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Culturable marine actinomycete diversity from tropical Pacific Ocean sediments

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Summary

Actinomycetes were cultivated using a variety of media and selective isolation techniques from 275 marine samples collected around the island of Guam. In total, 6425 actinomycete colonies were observed and 983 (15%) of these, representing the range of morphological diversity observed from each sample, were obtained in pure culture. The majority of the strains isolated (58%) required seawater for growth indicating a high degree of marine adaptation. The dominant actinomycete recovered (568 strains) belonged to the seawater-requiring marine taxon '*Salinospora*', a new genus within the family Micromonosporaceae. A formal description of this taxon has been accepted for publication (Maldonado *et al*., 2005) and includes a revision of the generic epithet to *Salinispora* gen. nov. Members of two major new clades related to *Streptomyces* spp., tentatively called MAR2 and MAR3, were cultivated and appear to represent new genera within the Streptomycetaceae. In total, five new marine phylotypes, including two within the Thermomonosporaceae that appear to represent new taxa, were obtained in culture. These results support the existence of taxonomically diverse populations of phylogenetically distinct actinomycetes residing in the marine environment. These bacteria can be readily cultured using low nutrient media and represent an unexplored resource for pharmaceutical drug discovery.

Introduction

As of 1988, approximately two-thirds of the known, naturally derived antibiotics, including many pharmaceuticals in current clinical use, were discovered as fermentation products from cultured actinomycetes (Okami and Hotta, 1988). Although the positive impact of actinomycete products on human health is clear, there is a perception that 50 years of intensive research by the pharmaceutical industry has exhausted the supply of compounds that can be discovered from this group. This perception has been a driving force behind the recent shift away from natural products as a source of small molecule therapeutics towards other drug discovery platforms including high throughput combinatorial synthesis and rational drug design (Blondelle and Houghten, 1996; Bull *et al*., 2000; Wijkmans and Beckett, 2002).

Historically, actinomycetes are best known as soil bacteria and were generally believed to occur in the ocean largely as dormant spores that were washed into the sea (Goodfellow and Haynes, 1984). Despite evidence to suggest that this may not be the case (Helmke and Weyland, 1984; Jensen *et al*., 1991; Takizawa *et al*., 1993; Moran *et al*., 1995; Colquhoun *et al*., 1998), the distributions and ecological roles of actinomycetes in the marine environment, and the extent to which obligate marine species occur, have remained an unresolved issue in marine microbiology.

Recently, we reported the cultivation from marine sediments of a major new group of marine actinomycetes (originally called MAR1) for which the generic epithet '*Salinospora*' was proposed (Mincer *et al*., 2002). The systematics of this taxon have now been studied in more detail and a formal description of two species, '*Salinospora arenicola*' and '*Salinospora tropica*', is forthcoming, including a revision of the generic epithet '*Salinospora*' to *Salinispora* gen. nov. (Maldonado *et al*., 2005). To date, in excess of 1000 *Salinispora* strains have been recovered from sediments collected from the subtropical Atlantic, the Red Sea and the Sea of Cortez suggesting a pan-tropical distribution. All strains tested have required seawater and, more specifically, sodium for growth indicating a high level of marine adaptation. In addition, the taxon has proven to be a productive source of structurally unique and biologically active secondary metabolites (Feling *et al*., 2003; Jensen *et al*., 2005). Thus, there is mounting evidence that marine actinomycetes represent an autochthonous yet little understood component of the sediment microbial community as well as a useful resource for pharmaceutical discovery.

In an effort to gain a better understanding of marine actinomycete diversity, a culture-dependant study was

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undertaken using samples collected around the island of Guam. The goals of this study were to determine whether *Salinispora* strains could be recovered from this Pacific Ocean location, to test new cultivation methods in an effort to discover new *Salinispora* diversity and to determine whether additional new marine actinomycete taxa could be recovered.

Results

Actinomycete isolation

A total of 288 samples were processed for actinomycete isolation of which 223 (77%) yielded actinomycete growth. The samples consisted largely of sediments (240); however, they also included a relatively small number of algae (33) and sponges (15). Samples were inoculated onto 1909 primary isolation plates of which 832 (44%) yielded actinomycete colonies. In many cases, actinomycete hyphae could be observed growing away from sand grains or shells (Fig. 1) suggesting that they were associated with particles. Microscopic examination (SEM) of these sands grains revealed branching filaments, a diagnostic characteristic of many actinomycetes, and spores borne singly on substrate mycelium, a morphological feature associated with the genus *Micromonospora* (Fig. 2). Many of these colonies, when growing on low nutrient media, could only be visualized with the aid of a stereomicroscope even after 2–3 weeks of incubation.

