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Comparative actinomycete diversity in marine sediments

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ABSTRACT: The diversity of cultured actinomycete bacteria was compared between near- and off-shore marine sediments. Strains were tested for the effects of seawater on growth and analyzed for 16S rRNA gene sequence diversity. In total, 623 strains representing 6 families in the order *Actinomycetales* were cultured. These strains were binned into 16 to 63 operational taxonomic units (OTUs) over a range of 97 to 100% sequence identity. The majority of the OTUs were closely related (>98% sequence identity) to strains previously reported from non-marine sources, indicating that most are not restricted to the sea. However, new OTUs averaged 96.6% sequence identity with previously cultured strains and ca. one-third of the OTUs were marine-specific, suggesting that sediment communities include considerable actinomycete diversity that does not occur on land. Marine specificity did not increase at the off-shore sites, indicating high levels of terrestrial influence out to 125 km from shore. The requirement of seawater for growth was observed among <6% of the strains, while all members of 9 OTUs possessed this trait, revealing a high degree of marine adaptation among some lineages. Statistical analyses predicted greater OTU diversity at the off-shore sites and provided a rationale for expanded exploration of deep-sea samples. A change in community composition was observed, with the number of *Micromonospora* OTUs increasing in the off-shore samples. UniFrac (see http://bmf2.colorado.edu/unifrac) statistics support a difference in community composition between near- and off-shore locations. Overall, 123 of 176 strains had distinct 16S rRNA gene sequences, indicating a high level of actinomycete diversity in marine sediments.

KEY WORDS: Marine actinomycetes · Marine sediments · Bacterial diversity · Seawater requirements · Phylogeny

INTRODUCTION

Bacteria belonging to the order *Actinomycetales*, commonly called actinomycetes, represent one of the most studied and exploited classes of bacteria due to their ability to make a wide range of biologically active metabolites (Goodfellow et al. 1988, Bull et al. 2000). These microorganisms have been recovered from diverse environments including rain forests (Wang et al. 1999), lake sediments (Terkina et al. 2002), estuaries (Takizawa et al. 1993), and deep ocean trenches (Colquhoun et al. 1998). With respect to the marine environment, it has long been known that actinomycetes can be cultured from ocean samples including sediments collected at depths >3 km (Weyland 1969). Marine-derived strains have more recently received attention as a resource for biotechnology (Bull et al. 2005, Fiedler et al. 2005). To date, taxonomic studies of marine-derived actinomycetes have led to the description of 3 new genera: *Salinibacterium*, *Serinicoccus* and *Salinispora* (Han et al. 2003, Yi et al. 2004, Maldonado et al. 2005a), the proposal of *Solwaraspora* as a fourth (Magarvey et al. 2004), and various new species (e.g. Helmke & Weyland 1984, Stach et al. 2004). These studies have made it clear that new taxa reside in the ocean; however, the extent to which actinomycete communities differ between the land and sea remains unknown.

Although actinomycetes are regularly recovered from marine samples, they are also common soil inhab-

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itants and are undoubtedly transported in large numbers from land into the sea. Considering that many species are salt tolerant and produce spores (Basilio et al. 2003), it is not surprising that some strains recovered from near-shore marine samples also occur on land (e.g. Gontang et al. 2007). To date, the component of the marine actinomycete community that appears to be specific to the marine environment has not been defined for any environmental samples. It has also yet to be determined which members of these communities are metabolically active in marine sediments (Goodfellow & Haynes 1984), although evidence of marine adaptation and growth in the marine environment has been reported (Moran et al. 1995, Mincer et al. 2002).

In an effort to gain a better understanding of marine actinomycete community composition, strains isolated form marine sediments collected near-shore (≤2 km from shore) were analyzed and compared to those isolated from samples collected 30 to 125 km from shore (off-shore). One goal was to test the hypothesis that the number of marine-specific strains would increase at sites more distant from shore, where terrestrial input was presumed to be less. The requirement of seawater for growth was also assessed as an indicator of marine adaptation and to determine if this trait could be used as a tool to enhance the recovery of new marine taxa. The results indicated that new actinomycete phylotypes can readily be cultured from marine samples, yet differences in community structure between near- and off-shore sites could not be linked to marine-specificity or seawater dependence.

MATERIALS AND METHODS

Sample collection and processing. Fifty-seven near-shore marine sediment samples were collected using a modified surface-deployed grab sampler (model #214WA110, Kahlsico) from a depth range of 30 to 150 m within a 0.5 km^2 quadrant centered 1.8 km off the coast of La Jolla, California, USA (32° 53.01′ N, 117° 16.22′ W) in August, September, and November 2001. Eleven off-shore San Diego sediment samples were collected from 6 different locations along an 85 km transect (32° 39.75′ N, 117° 16.083′ W to 32° 11.09′ N, 118° 18.02′ W) that extended to 125 km from shore. These samples were collected using an untethered coring device designed and constructed at the Scripps Institution of Oceanography (SIO) from which cores 20 to 30 cm in length were obtained. When possible, the cores were separated into 2 to 6 sections (ca. 5 cm each). Five additional off-shore samples were obtained from a 55 km^2 region that extended from 32 km off the Farallon Islands out to 60 km off the coast of San Francisco (37° 39.998′ N, 123° 27.001′ W to 37° 42.946′ N 123° 32.947′ W). All near-shore samples were placed in sterile 50 ml centrifuge tubes using an ethanol-sterilized spatula and transported to the SIO for processing (generally within 4 h). For the off-shore San Diego samples, core sections were placed in sterile Petri dishes and kept at 4°C prior to processing (generally within 1 wk). The San Francisco samples were collected using a 0.25 m^2 box corer (Sandia MK-III, Ocean Instruments) and approximately 10 ml of wet sediment was removed from the surface of each core using an alcohol sterilized stainless steel spoon, placed in sterile 50 ml centrifuge tubes, and maintained at 4°C for approximately 2 wk before being processed.

