995).

Effects of seawater on growth

All strains were examined to determine the effects of replacing seawater with deionized water in the growth medium. Using a sterile loop, cells from a well-defined colony were removed from an A1 plate prepared with 100 % natural seawater and streaked onto plates of A1 prepared with seawater and deionized water. Plates were incubated at 25–28 °C and growth was monitored at up to 60× magnification for 8 weeks.

16S rRNA gene amplification and sequencing

The strains were primarily grouped based on the presence or absence of aerial mycelium and the effects of seawater on growth. Within these categories, strains were additionally grouped according to colony color, morphology, and diffusible pigment production. Representatives from each group were selected for 16S rRNA gene sequencing and phylogenetic analysis. Strains were cultured in 25 ml of medium A1 and

shaken at 215 rpm and 28 °C for 7 days. Cells were pelleted by centrifugation and genomic DNA extracted using previously described methods (Gontang et al. 2007). 16S rRNA genes were PCR amplified using the primers FC27 (5′–3′AGAGTTTGATCCTGGCTCAG) and RC1492 (5′–3′TACGGCTACCTTGTTACGACTT) and the following conditions: initial denaturalization at 95 °C for 15 min followed by 32 cycles at 95 °C for 1 min, 61 °C for 1 min and 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. PCR products were purified with a Qiagen QIAquick PCR cleanup kit, using protocols provided by the manufacturer (Qiagen Inc., Chatsworth, Calif.). Sequences were obtained using the primers listed above at SeqXcel, INC (<http://www.seqxcel.com/>) using the BigDye Terminator Cycle Sequencing Chemistry 3.1 technique and a Genetic Analyzer ABI Prism 3100 (Applied Biosystem).

Phylogenetic analysis

The forward and reverse 16S rRNA sequences obtained from each strain were assembled and analyzed using BLAST (Basic Local Alignment Search Tool) (Altschul et al. 1990) available on the NCBI website (<http://www.ncbi.nlm.nih.gov/>). Sequences were aligned using ClustalX and imported into the Bioedit program (Hall and Brown 2001) for manual alignment. A neighbor-joining phylogenetic tree was constructed from 1300 base pairs using the program MEGA4 (Tamura et al. 2007) with 1000 bootstrap replicates. Sequences were deposited in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/index.html>) under the accession numbers HQ873926–HQ873952 and HQ877423–HQ877448.

Operational taxonomic units (OTUs)

Aligned 16S rRNA gene sequences (1300 bp) were grouped into OTUs based on 98, 99 and 100 % sequence identity using the program Clusterer (www.bugaco.com/mioritir/cluster_jlp.php). An OTU was considered ‘new’ if all of the members shared ≤ 98 % sequence identity with any previously cultured strain for which sequences had been deposited in GenBank. The OTU was designated ‘marine’ if all GenBank sequences that shared >98 % sequence identity had been cultured from marine sources. The strain in each 98 % OTU with the lowest similarity to the closest type strain was selected for the construction of a phylogenetic tree.

KS domain amplification and sequencing

Ketosynthase (KS) sequences were PCR amplified from 20 to 50 ng of genomic DNA using the degenerate primers PKSRb (5′-GTSCCSGTSCCGTGGCGCTC SA-3′) and PKSFa (5′-CCSCAGSAGCGCSTTTSC TGG A-3′) (Edlund et al. 2011). The conditions consisted of an initial denaturalization at 95 °C for 15 min, followed by one cycle of 95 °C for 1 min, 65 °C for 1 min and 72 °C for 1 min, and then 31 cycles of 1 min at 95 °C, 62 °C for 1 min and 72 °C for 1 min, followed by a 7 min final extension at 72 °C. The PCR products were purified using the Qiagen QIAquick MinElute Gel Extraction kit following the manufacturer protocols.

The purified PCR products were cloned using the Topo TA cloning kit (Invitrogen, Catalog nos. C4040-10) following the manufacturer’s protocol. Transformed clones were inoculated into 10 ml Falcon tubes containing 3 ml of Luria-Bertoni culture media with kanamycin (50 $\mu\text{g ml}^{-1}$). Plasmid DNA was extracted using the QiaPrep[®] MiniPrep extraction kit, following the manufacturer’s instructions (Qiagen, Cat. No: 27106). Plasmid DNA was subjected to a restriction digest using BstX I (New England BioLabs) and the product run on a 1 % agarose gel to confirm the presence of the appropriate size insert. Select plasmids were sequenced at SeqXcel (La Jolla) using the M13 primer. KS sequences were deposited in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/index.html>) under the accession numbers HQ877411–HQ877422.