The total number of actinomycete colonies observed on all primary isolation plates was 6425. On average, we observed 3.4 actinomycete colonies per plate with that number increasing to 7.7 per plate when only considering those plates that yielded actinomycetes. Of the total (6425) actinomycetes colonies observed, 2772 (43%) could be tentatively grouped with the genera *Salinispora* (Mincer *et al*., 2002) and *Micromonospora* based on colony morphology. Many of these colonies were subsequently isolated and their precise generic affiliations confirmed by SSU rRNA gene sequencing and by testing for a requirement of seawater for growth, a consistent feature of the *Salinispora* clade that has yet to be reported for any other member of the Micromonosporaceae.

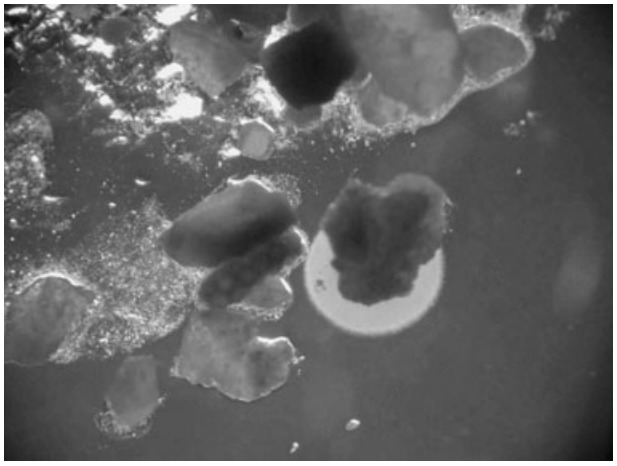

Fig. 1. Light micrograph (64×) of an actinomycete colony growing away from a sand grain.

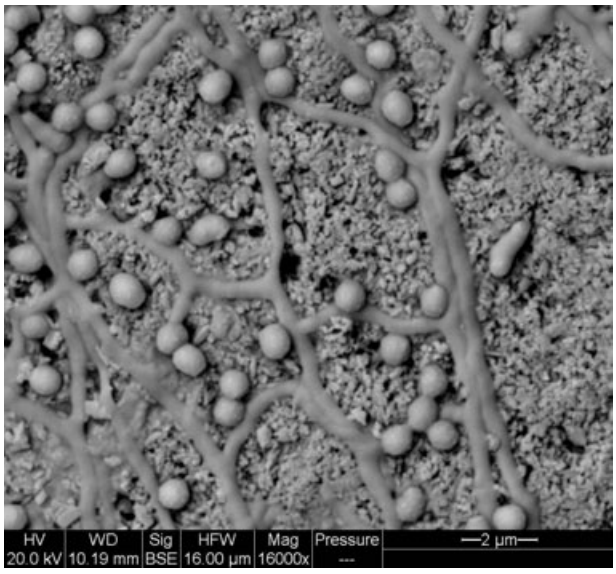

Fig. 2. Microscopic examination (SEM) (16 000×) of an actinomycete growing on a sand grain. Branching filaments, a diagnostic characteristic of many actinomycetes, and spores are clearly evident. HV, high voltage; WD, working distance, Sig, signal; HFW, horizontal field width; Mag, magnification.

Representatives of all actinomycete morphotypes observed from each sample were obtained in pure culture resulting in the isolation of 983 individual strains (15.3% of the colonies observed). Once isolated, 643 (65%) were tentatively assigned to the *Salinispora*/*Micromonospora* group based on morphological features supporting our initial observation that these two genera represented the majority of the actinomycetes cultured. On nutrient-rich media (e.g. medium 1), both *Salinispora* and *Micromonospora* spp. generally produce orange-pigmented colonies that lack aerial hyphae and black spores that darken the colony surface thus making them difficult to differentiate based on colony morphology alone. Five hundred and sixty-eight (88%) of the 643 strains that were grouped in these two genera required seawater for growth suggesting that they were *Salinispora* species. This suggestion was confirmed for 57 strains by partial SSU rRNA gene sequence analyses. All of these strains, encompassing a range of *Salinispora* morphotypes derived from diverse samples, demonstrated a clear phylogenetic affiliation with the *Salinispora* clade and possessed all four of the signature nucleotides reported for the first 600 base pairs of the SSU rRNA gene (Mincer *et al*., 2002).