All samples were processed using previously described heat-shock and drying methods (Mincer et al. 2002) and inoculated onto medium M1 (10 g starch, 4 g yeast extract, 2 g peptone, 18 g agar, 1 l of natural seawater). Medium M1 was amended with cycloheximide (100 µg ml^{-1} final concentration) and either rifamycin (5 µg ml^{-1} final concentration) or gentamicin (10 µg ml^{-1} final concentration). Briefly, for the heat-shock method, 1 ml of wet sediment was added to 4 ml of sterile seawater (for San Diego off-shore samples, 2 ml of wet sediment was used), heated for 6 min at 55°C, vigorously shaken, further diluted (1:100 and 1:1000) in sterile seawater, and 50 µl of each dilution was inoculated onto medium M1 by spreading with a sterile glass rod. For the stamping method, approximately 10 ml of wet sediment was aseptically placed into a sterile petri dish and dried (ca. 24 h) in a laminar flow hood. A sterile foam plug (14 mm diameter) was pressed into the dried sediment and used to inoculate the medium by stamping 8 or 9 times in a circular fashion, giving a serial dilution effect. Due to the low yield of actinomycete colonies obtained from the San Diego off-shore sediments, these samples were also stamped onto $\frac{1}{4}$th and $\frac{1}{8}$th strength M1 medium.

Actinomycetes were recognized based on colony and microscopic morphology. Colonies with a tough leathery texture, dry or folded appearance, and branching filaments with or without aerial hyphae that could be observed either unaided by eye or using a dissecting microscope (64× magnification) were transferred from the primary plates onto new media. Hence, this study was limited to filamentous bacteria within the order *Actinomycetales*. An attempt was made to obtain in pure culture all accessible actinomycete-like colonies observed on the plates. Actinomycetes from near-shore sediments generally appeared after 2 to 6 wk of incubation at 25 to 28°C, while colonies from the off-shore samples took 8 to 12 wk to appear.

Effects of seawater on growth. All strains were tested for the effects of seawater on growth using medium M1 prepared with either seawater or de-

ionized water. A single colony from each isolate was carefully transferred from a culture growing on medium M1 (seawater) and dilution streaked onto plates of the same medium prepared with either seawater or de-ionized water. In cases where only a few colonies appeared on the de-ionized water plates, the process was repeated using one of these colonies to ensure that growth was not due to salts carried over from the initial transfer. Based on the results of these tests, strains were categorized as either seawater dependent (no growth on M1/de-ionized water) or non-seawater dependent (growth on both media). The number of strains for which the relative amount of visually assessed growth was reduced in the absence of seawater was also recorded.

16S rRNA gene amplification and sequencing. The PCR amplification of the 16S rRNA gene was carried out with primers FC27 (5′ to 3′ AGAGTTTGATCCTGGCTCAG) and RC1492 (5′ to 3′ TACGGCTACCTTGTTACGACTT). PCR products were purified using a Qiagen QIAquick PCR cleanup kit using the manufacturer's protocols (Qiagen). Partial gene sequences were obtained using the FC27 primer, and nearly full sequences (1484 bp, *Escherichia coli* positions 8 to 1492) in both the forward and reverse directions were obtained for select strains using the additional sequencing primers F514 (5′ to 3′ GTGCCAGCAGCCGCGGTAA), F1114 (5′ to 3′ GCAACGAGCGCAACCC), RC1492 (5′ to 3′ TACGGCTACCTTGTTACGACTT), R936 (5′ to 3′ GTGCGGGCCCCCGTCAATT), and R530 (5′ to 3′ CCGCGGCTGCTGGCACGTA). Forward and reverse strand contigs were assembled using Sequencher (version 4.5, Gene Codes).

Phylogenetic analysis. Partial and nearly complete 16S rRNA gene sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) on the NCBI website (www.ncbi.nlm.nih.gov/) and the percent identity of each sequence to the closest cultured strain recorded. Operational taxonomic units (OTUs) were calculated for all partial sequences (573 bp near-shore, 642 bp off-shore) at 97, 98, 99, and 100% sequence identity using the program Clusterer (www.bugaco.com/mioritic/clusterer_jlp.php) by setting the distance parameter to the number of nucleotides required to give the desired consensus groups. If all of the strains in an OTU shared ≤98% 16S rRNA gene sequence identity with any previously cultured strain for which sequence data was available, it was defined as a 'new' OTU. OTUs were otherwise considered 'known' or, if all NCBI sequence deposits sharing >98% sequence identity to strains within an OTU were from marine sources, the OTU was further considered 'known marine'. One representative of each OTU in the 98% consensus group was selected for nearly complete 16S rRNA gene sequencing. Assembled sequences were imported into the ARB database (www.arb-home.de/), automatically then manually aligned along with their closest BLAST matches, and exported into PAUP (Swofford 2002) for phylogenetic analysis. Neighbor-joining distance trees were created using 1378 base pairs and default settings with uncorrected 'p'. Bootstrap analyses were performed with 1000 re-samplings.