KS sequences were analyzed using BLAST and the program NaPDoS (<http://napdos.ucsd.edu>). Sequences were aligned using ClustalX and imported into the Bioedit program (Hall and Brown 2001) for manual alignment. A neighbor-joining phylogenetic tree was constructed from 86 amino acids using the program MEGA4 (Tamura et al. 2007) with 1000 bootstrap replicates.

Fermentation and chemical analysis

Strains AMS515, AMS22 and AMS300 were inoculated from frozen stocks into 25 ml of medium A1 and shaken at 215 rpm and 28 °C for 3 days. The cultures were then transferred to 2.8-l Fernbach flasks containing 1 l of the same medium. On days 3, 5 and 7, 25 ml of the culture was removed and extracted with 50 ml of ethyl acetate. The organic layers were separated, dried over anhydrous sodium sulfate, decanted and concentrated under vacuum. The crude extracts were

Table 1 Numbers of Actinobacteria isolated from marine sediment samples collected in the Gulf of California

Site	Sample (depth)	No. samples	Strains isolated (cryopreserved)	Average strains per sample
San Felipe Bay	S1 (10 m)	8	25 (25)	3.1
Los Angeles Bay	S2 (20 m)	9	108 (55)	12.0
Concepción Bay	S3 (0–15 m)	41	185 (166)	4.5
Loreto				
Mainland beach	S4 (0–15 m)	32	415 (80)	12.9
Island beach	S5 (0–10 m)	11	80 (58)	7.2
Around Danzante Island	S6 (200 m)	11	147 (125)	13.4
Around Danzante Island	S7 (300 m)	14	537 (411)	38.3
Total		126	1497 (920)	12.2

dissolved in methanol and analyzed in a Hewlett-Packard MSD 1100 liquid chromatography-mass spectrophotometer (LC–MS) system using a C-18 reversed-phase column and a 10–100 % acetonitrile in water gradient. Fractions that showed masses characteristic of compounds in the rifampicin class were analyzed by high-resolution (HR) electrospray ionization (ESI)-mass spectrometry (MS) in positive mode using a Thermo Scientific LTQ Orbitrap XL Mass Spectrometer.

Results

Cultivable Actinobacteria

One hundred 26 sediment samples were collected at depths ranging from 0 to 300 m from four different locations in the Gulf of California (Fig. 1). From these samples, 1497 bacterial strains with colony morphologies indicative of the Order Actinomycetales were obtained in pure culture by repeated streaking on agar media. Many of the actinomycete colonies were visible by the fourth week of incubation however some took as long as 12 weeks to appear. The largest number of strains per sample originated from sediments collected at depths from 200 to 300 m at sites S6 and S7 (Table 1) around Danzantes Island, Loreto Bay (Table 1). Los Angeles Bay site S2 and Loreto Bay mainland beach site S4 also yielded high numbers of strains per sample. On average, 3.1–7.2 strains were isolated from sediments collected at sites S1, S3, and S5 while 12.0–38.3 were obtained from sites S2, S4,

S6 and S7 (Table 1). It should be mentioned that sites S1, S3 and S4 are all <1 km from the mainland and yielded many Gram-negative bacteria that may have out-competed slower growing Actinobacteria for space and nutrients. This was not the case for sites S2, S5–S7, which were 5–10 km from shore and yielded relatively few Gram-negative bacteria.

Interestingly, 44–91 % of the Actinobacteria cultured from Los Angeles Bay and Loreto Bay sites S2, S5, S6 and S7 possessed colony morphologies characteristic of the genera *Micromonospora* or *Salinispora*. These include orange-pigmented colonies that lack areal hyphae and spores that blacken the colony surface. The majority of these strains failed to grow when seawater was replaced with deionized water in the growth medium providing preliminary evidence to support their inclusion in the genus *Salinispora* (Maldonado et al. 2005). The relative abundance of seawater dependent strains was greatest at sites S2 and S5–S7 (Fig. 2). Overall, only 16 of 625 Actinobacteria cultured from shallow (0–15 m) sediment samples collected from near-shore Baja California sites (S1, S3 and S4) failed to grow when seawater was replaced with deionized water in the growth medium. While near-shore sites S1 and S2 yielded many isolates (48 and 40 % of total, respectively) with colony morphologies characteristic of the genera *Micromonospora* or *Salinispora*, all of these grew in the absence of seawater as is typical of the genus *Micromonospora*. Sites S1, S3, and S4 also yielded large numbers of actinomycetes with well-developed aerial hyphae that did not require seawater for growth (52, 90 and 91 % of total respectively) as might be expected from strains that were introduced from land (Fig. 2).