Likewise, the remaining 75 strains, initially placed in the *Salinispora*/*Micromonospora* group, did not require seawater for growth and therefore were presumed to be *Micromonospora* spp. Five of these strains were subjected to partial SSU rRNA gene sequencing and all five were from 99% to 100% identical with *Micromonospora* spp. including strain CNH394 (AY221497) which was previously isolated from marine sediments collected in the Bahamas (Mincer *et al*., 2002). Thus, *Salinispora* was the most common actinomycete cultured (58% of the total 983 strains isolated) while *Micromonospora* strains were relatively uncommon. In addition, a demonstrable requirement of seawater for growth remains a rapid and accurate method to distinguish between these two genera.

Excluding the 568 *Salinispora* strains obtained in pure culture, only seven additional seawater-requiring actinomycetes were recovered. The requirement of seawater for growth was first reported in the actinomycetes for *Salinispora* strains (Mincer *et al*., 2002) and, outside of this group, thus far appears to be uncommon among marine-derived actinomycetes. Of the non-seawater-requiring strains, 61 grew poorly in the absence of seawater while the remainder grew equally well when seawater was replaced with purified water in a complex nutrient medium (medium 1). When considering the total number of actinomycetes obtained in pure culture (983), 58% required seawater for growth indicating that the majority of actinomycetes recovered were highly adapted to growth in the marine environment.

From the algal and sponge samples processed, there were dramatically different rates of actinomycete recovery. From 33 algal samples, a total of 343 actinomycete colonies were observed (on average, 2.9 per primary isolation plate), 55% of which were assigned to the *Salinispora*/*Micromonospora* group. Of the 15 sponges processed, only four actinomycete colonies were observed (on average, <0.1 colony per plate), and only one of these was ascribed to the *Salinispora*/*Micromonospora* group. Although different processing methods were used, it appears that actinomycetes are less abundant in sponges than on algal surfaces or at least more difficult to recover.

Phylogenetic diversity

One of the objectives of this study was to determine if new *Salinispora* diversity could be cultured as a result of sampling new locations and testing new culture techniques. Based on the partial SSU rRNA gene sequence data obtained from the 57 *Salinispora* strains discussed above, 13 strains were selected for full SSU rRNA gene sequencing and three of these (CNR040, CNR107 and CNR425) are presented in Fig. 3. These strains all fall within the *S. arenicola* (CNR425, CNR107) and *S. tropica* (CNR040) clades, possess the five previously reported *Salinispora*-specific signature nucleotides (207 = A, 366 = C, 467 = U, 468 = U, 1456 = G, *Escherichia coli* numbering; Mincer *et al*., 2002), are ≥ 99.0% similar to previously reported *Salinispora* strains (Mincer *et al*., 2002) and are from 98.3% to 98.6% similar to *Micromonospora olivasterospora*, the most closely related non-*Salinispora* species. Given that sequence differences of ≤1% have been used to define an operational taxonomic unit (reviewed by Hughes *et al*., 2001), and the inconsistent correlation between genomic DNA–DNA hybridization results and SSU rRNA sequence similarities (Rossell-Mora and Amann, 2001), it remains possible that new *Salinispora* species were cultivated. However, despite the large number of strains examined, it is clear that no significant new SSU rRNA-based phylogenetic diversity was recovered within the *Salinispora* clade.

A second objective of this study was to determine whether additional new actinomycete taxa could be cultured from marine samples, and in this regard, the results were highly encouraging. Thus far, we have focused our analyses on seawater-requiring strains and strains that grew poorly in the absence of seawater. Based on phylogenetic relationships inferred from partial SSU rRNA sequence data, 13 non-*Salinispora* strains were sequenced in full (Fig. 3). These strains form two major new clades within the Streptomycetaceae that have tentatively been called MAR2 and MAR3. The four MAR2 strains (CNQ695, CNQ703, CNQ732 and CNR252), which grew poorly or not at all in the absence of seawater (Table 1), share from 96.2% to 96.9% similarity with *Streptomyces alkalophilus* (AY331685), the most closely related sequence based on an NCBI BLAST (BLASTN) search, and appear to represent a new genus. The six members of the MAR3 clade (CNQ530, CNQ687, CNQ698, CNQ719, CNQ857, CNR530) possess from 97.2% to 98.3% sequence similarity to an unidentified *Streptomyces* sp. (AY236339) and similarly may represent another new genus within the family. With the exception of CNR530, all six of the MAR3 strains required seawater for growth (Table 1). A MAR3 intraclade similarity of 96.8% suggests that this group is comprised of multiple species.