Statistical analyses. Non-parametric diversity estimators as well as Shannon and Simpson indexes were calculated using EstimateS (Version 7, Colwell 2004 http://purl.oclc.org/estimates). For the 111 near-shore strains sequenced, coverage was calculated using the equation: $C = 1 - (n/N) \times 100$, where n = number of unique OTUs and N = total number of OTUs examined (Good 1953). The UniFrac web tool (http://bmf2.colorado.edu/unifrac/index.psp) is a multivariate statistic that was employed to further analyze and compare the phylogenetic diversity between the near- and off-shore actinomycete communities.

RESULTS

Fifty-seven near-shore marine sediment samples were collected at depths of 30 to 150 m and distances of 1.5 to 2.0 km off the coast of San Diego (Table 1). Sixteen additional samples were collected from 2 off-shore locations (depths 1.1 to 3.1 and 30 to 125 km from shore); 11 of these came from a site off San Diego and 5 from a site off San Francisco (Table 1). All 73 samples were processed for the selective cultivation of actinomycete bacteria and an attempt was made to obtain in pure culture all accessible actinomycete colonies observed on the isolation plates.

In total, 623 actinomycete strains were isolated, the vast majority of which (557) were obtained from the near-shore locations (Table 1). Although more samples were collected from the near-shore sites, they yielded ca. 2 and 5 times as many strains per sample as the off-shore San Diego and San Francisco sites, respectively. Of the 623 strains cultured, 32 (5.1%) required seawater for growth, while 39 (6.3%) showed reduced growth when seawater was replaced with de-ionized water in the culture medium. All strains for which the absence of seawater in the culture medium affected growth originated from the near-shore samples. The vast majority (88.3%) of the actinomycetes cultured grew equally well when seawater was replaced with de-ionized water in the cultivation medium.

Partial 16S rRNA gene sequences were obtained for all of the strains isolated from samples collected off-shore San Diego (57 strains) and San Francisco (9 strains) (Table 1). For the near-shore samples, 111 strains were selected for sequencing based on their

Table 1. Marine sediment sampling sites, ocean depths, distances from shore, number of actinomycetes isolated, and the effects of seawater on growth. NA: not applicable

Site	Depth (km)	Distance (km)	No. samples	Strain isolated (sequenced)	Strains per sample	Seawater requirement		
						Yes	No	No[a]
San Diego								
Near-shore	0.03–0.15	1.5–2.0	57	557 (111)	9.8	32	486	39
Off-shore[b]	1.1–2.0	30–125	11	57 (57)	5.2	0	56	0
San Francisco								
Off-shore	2.3–3.1	32–40[c]	5	9 (9)	1.8	0	8	0
Total	NA	NA	73	623 (177)	8.5	32	550	39

[a]Reduced growth in the absence of seawater
[b]2× material inoculated and 2 additional media used
[c]Distances are to the Farallon Islands, distances to the mainland range from 50 to 60 km

response to the replacement of seawater with de-ionized water in the growth medium. These included all 32 strains that required seawater for growth, all 39 strains that displayed reduced growth without seawater, and 40 randomly chosen strains that grew equally well on both media. In total, partial 16S rRNA gene sequence data were obtained for 177 of the 623 strains obtained in culture.

Given that various 16S rRNA gene sequence identity values have been used to assess species-level diversity (e.g. Hagström et al. 2002), OTUs were calculated over a range from 97 to 100% sequence identity. These analyses resulted in the detection of 18 to 60 OTUs from the near-shore sediments and 16 to 63 OTUs from the off-shore sediments (Table 2). In near-shore sediments, new OTUs ranged from 17 to 32% of the total while in off-shore sediments the range was 16 to 25% (Table 2). On average for all sequence identity groups, 26% of the near-shore and 20% of the off-shore OTUs were new to this study. In all but the 97% consensus group, new OTUs were more common in the near-shore samples. Known marine OTUs ranged from 6 to 12% of the various near-shore and from 12 to 16% of the various off-shore consensus group totals (Table 2). Overall, the number of OTUs recovered in this study that are marine specific (i.e. have only been reported from marine sources) averaged 35% over all consensus groups for the near-shore samples and 32% for the off-shore samples. All of the new OTUs recovered from the off-shore San Diego samples originated from the 5 to 10 cm sections of the cores (data not shown), supporting the need for further studies of vertical actinomycete profiles in marine sediments. Only 2 strains were isolated from below the top 10 cm of any core despite the fact that actinomycetes have been detected down to 46 cm when culture-independent techniques were applied to a deep-sea core (Stach et al. 2003).

Based on BLAST analyses of partial 16S rRNA gene sequence data, the 177 strains sequenced were most closely related to 8 genera (Table 3). The majority of these (85%) were affiliated with the genus *Streptomyces* (108 strains) or *Micromonospora* (42 strains). The relative abundance of *Micromonospora* to *Streptomyces* strains increased by greater than 10-fold in the off-shore San Diego sites. Both near- and off-shore sediments yielded 1 to 3 strains related to the genera *Actinomadura*, *Saccharomonospora*, and *Verrucosispora*, while *Nocardiopsis*, *Streptomonospora*, and *Streptosporangium* were only recovered from sediments collected near-shore. Of the 5 OTUs, 2 belong-