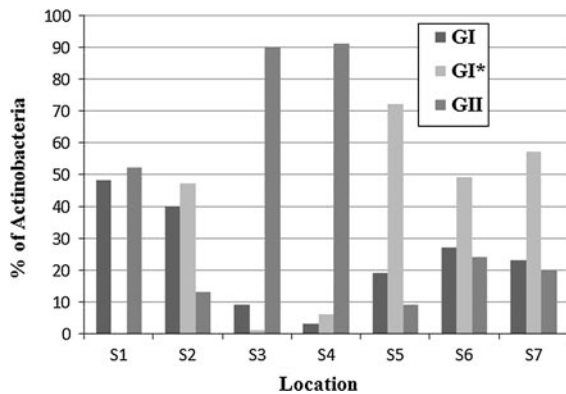


Fig. 2 Actinobacterial colony morphology and seawater requirements. GI = substrate mycelium only. GI* = substrate mycelium only and requires seawater for growth. GII = aerial hyphae. S1 = San Felipe Bay, S2 = Los Angeles Bay, S3 = Concepción Bay, S4 = Mainland beach, S5 = Danzantes Island beach, S6 = Around Danzante Island site a and S7 = Around Danzante Island site b

Actinobacteria diversity

Actinobacterial diversity was evaluated based on the partial 16S rRNA gene sequences of 136 strains that were selected to represent a diversity of seawater requirements and colony morphologies from each location. These strains included a large number that failed to grow on media prepared with deionized water. In total, 85 of the strains sequenced originated from 200 to 300 m (sites 6 and 7) while the remaining

strains were obtained from 0 to 20 m (sites 1–5). BLAST analyses revealed they were most closely related to 8 genera (Table 2). Those strains with closest BLAST matches to *Micromonospora*, *Nonomuraea*, *Salinispora* and *Verrucosisspora* all lacked aerial hyphae and produced orange colonies and black spores, yet among these, only the strains with top matches to *Salinispora* spp. failed to grow when seawater was replaced with deionized water in the growth medium. Bacteria with well-developed aerial hyphae (53 strains) were related to four genera: *Actinomadura* (7.1 %), *Saccharomonospora* (4.2 %), *Streptomyces* (76.8 %) and *Nocardiopsis* (1.7 %). Of these, only strains related to *Streptomyces* spp. failed to grow when seawater was replaced with deionized water in the growth medium.

Operational taxonomic units (OTUs) calculated based on sequence identities of 98 and 99 % revealed 26 and 37 OTUs, respectively. Within the 98 % group, there are three new OTUs (Table 2) that potentially represent new taxa within the genera *Streptomyces* and *Actinomadura*. Forty-one of the 56 OTU's identified at 100 % sequence identity had not previously been reported (Table 2). The most distinct OTU (no. 20, Supplemental Table 1) is represented by strain AMS37 and shared only 95.4 % 16S rRNA sequence identity to the nearest *Streptomyces* type strain. All three of the strains in this OTU originated from site S6 (200 m). Additionally, all of the strains in OTUs 13–21 share

Table 2 Generic affiliation of Actinobacterial strains and OTUs recovered from samples as delineated by 100–98 % 16S rRNA gene sequence identity

Genera	Colonial morphology	No. strains	OTU's								
			100 %			99 %			98 %		
			Total	New	Marine	Total	New	Marine	Total	New	Marine
<i>Salinispora</i>	GI	60	5 ^a	1 ^a	4 ^a	1 ^a	0	1 ^a	1 ^a	0	1 ^a
<i>Micromonospora</i>	GI	15	9	6	1	7	0	1	2	0	0
<i>Nocardiopsis</i>	GII	1	1	0	0	1	0	–	1	0	0
<i>Saccharomonospora</i>	GII	8	8	8	0	5	2	1	3	0	1
<i>Streptomyces</i>	GII	43	25	20	3	15	4	6	12	1 ^{a,b}	6
<i>Verrucosisspora</i>	GI	4	3	2	1	3	1	1	2	0	0
<i>Nonomuraea</i>	GI	1	1	0	0	1	0	0	1	0	0
<i>Actinomadura</i>	GII	4	4	4	0	4	2	1	4	2	1
Total		136	56	41	9	37	9	11	26	3	9