The tree topology illustrated in Fig. 3 was maintained using multiple treeing methods with the exception that strain CNR530 fell outside of the MAR3 clade following parsimony analysis. As mentioned, this is the only strain among the six MAR3 clade members that did not require seawater for growth. In addition to the MAR2 and MAR3 clades, CNQ766 also falls within the Streptomycetaceae and, based on its requirement of seawater for growth and sequence similarity of 98.6% to *Streptomyces kasugaensis* (AB024442), this strain may also represent a new taxon that we have provisionally called MAR4. In addition to the new phylogenetic diversity observed within the Streptomycetaceae, CNR363 and CNR431 fall within

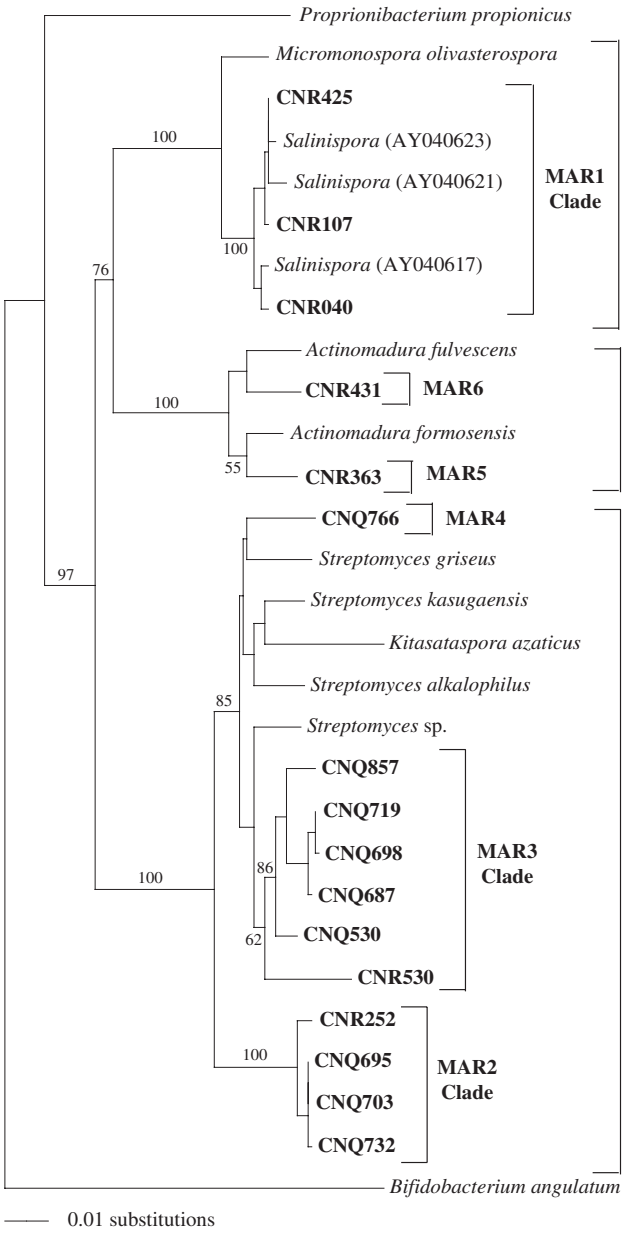

Fig. 3. Phylogenetic relationships among nearly complete (1476 nucleotide positions) SSU rRNA gene sequences of cultured marine actinomycetes (in bold) and closely related sequences obtained from an NCBI BLAST (BLASTN) search. MAR1–MAR6 are tentative designations for new marine actinomycete phylotypes. The generic epithet *Salinispora* (revised to *Salinispora*) has been proposed for the MAR1 clade which currently consists of two species *S. arenicola* (CNR425, CNR107) and *S. tropica* (CNR040). The tree was constructed using the neighbour-joining method with the percentage of bootstrap replicates (1000 re-samplings) supporting the proposed branching order shown at the relevant nodes (values below 55% not shown). *Bifidobacterium angulatum* and *Propionibacterium propionicus* were used as outgroups.

the family Thermomonosporaceae. These strains are 97.8% and 96.7% similar to *Actinomadura formosens* (AF0420140) and *A. fulvescens* (AJ420137), respectively, and appear to represent new taxa that have tentatively been named MAR5 and MAR6. Thus, strains belonging to six new marine actinomycete phylotypes (MAR1–MAR6), representing three families within the order Actinomycetales, have been successfully cultivated from marine samples collected around the island of Guam.