Table 2. Number of operational taxonomic units (OTUs) recovered from near-shore (NS) and off-shore (OS) marine sediments calculated using consensus groups ranging from 97 to 100% 16S rRNA gene sequence identity (% of total given in parentheses). OTUs were identified as new if all strains shared ≤98% 16S rRNA gene sequence identity with previously cultured strains for which sequence data were available. Otherwise, OTUs were considered known (previously cultured), or known marine (previously cultured from marine sources)

OTUs	% Sequence identity							
	97 NS	97 OS	98 NS	98 OS	99 NS	99 OS	100 NS	100 OS
New	3 (17)	4 (25)	9 (26)	6 (16)	14 (32)	9 (18)	18 (30)	12 (19)
Known marine	1 (6)	2 (13)	4 (11)	6 (16)	4 (9)	6 (12)	7 (12)	8 (13)
Known	14 (78)	10 (63)	22 (63)	25 (68)	26 (59)	36 (71)	35 (58)	43 (68)
Total	18	16	35	37	44	51	60	63

Table 3. Generic affiliation and abundance of actinomycete strains and OTUs recovered from near and off-shore samples as delineated by 98% 16S rRNA gene sequence identity. Number of new OTUs are given in parentheses and those requiring seawater for growth in italics. Total number of unique OTUs among the 3 sampling sites is 54 and between the 2 off-shore sites is 37 (i.e. 1 off-shore OTU represented by CNS-635 [Fig. 2b] is shared between San Diego and San Francisco)

Genus	San Diego				San Francisco	
	Near-shore		Off-shore		Off-shore	
	Strains	OTUs	Strains	OTUs	Strains	OTUs
Actinomadura	1	1	0	0	1	1
Micromonospora	11	5	31	15, (1)	0	0
Nocardiopsis	9	2, (1), *2*	0	0	0	0
Saccharomonospora	3	1, *1*	1	1	0	0
Streptomonospora	5	2, (1), *2*	0	0	0	0
Streptomyces	79	22, (7), *4*	22	12, (3)	7	7, (2)
Streptosporangium	1	1	0	0	0	0
Verrucosispora	2	1	3	1	1	1
Total	111	35, (9), *9*	57	29, (4)	9	9, (2)

ing to these latter 3 groups were new to this study (Table 3). The OTU to sample ratio was considerably higher for off-shore (38:16) compared to near-shore samples (35:57), yet the total number of genera observed was greater in the near-shore samples (8 vs. 5) as was the number of strains per OTU (3.2 vs. 1.7). BLAST identity scores for all strains ranged from 91.3 to 100% (mean = 98.4 ± 1.4%) and averaged 96.6% for the strains in the new OTUs (98% consensus group).

Considering the total diversity observed in this study, the number of OTUs in which all strains required seawater for growth ranged from 14 of 126 (11%) within the 100% consensus group to 6 of 34 (18%) within the 97% consensus group (Fig. 1). There was no evidence for an increase in the probability of an OTU being new or 'known marine' based on the requirement of seawater for growth (T-test of proportions, p = 0.2505). Each of the 4 sequence identity groups (97 to 100%) contained 3 or 4 OTUs in which the requirement of seawater for growth varied among strains. All of these 'variable' OTUs were observed in the near-shore samples, affiliated with the genus *Streptomyces*, and in most cases only reported from marine sources. Of the 9 seawater-requiring OTUs identified within the 98% consensus group (Table 3), 5 were previously reported form non-marine sources.

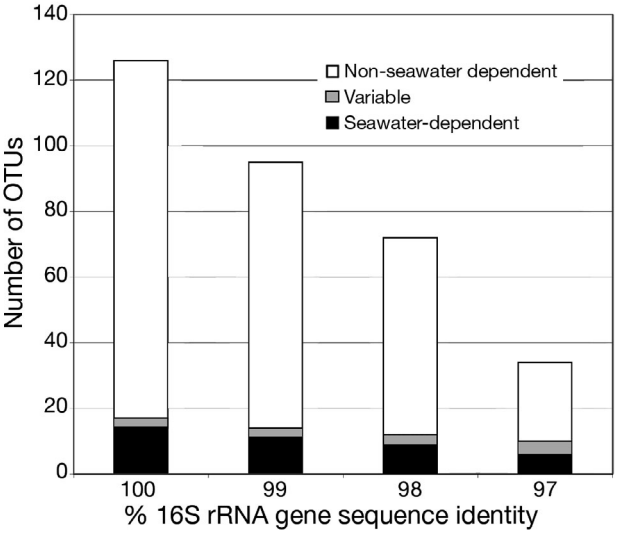

Fig. 1. Number of seawater-dependent, non-seawater-dependent, and variable operational taxonomic units (OTUs) at consensus groups ranging from 97 to 100% for combined near- and off-shore data sets

Representatives of the 35 near-shore and 37 off-shore OTUs delineated at 98% sequence identity (Table 2) were chosen for nearly complete 16S rRNA gene sequencing and phylogenetic analysis (Fig. 2). The increased abundance and diversity of *Micromonospora* relative to *Streptomyces* in the off-shore communities is evident when comparing the trees generated from these 2 sites. New OTUs are scattered throughout the near-shore tree (Fig. 2a), while at the off-shore sites new diversity is restricted to the genus *Streptomyces* with the exception of one *Micromonopora* OTU. Among the near-shore strains, 2 distinct marine clades related to the genus *Streptomyces* are evident (Fig. 2a). The clade at the top of the tree comprises 24 strains that fall into 5 OTUs, 3 of which are new. All OTUs within this clade and all NCBI reference strains that share >98% sequence identity were derived from marine sources, making this a highly diverse marine lineage within the genus *Streptomyces*. Seventeen strains belonging to this clade were also isolated from the off-shore samples (Fig. 2b, represented by strain CNS-775). These strains fall into 9 OTUs, all of which are marine and 3 of which are new. There is strong bootstrap support for the delineation of the second marine clade represented by strain CNQ-259. Members of this group, which have previously been referred to as MAR2, were also observed in both the near- and off-shore communities. The relationship of MAR2 as a sister lineage to the genus *Streptomyces* indicates that it may represent a new genus. This clade has proven to be a rich source of macrolide antibiotics (Fenical & Jensen 2006).