^a OTU that requires seawater for growth. GI = substrate mycelium only. GII = aerial hyphae. Marine OTUs include strains that have previously been isolated before from marine sources. New OTUs include strains that were reported for the first time here

^b Mycelium substrate colony

<98.5 % sequence identity with the nearest type strains and thus may also represent new taxa (Rossello-Mora and Amann 2001). Eight of these new OTUs originated from samples collected at depths of 200–300 m.

Many of the OTUs were designated as marine if they had previously been isolated from marine sources and all publically available sequences in the OTU were from marine sources (Table 2). As the number of new OTUs decreases with decreasing levels of OTU 16S sequence identity, the relative proportion of known marine OTUs increases indicating the existence of diverse lineages that have only been reported from marine sources. To date, eight of these lineages have been formally described as new genera (*Salinispora*) or species (*Micromonospora krabiensis*, *Saccharomonospora marina*, *Streptomyces fenuangensis*, *Streptomyces marinus*, *Streptomyces nanshensis*, *Verrucosipora maris* and *Verrucosipora sediminis*).

A phylogenetic tree reveals that the five strains belonging to OTU number one (Supplemental Table 1), which represent the 60 seawater-requiring strains that possessed *Micromonospora* or *Salinispora*-like morphologies, all claded with *Salinispora* spp. (Fig. 3). Fifty-three of the 60 *Salinispora* strains were identified as *S. arenicola* and the remaining seven as “*S. pacifica*”. All of the *S. arenicola* strains were identical to those previously reported as sequence types “A” or “B” (Jensen and Mafnas 2006). Interestingly, the “standard” *S. arenicola* sequence type (i.e., the first 16S sequence type reported for the species and represented by strain CNS-205 in Fig. 3) was not observed. *S. arenicola* sequence type “A” was the most abundant *Salinispora* sequence type, representing 86.6 % (52 strains) of the 60 *Salinispora* strains examined in this study while only one strain belonging to sequence type “B” was recovered. The seven strains identified as “*S. pacifica*” fell into three OTUs at 100 % sequence identity (Fig. 3) and included the “standard” “*S. pacifica*” sequence type (AMS365), sequence type “C” (AMS178) and one sequence type that has not previously been reported (AMS301). We have designated this new sequence type, which shares 99.84 % sequence identity with the standard “*S. pacifica*” sequence type, as sequence type “K”.

Effects of seawater on growth

Six OTUs identified based on 100 % sequence identity failed to grow when seawater was replaced with

deionized water in the culture medium. Five of these OTUs were affiliated with the genus *Salinispora* (strains AMS300, AM360, AMS301, AMS365 and AMS178) and the sixth with the genus *Streptomyces* (strain AMS37, Supplemental Table 1). The *Salinispora* isolates were recovered from Los Angeles Bay to Loreto Bay and were collected from depths of 20–300 m demonstrating a broad distribution in the Gulf of California.

Analysis of biosynthetic genes sequences

Ketosynthase (KS) domains were cloned and sequenced from three *S. arenicola* (AMS300, AMS22 and AMS515) and two “*S. pacifica*” (AMS301 and AMS178) strains. These domains are associated with polyketide synthase (PKS) genes and their phylogeny has proven informative in terms of making prediction about secondary metabolite production (Ginolhac et al. 2005; Gontang et al. 2010). The three *S. arenicola* sequence type “A” strains yielded similar results with the detection of KS sequences that share a high level of identity to those associated with rifamycin biosynthesis (*rifA* and *rifE*) and both ten- (*Cal05*) and nine-membered (*NcsE*) enediyne biosynthesis (Fig. 4). All of these sequences were observed previously in the *S. arenicola* strain CNS-205 genome sequence (Penn et al. 2009) or in other related work (Gontang et al. 2010). “*S. pacifica*” sequence type “K” had not previously been examined for KS sequence diversity. This strain yielded a KS sequence that shared a high level of identity to that associated with lymphostin biosynthesis, as has previously been observed in both *S. arenicola* and *S. tropica* (Penn et al. 2009) in addition to a *Streptomyces* sp. (Nagata et al. 1997). Thus, lymphostin production may be a common feature of the genus. The other three KS sequences shared 55 % similarity with *CurI*, which is part of the curacin biosynthetic pathway observed from *Lyngbya majuscula* (Chang et al. 2004). Only one KS domain was detected from “*S. pacifica*” sequence type “C” (strain AMS178). It possessed 56.57 % identity to *EpoC*, which is involved in epothilone biosynthesis in *Sorangium cellulosum* (Walsh et al. 2003) (Supplementary Table 2). The low levels of sequence identity for KS sequences observed in the “*S. pacifica*” sequence type “C” and “K” suggest that the associated biosynthetic pathways have yet to be characterized.