Cultivation techniques

The percentage of plates yielding actinomycete colonies ranged on the low end from 16% to 25% for medium 2 [nutrient-poor sediment (NPS)] and medium 3 [nutrient-rich sediment (NRS)], respectively, to a maximum of 69% for medium 1 (Table 2). Interestingly, although NPS and NRS, which contain no added organics other than those present in seawater, noble agar and the sediment extracts, yielded the lowest percentage of plates with actinomycetes, they yielded the highest percentage (82–91%) of actinomycetes that required seawater for growth. Medium 6, which contained low concentrations of mannitol and peptone, also produced a high percentage of seawater requiring strains (79%). As the majority of these strains were *Salinispora*, these media proved to be among the most effective for isolating members of that group. Medium 1, which was the only high nutrient

Table 1. New actinomycete phylotypes cultured from marine sediments.

Strain No. (Accession No.)	Phylotype	Family	Collection depth (m)	Sample number	Isolation method	Medium	Seawater requirement
CNR425 (AY464533)	MAR1	Micromonosporaceae	115	GU02-184	5	9 (SRC)	Yes
CNR107 (AY464534)	MAR1	Micromonosporaceae	40	GU02-313	1	6 (SMP)	Yes
CNR040 (AY464535)	MAR1	Micromonosporaceae	42	GU02-246	1	6 (SMP)	Yes
CNR252 (AY464536)	MAR2	Streptomycetaceae	3	GU02-290	6	6 (SMP)	No
CNQ695 (AY464537)	MAR2	Streptomycetaceae	500	GU02-178	1	9 (SRC)	Yes
CNQ703 (AY464538)	MAR2	Streptomycetaceae	75	GU02-316	1	6 (SMP)	No
CNQ732 (AY464539)	MAR2	Streptomycetaceae	3	GU02-284	4	12 (SMY)	No
CNR530 (AY464540)	MAR3	Streptomycetaceae	45	GU02-225	1	9 (SRC)	No
CNQ530 (AY464541)	MAR3	Streptomycetaceae	50	GU02-39	1	5 (SMC)	Yes
CNQ687 (AY464542)	MAR3	Streptomycetaceae	<1	GU02-172	4	5 (SMC)	Yes
CNQ698 (AY464543)	MAR3	Streptomycetaceae	3	GU02-290	1	3 (NRS)	Yes
CNQ719 (AY464544)	MAR3	Streptomycetaceae	<1	GU02-164	1	8 (SPC)	Yes
CNQ857 (AY464545)	MAR3	Streptomycetaceae	10	GU02-194	1	10 (SSC)	Yes
CNQ766 (AY464546)	MAR4	Streptomycetaceae	500	GU02-178	1	9 (SRC)	Yes
CNR363 (AY464547)	MAR5	Thermomonosporaceae	3	GU02-292	4	9 (SRC)	No
CNR431 (AY464548)	MAR6	Thermomonosporaceae	500	GU02-178	1	9 (SRC)	No

medium tested and contained more than 20 times the nutrient concentration of any other formulation, yielded the lowest percentage (29%) of seawater-requiring strains.

Of the three anti-bacterial agents compared in media 7–9, the highest percentage of plates yielding actinomycetes occurred on those containing novobiocin (Table 2, medium 7, 61%), and this antibiotic also yielded the second highest average number of colonies per plate (6.1). Medium 6 also yielded good actinomycete recovery; however, none of the combinations of organic substrates tested yielded dramatically improved actinomycete culturability. Although the vast majority of the strains reported in this study were cultivated on media containing relatively low nutrient concentrations, all of these strains were capable of growth on the high nutrient medium 1 suggesting that obligate oligotrophy is not common among marine actinomycetes and that the effectiveness of low nutrient formulas in this case may result from a reduction in growth by non-actinomycete bacteria.