Seawater-requiring OTUs belonging to clades related to the genera *Streptomonospora* and *Nocardiopsis* were also observed (Fig. 2a). Interestingly, the most closely related reference strains are either

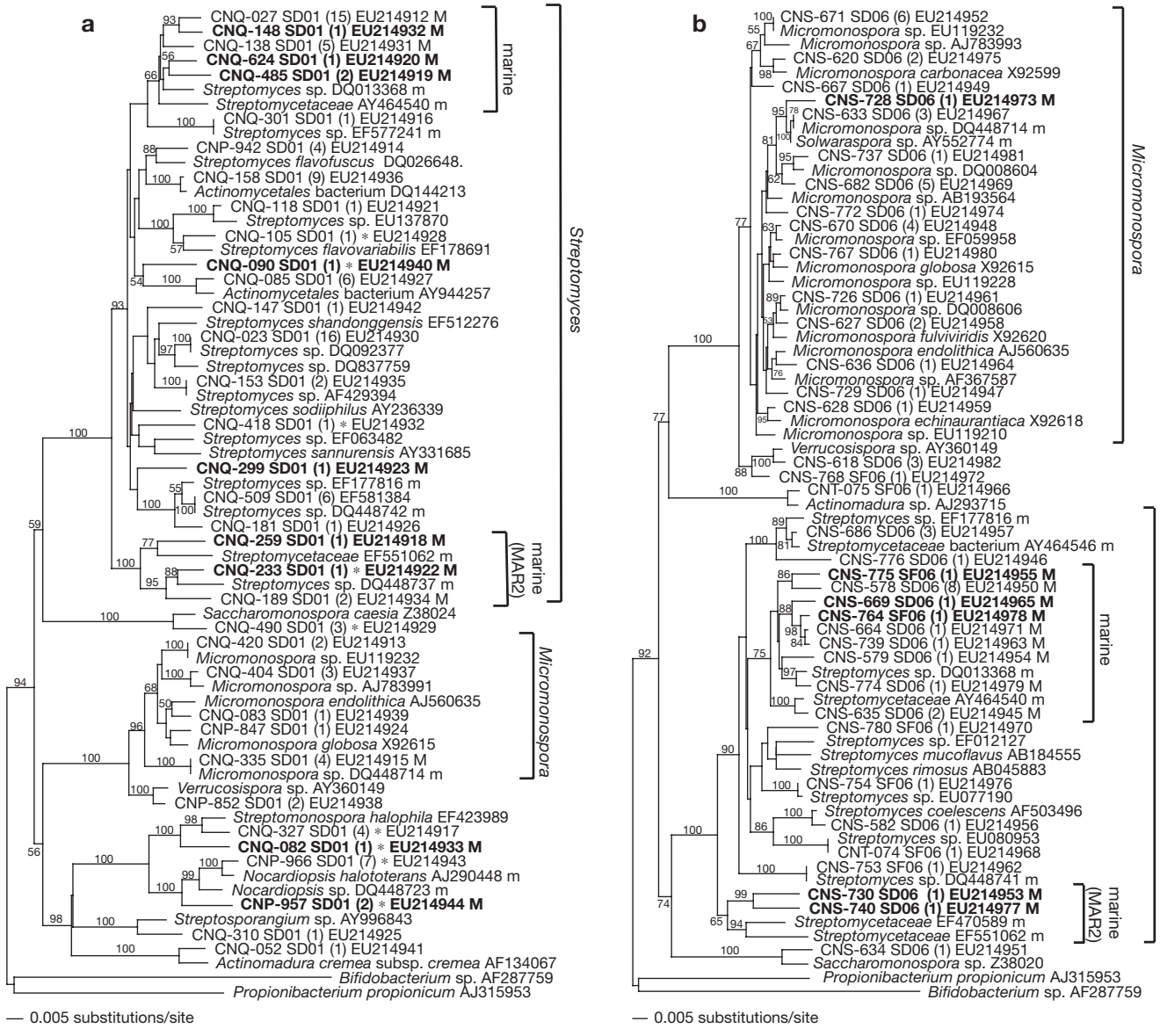

Fig. 2. Neighbor-joining distance trees detailing the phylogenetic relationships among OTU representatives (based on 98% sequence identity, 1378 bp) cultured from (a) near-shore and (b) off-shore marine sediments. The closest BLAST matches to each OTU are included with strain name and GenBank accession number. OTU representatives are shown with strain number (e.g. CNQ-082), collection location (SD = San Diego, SF = San Francisco), and year (e.g. 01 = 2001). The number of strains in each OTU is presented in parentheses. Bold = new OTUs, * = seawater-dependent OTUs, italics = variable OTUs (i.e. OTUs containing both seawater-dependent and non-dependent strains), M = marine OTUs (all NCBI reference sequences sharing >98% sequence identity are from marine sources), m = reference sequence derived from a marine source. *Propionibacterium propionicum* and *Bifidobacterium* sp. were used to root the trees. Bootstrap values greater than 50 are placed at their respective nodes

from marine or high salt environments. Three additional seawater-requiring OTUs are scattered throughout the genus *Streptomyces* (Fig. 2a). All but one of these (CNQ-090) is associated with an OTU that has also been reported from non-marine sources. One additional seawater-requiring OTU is related to the genus *Saccharomonospora*. None of the OTUs from the genus *Micromonospora* required seawater for growth, while the related, seawater-dependent genus *Salinispora*, which has been consistently cultured from tropical and subtropical sediments (Jensen & Mafnas 2006), was not observed despite the use of cultivation methods known to support the growth of this taxon.