All of the KS domains had an active site cysteine, a conserved histidine residue 135 nucleotides downstream from the active site and the largely conserved active

Fig. 3 **A** Phylogenetic tree based on 16S rRNA gene sequences from 26 *Actinomycetales* OTUs (calculated using a sequence identity value of $\geq 98\%$) observed in this study (AMS numbers) and the nearest type strains. The isolates include 9 OTUs that appear to represent new taxa and 8 OTUs with nearest type strains isolated from marine sources. *Rubrobacter radiotolerans* was used to root the tree. **B** Phylogenetic tree based on 16S rRNA gene sequences from 5 *Salinispora* OTUs (calculated using a sequence identity value of 100) observed in this study (AMS numbers) and previously reported “*S. pacifica*”, *S. arenicola* and *S. tropica* strains. Letters after the species name designate 16S “sequence types”. Those without letters represent the “standard” sequence type (i.e., the first sequence type observed for the species). The trees were constructed using the neighbor-joining method and the program MEGA4 (1000 bootstrap replicates). Accession numbers (in parentheses). M = marine source, * Actinobacteria that require seawater for growth

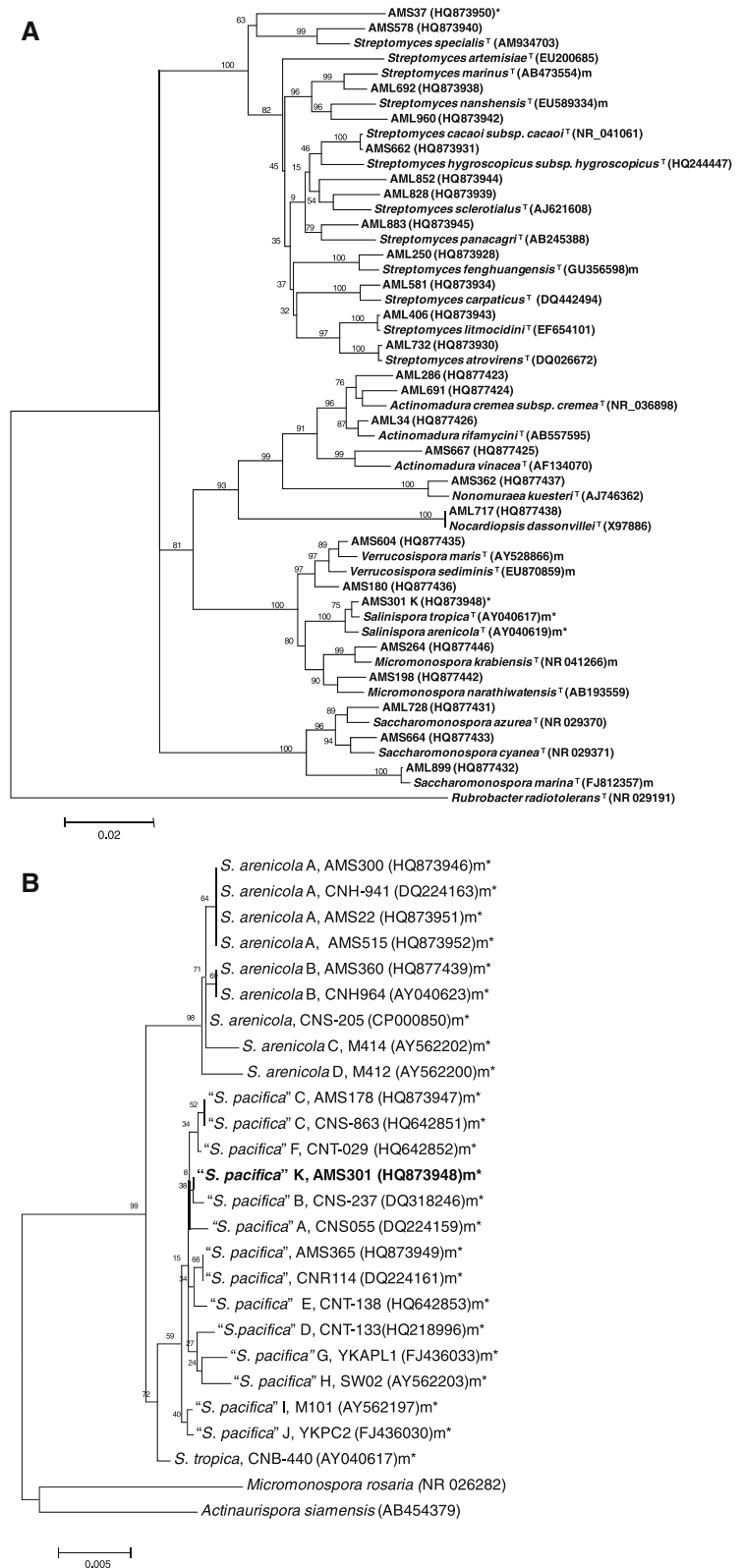
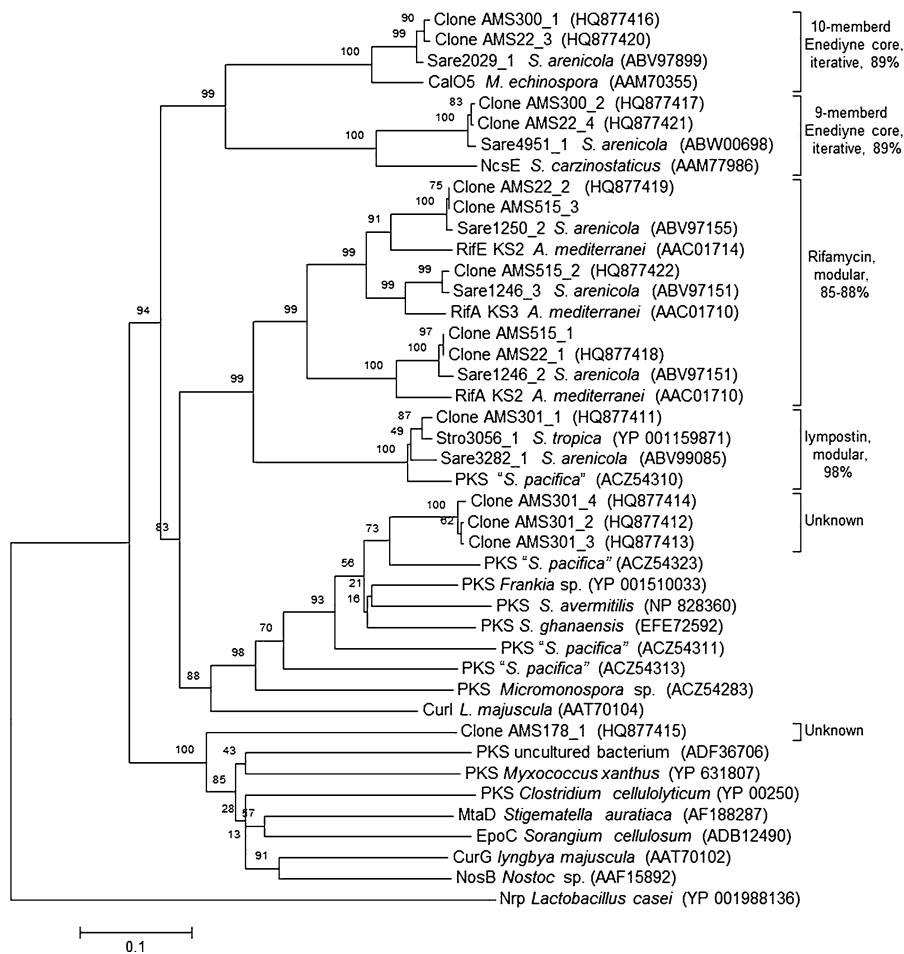


Fig. 4 *Salinispora* KS phylogeny. Neighbor-joining distance tree constructed using 86 aligned amino acids from strains AMS22, AMS300, AMS515, AMS301 and AMS178. The tree was created using the program MEGA4 (Tamura et al. 2007) with 1000 bootstrap replicates. KS sequences were classified using NaPDoS (<http://napdos.ucsd.edu/>)



site domain sequence VDTACSSSLVA (Aparicio et al. 1996; Bevitt et al. 1992; Donadio and Katz 1992). Exceptions include clones 2–4 from “*S. pacifica*” sequence type “K” (strain AMS301) in which the valine at position 94 is replaced with an alanine, and one clone from sequence type “C” (strain AMS178) in which glutamine and threonine are observed at positions 85 and 91, respectively (Supplementary Fig. 1).