The majority of the samples were processed using methods 1 (dry/stamp) and 4 (dilute/heat). These methods yielded good actinomycete recovery with 44% and 47%, respectively, of the plates yielding actinomycete colonies (data not shown). Interestingly, although method 2 (dry/scrape) was only used on 13 samples, 9 of these yielded actinomycetes suggesting that rock surfaces may be a good source from which to isolate these bacteria. Samples processed using method 6 yielded the highest rate of actinomycete recovery (70%) suggesting that increasing the amount of material inoculated could further improve recovery rates. Freezing as a selective pre-treatment (method 7) was relatively ineffective with 20% of the plates yielding actinomycetes and a 48 h post-thaw incubation (method 8) further reduced recovery rates to the lowest levels observed with only 2% yielding actinomycetes. Drying followed by dilution (method 3) and two cycles of heating (method 5) were among the least effective methods employed yielding actinomycetes on 8% and 18% of the plates respectively.

Table 2. Actinomycete recovery and seawater requirements using various isolation media.

Medium	No. of plates inoculated	No. of plates with actinomycetes (%)	Total No. of actinomycetes observed	Mean No. of actinomycetes per plate	No. of actinomycetes isolated	No. of seawater requiring actinomycetes (%)
1 (AMM)	48	33 (69)	196	4.1	30	9 (30)
2 (NPS)	217	35 (16)	191	0.9	47	43 (91)
3 (NRS)	218	54 25	236	1.1	72	59 (82)
4 (SHG)	97	44 (45)	303	3.1	37	10 (27)
5 (SMC)	94	49 (52)	354	3.8	61	27 (44)
6 (SMP)	172	96 (56)	1199	7.0	180	143 (79)
7 (SNC)	303	184 (61)	1839	6.1	233	137 (59)
8 (SPC)	93	52 (56)	395	4.2	50	26 (52)
9 (SRC)	376	175 (47)	1139	3.0	196	94 (48)
10 (SSC)	97	35 (36)	178	1.8	25	12 (48)
11 (STC)	93	33 (35)	131	1.4	14	4 (29)
12 (SMY)	101	42 (42)	264	2.6	38	17 (45)

The unique actinomycete phylotypes that were recovered (Table 1) came from a wide range of depths (3–500 m) and samples. Sample GU02-178, which was among the deepest samples obtained (500 m), yielded 35 actinomycete strains that included members of three unique phylotypes (CNQ695 = MAR2, CNQ766 = MAR4, CNR431 = MAR6) in addition to a relatively low percentage (7%) of *Salinispora* isolates. This sample was collected off the southwestern corner of the island at one of the few deep sites where mud samples were successfully retrieved as most other areas >100 m appeared to be dominated by hard, rocky bottom. This result suggests that further efforts to sample deep sediments may yield new actinomycete diversity.

Although the actinomycetes cultivated in this study span three families within the order Actinomycetales, the majority of the new diversity (three phylotypes) falls within the Streptomycetaceae. With the exception of the *Salinispora* clade, few strains were recovered for any of the new phylotypes (MAR2–MAR6) indicating that members of these groups are either rare or not readily cultured with the methods employed. Media 6 and 9 yielded the highest numbers of new phylotypes along with method 1; however, this method was applied to the largest number of plates so it is not clear that it is more effective for the cultivation of new taxa.

Discussion

It has long been known that actinomycetes can be recovered from marine sediments (Weyland, 1969) raising the possibility that these bacteria, like their terrestrial counterparts in soils, play important roles in the decomposition of recalcitrant organic matter in the sea floor. More recently, marine-derived actinomycetes have become recognized as a source of novel antibiotics and anti-cancer agents (Faulkner, 2002 and references cited therein) suggesting that they represent a new resource for natural product drug discovery (Bull *et al.*, 2000; Jensen and Fenical, 2000). For this to be correct, actinomycetes must be metabolically active in the marine environment and this activity must lead to the production of compounds that are not observed from terrestrial strains. Thus, to understand the importance of marine-derived actinomycetes in ecological terms and as a resource for biotechnology, we must understand the extent to which they are capable of growth in the ocean, the degree to which they display specific marine adaptations and the extent to which these adaptations have affected secondary metabolite production. Although prior evidence has been presented for the existence of indigenous marine actinomycete populations (Jensen *et al.*, 1991; Takizawa *et al.*, 1993; Colquhoun *et al.*, 1998) and for *in situ* metabolic activity (Moran *et al.*, 1995), we have only begun to define the extent to which marine-adapted actinomycetes differ from their terrestrial relatives.