Using the OTUs delineated at 98% sequence identity, the percent coverage (C) of the near-shore collection was calculated to be 74%, suggesting that the diversity within the 557-strain collection was well

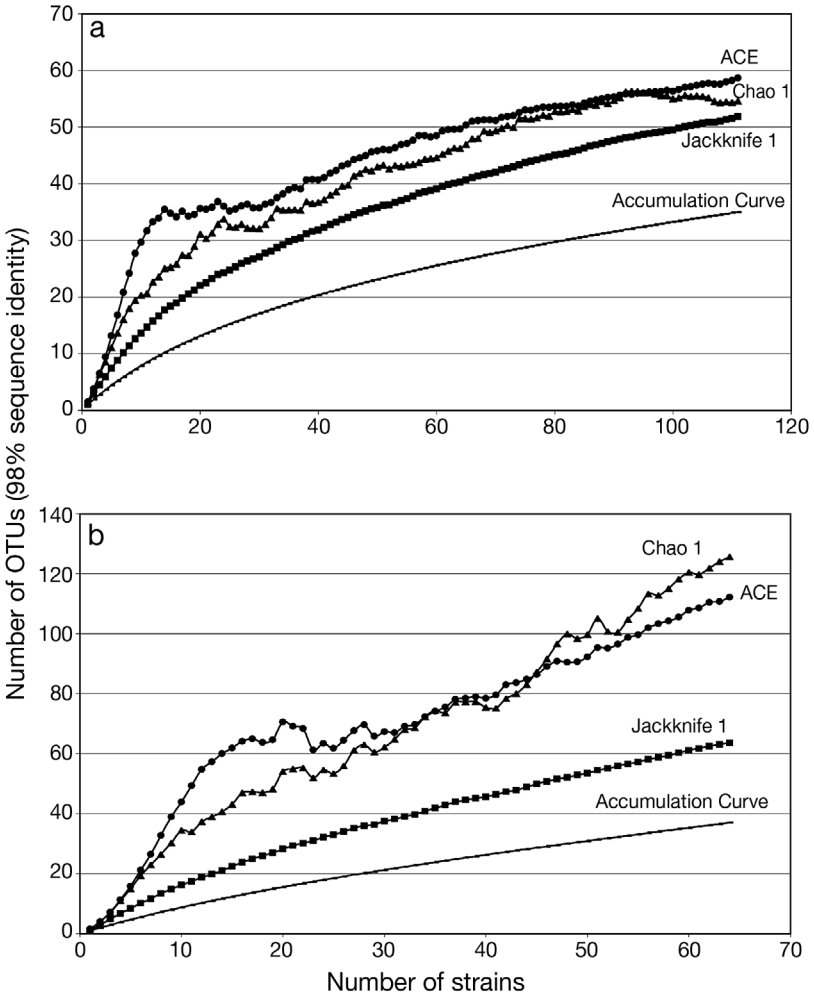

Fig. 3. OTU accumulation curves and diversity estimates calculated using Chao 1 (▲), Abundance-based Coverage Estimator (ACE) (●), and Jackknife 1 (■) for (a) near-shore and (b) off-shore actinomycete communities

represented by the 111 strains sequenced. Since all of the strains isolated from the off-shore sediments were sequenced, a coverage calculation was not performed. Accumulation curves were generated and 3 diversity estimators used to predict the total number of OTUs that occur in the near-shore (Fig. 3a) and off-shore (Fig. 3b) samples. The non-linearity of the curves reflects well-sampled communities (Martin 2002), and the fact that saturation was not reached in either environment indicates that additional processing would yield more diversity. The diversity estimators Abundance-based Coverage Estimator (ACE), Chao 1, and Jackknife all predict greater diversity in the off-shore (112.2, 125.6, 63.4, respectively) compared to near-shore samples (58.6, 54.6, 51.9, respectively), with the first 2 methods predicting approximately twice as many OTUs off-shore. Species richness (35 vs. 37) and the Shannon index (3.1 vs. 3.4) were approximately equal for both environments, while the Simpson index was considerably greater for the off-shore sediments (17.7 vs. 31.5). Although greater diversity is predicted for the off-shore samples, actinomycete colonies were recovered less frequently from this environment, suggesting that accessing this diversity will require the application of additional cultivation procedures.