Analysis of the LC/MS data

Secondary metabolite production has been successfully predicted in cases where KS amino acid sequence identity is $\geq 85\%$ to sequences associated with experimentally characterized biosynthetic pathways (Gontang et al. 2010). Based on this, it was predicted that *S. arenicola* strain AMS515 produces compounds in the rifamycin class. LC/MS analysis of organic extracts of a

culture grown for nine days led to the detection of a compound in the rifamycin class with UV absorbance maxima (λ_{max}) at 220, 300 and 420 nm (Supplementary Fig. 2).

Discussion

Marine Actinobacteria have become an important source of medically relevant secondary metabolites, such as antibiotics, antitumor, anti-inflammatory, and antifungal compounds (Bull and Stach 2007; Fenical and Jensen 2006; Olano et al. 2009). This creates a rationale to explore poorly studied marine ecosystems as a source of new actinobacterial strains and taxa. In the present study, 1497 actinomycete strains were cultured from marine sediments collected at three sites in the Gulf of California. Using a 16S rRNA sequence identity of 98% to cluster strains into OTUs, three potentially

new taxa were found (Table 2). These OTUs belong to the genera *Streptomyces* and *Actinomadura* and support further taxonomic studies of marine actinomycetes from the Gulf of California. Of these, OTU 20 is the most unique, sharing only 95.4 % sequence identity with the nearest type strain (Supplemental Table 1). This OTU is comprised of three strains, all of which require seawater for growth, and could potentially represent a new genus-level taxon in the family *Streptomycetaceae*. In addition to the three potentially new taxa, several previously described marine species were isolated including *Micromonospora krabiensis* (AMS264, 99.06 % similarity), *Saccharomonospora marina* (AML899, 99.54 % similarity), *Streptomyces fenhuangensis* (AML250, 98.71 % similarity), *Verrucosipora maris* (AMS604, 99.42 % similarity) and *Verrucosipora sediminis* (AMS180, 98.76 % similarity) suggesting that these species may have broad geographic distributions.

A majority of the strains sequenced (60) were identified as members of the marine actinomycete genus *Salinispora*. Of these, 53 were identified as *S. arenicola* sequence types “A” or “B” while the rest were identified as “*S. pacifica*”. *S. arenicola* sequence types “A” and “B” were previously reported from the Gulf of California (Jensen and Mafnas 2006; Maldonado et al. 2009) and off the Pacific coast of Costa Rica (Solano et al. 2009). The absence of the “standard” sequence type, which has been reported from the Bahamas, Palau, the Red Sea, Guam and Fiji (Freel et al. 2011; Jensen and Mafnas 2006), but has yet to be reported from the Gulf of California, suggests there are distinct geographical boundaries associated with the three *S. arenicola* sequence types. While only seven “*S. pacifica*” strains were identified, they include the “standard” sequence type (Strain AMS363), sequence type “C” (Strain AMS178), and the first report of sequence type “K” (strain AMS301, HQ873948), which represents the twelfth sequence type observed to date for this species (Freel et al. 2012).

The actinomycetes cultured from the Gulf of California provide opportunities to explore these strains for the genetic potential to produce secondary metabolites. Targeting KS domains provides a rapid method to assess PKS diversity and novelty within individual strains. The results revealed evidence of common pathways shared with other *Salinispora* strains but also sequences that share low levels of identity with any characterized pathways and thus may be associated with the production of new secondary metabolites. It is

noteworthy that the new sequence type “K” (strain AMS301) also possesses a KS sequence that has not previously been observed in “*S. pacifica*”.

This study presents an analysis of the culturable actinomycete diversity obtained from marine sediments collected off the western margin of the Gulf of California. The results add to the diversity observed within the genus *Salinispora* and to the growing diversity of *Streptomyces* spp. that have been recovered from marine samples. The unique characteristics of the Gulf of California make it an ideal location to search for new actinomycete diversity that can be incorporated into natural product screening programs.

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