In the present study, actinomycetes were cultivated from the majority of the samples collected (77%) indicating that these bacteria were widely dispersed in marine sediments around the island of Guam and that the isolation methods employed were largely appropriate for the selective cultivation of these slow growing bacteria. As has been our experience with tropical marine sediments, the most abundant actinomycete recovered belonged to the MAR1 clade for which the generic epithet *Salinispora* (Mincer *et al.*, 2002) and the species *S. arenicola* and *S. tropica* (Maldonado *et al.*, 2005) have been proposed. In total, 568 (58%) of the 984 strains obtained in pure culture could be confidently assigned to this genus based on a requirement of seawater for growth, which they all possessed, and a phylogenetic analysis of a subset of 57 strains.

It is noteworthy that the island of Guam can now be added to the Bahamas, the US Virgin Islands, the Red Sea and the Sea of Cortez as sites from which we have thus far recovered *Salinispora* strains from marine sediments. Guam being the first tropical Pacific site sampled adds support for a pan-tropical *Salinispora* distribution. To date, we have failed to isolate *Salinispora* strains from more temperate locations in the Pacific Ocean off La Jolla, CA, and from sediments collected off Alaska suggesting distinct latitudinal distribution limits. We have successfully cultivated *Salinispora* at 10°C but not at 4°C suggesting that temperature may be an important variable affecting their distribution.

Early reports describing *Micromonospora* from temperate and polar marine sediments (Weyland, 1981) raised the possibility that these isolates actually belong to the *Salinispora* clade. However, phylogenetic analysis of two North Atlantic isolates (provided by E. Helmke) collected at depths of 700 m and 2970 m between 45°N and 47°N latitudes off the coast of France (data not shown) clearly placed these organisms within the genus *Micromonospora* thus adding further support for the absence of *Salinispora* from colder biomes. As part of the present study, we successfully recovered *Salinispora* strains from the deepest site sampled (570 m) so a lower depth limit has yet to be determined for this group.

The observation that the majority of actinomycetes cultured in this study required seawater for growth is remarkable considering that until recently there has been little support for the existence of autochthonous marine actinomycete populations. The requirement of seawater for growth is a well-defined marine adaptation (Macleod, 1965) that cannot be accounted for by the hypothesis that these bacteria were washed in from shore and reside in marine sediments merely as dormant spores. In addition, all *Salinispora* strains tested to date have a demonstrable

requirement of sodium for growth (Jensen *et al*., 1991), a defining characteristic of many marine bacteria. Based on the results obtained from the present study, it can be concluded that the majority of actinomycetes isolated are highly adapted to life in the sea and that the recovery of 'washed-in' strains may be the exception rather than the rule. Given that the vast majority of the seawater-requiring strains cultivated belonged to the *Salinispora* clade, this taxon may play important microbiological roles in marine sediments, e.g. the recycling of recalcitrant organic materials.

Despite performing phylogenetic analyses on 57 *Salinispora* strains obtained using a variety of isolation techniques, relatively little new diversity was observed within this taxon. In contrast, significant new diversity was observed within the Streptomycetaceae in the form of two new, well-delineated clades (MAR2 and MAR3). Although these clades are comprised of relatively few strains (four and six respectively), the within-clade sequence dissimilarity is high ranging from 1.7% for MAR2 to 3.3% for MAR3. Considering that *Streptomyces* and *Kitasataspora* are the only recognized genera in the family (Anderson and Wellington, 2001), the addition of two new marine genera would add considerably to the extant diversity within this family. In addition, considering the historical significance of the genus *Streptomyces* as a source of novel antibiotics, these new taxa may represent a useful natural product resource. Thus far, preliminary chemical studies of one MAR2 clade member have revealed the production of a series of structurally unprecedented macrolide antibiotics called marinomycins (to be published elsewhere) further supporting the concept that marine actinomycetes represent a new resource for pharmaceutical discovery.

To date, *Salinispora* forms the only multispecies actinomycete taxon within which all of the individuals thus far cultivated require seawater for growth. The observation that seawater requirements varied among members of the MAR2 and MAR3 clades warrants further study into the genetic basis of this trait and the rates at which individual strains can adapt to varying salt concentrations. It will be important to isolate additional members of the MAR2–MAR6 phylotypes to better assess the intragroup variability of this physiological requirement. It is also evident that non-seawater-requiring strains must also be examined if we are to gain a more complete understanding of actinomycete diversity in the marine environment. Likewise, any attempt to define marine bacteria by specific physiological characteristics such as a requirement of seawater for growth may overlook unique and environmentally important taxa.