Rank abundance plots were generated for near- and off-shore communities to assess the richness and evenness of the pool of sequenced strains (Fig. 4). For both analyses, the plots show a long right-hand tail as is typical of highly diverse communities (Hughes et al. 2001), with 50% or more of the OTUs comprising a single strain. For the near-shore community, 29% of the strains fell into the 2 most populated OTUs, both of which are most closely related to the genus *Streptomyces*. One of these (OTU number 2, Fig. 4a) is a 'variable' OTU comprising 5 seawater-dependent and 10 non-seawater-dependent strains. Phylogenetic analysis of all strains within this variable OTU did not delineate seawater-dependent from non-dependent lineages (data not shown). The most populated seawater-dependent OTU (number 4) comprises 7 strains and is most closely related to the genus *Nocardiopsis*. This OTU was not defined as 'new' or 'marine-specific' using the 98% sequence identity criterion. The seawater requirements of the poorly populated OTUs must be considered tentative until additional strains can be isolated and tested.

When statistical comparisons were made between near- and off-shore communities, the UniFrac measurement was significant ($p = 0.01$) but the P-test was not ($p = 0.33$), suggesting that the phylogenetic distribution of the OTUs is similar for both environments yet the OTUs that comprise them are distinct. When strains from both environments are pooled, 54 OTUs are delineated at the 98% consensus level (data not shown), with 40 of these being specific to either near- or off-shore sites (20 each). Although these results provide further evidence of differences between the 2 habitats, 13 of the near-shore and 17 of the off-shore specific OTUs are populated by only 1 strain (data not shown), suggesting that these distinctions may be an artifact of small sample size.

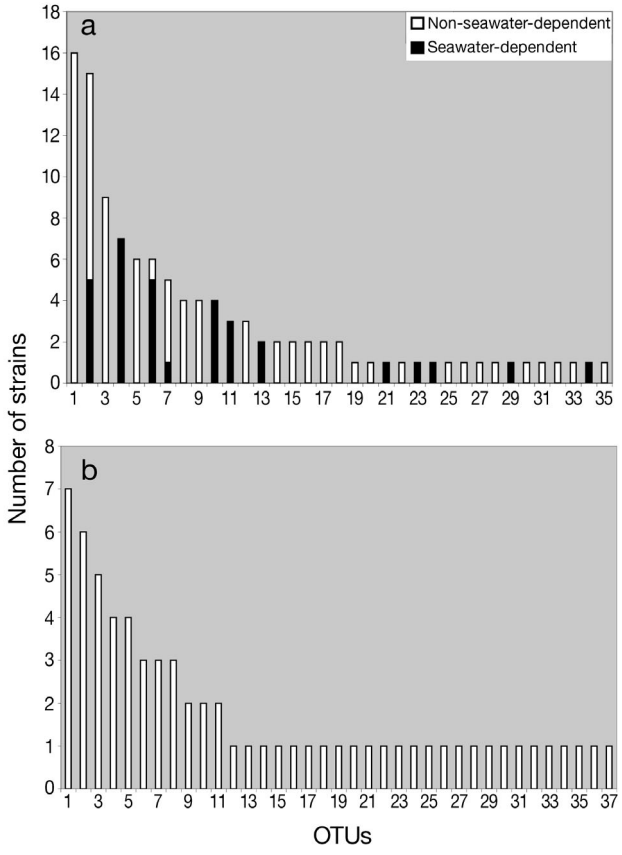

Fig. 4. Rank abundance plots for OTU diversity at 98% sequence identity from (a) near-shore and (b) off-shore sediments

DISCUSSION

Actinomycetes are regularly isolated from marine samples (e.g. Maldonado et al. 2005b, Stach & Bull 2005), yet the extent to which these bacteria are genetically distinct from those recovered from non-marine sources remains unknown. The main objectives of this study were to assess the culture-dependent diversity, phylogenetic novelty, and seawater requirements of filamentous, mycelium-forming actinomycetes cultured from marine sediments collected off the coast of California. These parameters were then compared among strains isolated from near- and off-shore locations to test the hypotheses that marine specificity and the requirement of seawater for growth would be encountered more commonly at sites more distant from shore. In addition, we tested for a correlation between the requirement of seawater for growth and phylogenetic novelty in an effort to determine if this trait could be used to guide the isolation of new marine taxa and to assess the level of marine adaptation in sediment-derived actinomycete communities.

Phylogenetic analyses were performed on 177 strains representing 66 off-shore and 111 near-shore isolates. The near-shore isolates comprised 32 to 40 representatives from each of 3 groups delineated based upon the effects of seawater on growth. In total, actinomycetes related to 8 genera within 6 families were isolated, indicating that considerable actinomycete diversity was recovered from marine sediments including representatives of 5 genera (*Nocardiopsis*, *Actinomadura*, *Saccharomnospora*, *Streptosporangium* and *Verrucosispora*) that have seldom been reported from the marine environment.

Various 16S rRNA gene sequence identity values have been used to delineate bacterial OTUs in sequence-based diversity studies (e.g. Hagström et al. 2002). Although the highly conservative value of 97% has been used in the past, it is now clear that this level of sequence similarity underestimates species-level diversity. For example, it has been shown for *Actinobacteria* that assigning OTUs based on >99% sequence similarity minimized the inclusion of strains with DNA-DNA re-association values of <70% and incorporated 70% of the values >70% (Stackebrandt & Goebel 1994, Stach et al. 2003). To contrast the diversity realized using different sequence identity values, strains were binned into consensus groups over a range from 97 to 100% similarity (Table 2). These analyses revealed considerable OTU diversity at 99% sequence identity with 111 near-shore and 66 off-shore strains (Table 1) falling into 44 and 51 OTUs, respectively (Table 2). Comparatively, only 18 near-shore and 16 off-shore OTUs were resolved using 97% sequence identity. Given the slow rate of change in the 16S rRNA gene (Woese 1987), it is likely that less conservative consensus groupings are more accurate predictors of taxonomic diversity in these communities.

For this study, OTUs generated based on 98% sequence identity were used for statistical analyses and considered 'new' if all of the strains within an OTU shared ≤98% sequence identity with NCBI reference sequences. Using this criterion, new OTUs accounted for 9 of 35 near-shore OTUs and 6 of 37 off-shore OTUs (Table 2). Even when OTUs previously reported from marine sources ('known marine') were considered, the majority of the OTUs detected in this study were previously reported from non-marine sources. Given the relatively high abundance of actinomycetes in soil and the preponderance of material being introduced from land into the sea, it can be suggested that many of these actinomycetes ultimately originated from land. Surprisingly, neither the level of phylogenetic novelty nor marine specificity increased in the off-shore sites (Tables 2 & 3). There was also no evidence that the San Francisco off-shore site was more highly influenced by terrestrial input despite the fact that a large percent-

age of California's freshwater drains through the San Francisco Bay estuary relative to San Diego Bay, which is a low inflow estuary. Most of the actinomycete diversity observed in this study was not marine-specific, an observation that could be explained by a high level of terrestrial input and mixing out to 125 km from shore. This is not to preclude the possibility that higher levels of marine-specific diversity occur at sites more distant from shore and that some marine-specific diversity was missed due to limitations in the culturing techniques employed.

The requirement of seawater for growth was not a common physiological trait among the actinomycetes cultured, being observed in less than 6% of the strains isolated and 9 of the 54 unique OTUs delineated at 98% sequence identity. In addition, phylogenetic novelty was not correlated to this requirement, which may in part be due to the NaCl tolerance of soil actinomycetes (Basilio et al. 2003) or our inability to accurately resolve ecotypes using the 16S rRNA gene (Cohan 2002). The fact that one OTU (represented by strain CNP-966, Fig. 2a) comprised 7 seawater-requiring strains related to the genus *Nocardiopsis* was not recognized as 'new' or 'marine-specific' suggests that the criteria applied here to delineate marine taxa were overly conservative. It is also possible that the detection of a requirement of seawater for growth is confounded by the rapid loss or acquisition of this phenotype. However, we have not been able to eliminate this requirement following successive transfers onto media prepared with decreasing concentrations of seawater (data not shown), suggesting that this trait is fixed and possibly associated with a specific sodium ion requirement, as has been observed for Gram-negative marine bacteria (Niven & MacLeod 1980). The seawater-dependent strains cultured from the near-shore collection sites were scattered throughout the phylogenetic tree (Fig. 2a), suggesting that they did not arise from a common marine ancestor and that this trait had instead evolved independently in multiple lineages. Although the requirement of seawater for growth was not common, it was observed among all OTUs related to *Nocardiopsis* and *Streptomonospora*, suggesting a high level of marine adaptation in these groups (Fig. 2a).

A deeply rooted marine lineage has been identified within the phylum *Actinobacteria* (Rappé et al. 1999); however, members of this group have yet to be cultured. As in other cultivation-based studies of actinomycetes in marine sediments (e.g. Pisano et al. 1989, Colquhoun et al. 1998), the dominant genera recovered were *Streptomyces* and *Micromonospora*. As has also been previously observed (Weyland 1981, Jensen et al. 1991), there was a shift in community composition from *Streptomyces* to *Micromonospora* between near- and off-shore samples (Fig. 2). The cause of this shift remains unresolved as neither OTU novelty, seawater dependence, nor the relative abundance of marine OTUs increased among the off-shore *Micromonospora* strains. Marine lineages related to the genus *Streptomyces* however were observed in both near- and off-shore samples. From a phylogenetic perspective, these lineages appear to include multiple new species and represent at least one new genus. Given the breadth of phylogenetic diversity currently ascribed to the genus *Streptomyces*, revision of this taxon appears warranted.

Fewer actinomycetes were obtained per sample from the off-shore sites despite the fact that the amount of material inoculated was greater and 2 additional media were used. These results suggest that actinomycetes may be less common in the off-shore locations. However, ambient ocean temperatures of <5°C (National Oceanographic Data Center, www.nodc.noaa.gov/) at the shallowest off-shore site in comparison to ca. 15°C at the deepest near-shore site suggest that incubation temperatures may have played a role in the relatively low numbers of actinomycetes recovered. Despite the relative difficulty obtaining actinomycetes from the off-shore samples, the number of OTUs delineated from each community was nearly identical (Table 2) and the number of OTUs predicted from the off-shore sites was almost double that from near-shore sediments (Fig. 3).

Remarkably, little redundancy was observed among the cultures as ≥75% of the OTUs delineated at 100% sequence identity included only 1 strain. The large number of single strain OTUs made it difficult to confidently assign habitat specificity and the effects of seawater on growth. Shannon and Simpson's indices as well as UniFrac statistics (Lozupone & Knight 2005) show differences between shallow and deep actinomycete communities, supporting the need for further exploration of deep-sea environments, although cultivation at low temperature or high pressure may be required to improve the rate of strain recovery. In summary, phylogenetic novelty and marine specificity were not greater at the off-shore sites, suggesting high rates of mixing out to distances of 125 km from shore. Nonetheless, considerable new actinomycete diversity was obtained in culture and community diversity was predicted to be greater at the off-shore sites, encouraging continued sampling of deep-sea locations as the bacteria recovered from these sites are proving to be a valuable resource for biotechnology (Bull et al. 2000, Jensen et al. 2005).

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