A recent culture-independent study of actinobacterial diversity in marine sediments revealed the presence of numerous new phylotypes including many clones that were most closely related to *Streptomyces* sp. (Stach *et al*., 2003). Many of these clones possessed ≤ 97% identity with previously cultured species suggesting the existence of multiple new genera. NCBI BLAST searches of the new phylotypes cultured as part of the present study did not yield any of the accession numbers reported by Stach and co-workers indicating that additional marine actinomycete taxa remain to be cultured from marine sediments. Although major progress has been made recently in the development of innovative techniques for the cultivation of marine bacteria (e.g. Rappé *et al*., 2002), it is clear that continued improvements in taxa-specific cultivation methods have the potential to yield significant new marine actinomycete diversity.

Our results support previous observations that *Streptomyces* are metabolically active in marine sediments (Moran *et al*., 1995) and suggest that a lack of genetic mixing with terrestrial strains, coupled with the adaptations required for survival in the marine environment, has led to the evolution of obligate marine taxa within the Streptomycetaceae and other actinomycete families. Continued efforts to improve cultivation techniques, along with the application of culture independent methods, will help reveal the true extent of marine actinomycete diversity and the potential importance of these bacteria as a resource for pharmaceutical discovery.

Experimental procedures

Sample collection and processing

Two hundred and seventy-five marine samples were collected around the island of Guam in the Southern reaches of the Northern Mariana Islands from 10 to 26 January 2002. The samples consisted of 227 sediments (ranging from fine muds to small rocks), 33 algae and 15 sponges. Algae, invertebrates and shallow sediments were collected by divers from depths of 1–20 m. The remaining sediments were collected using a modified, surface-deployed sediment sampler (Kahlsico, El Cajon, CA, model #214WA110) to depths of 570 m. All samples were processed within a few hours of collection at the marine laboratory of the University of Guam using a variety of techniques designed to reduce the numbers of Gram-negative bacteria and to enrich for slow-growing, spore-forming actinomycetes. Samples were processed and inoculated onto various agar media using one, or in some cases (especially for the deeper sediments) as many as three, of the eight methods described below. All algal samples were processed using method 1 (with grinding) while all sponges were processed using method 3.

Method 1 (dry/stamp). Sediment was dried overnight in a laminar flow hood and, when clumping occurred, ground lightly with an alcohol-sterilized mortar and pestle. An autoclaved foam plug (2 cm in diameter) was pressed onto the sediment and then repeatedly onto the surface of an agar plate in a clockwise direction creating a serial dilution effect.

Method 2 (dry/scrape). This method was used for small rocks that had been dried overnight in a laminar flow hood and then scraped with a sterile spatula generating a powder that was processed as per method 1. In some cases, the powder was collected with a wet cotton-tipped applicator or the rock was rubbed directly with the applicator which was then used to inoculate the surface of an agar plate.

Method 3 (dry/dilute). Dried sediment (*c.* 0.5 g) was diluted with 5 ml of sterile (autoclaved) seawater (SSW). The diluted sample was vortex mixed, allowed to settle for a few minutes, and 50 μl of the resulting solution inoculated onto the surface of an agar plate and spread with an alcohol-sterilized glass rod.

Method 4 (dilute/heat). Dried sediment was volumetrically added to 3 ml of SSW (dilutions 1:3 or 1:6), heated to 55°C for 6 min, and 50–75 μl of the resulting suspension inoculated onto an agar plate as per method 3.

Method 5 (dilute/heat/2). Dried sediment was treated as per method 4 (dilution 1:6) with the addition of a second heat treatment at 60°C for 10 min.

Method 6 (dry/stamp + dilute/heat). The surface of an agar medium was inoculated using a sample treated as per method 1. The dried sediment was then processed using method 4 and the same agar plate inoculated a second time with the heat-treated samples.

Method 7 (freeze/dilute). Wet sediment was frozen at –20°C for at least 24 h, thawed, volumetrically diluted in SSW (1:3–1:120 depending on particle size), and 50 μl of the resulting suspension inoculated onto the surface of an agar plate as per method 3.

Method 8 (freeze/dilute/2). Wet sediment was treated as per method 7 except that the thawed and diluted sample was incubated at room temperature for 48 h before inoculation onto the surface of an agar plate.

Processed samples were inoculated as described above onto the surface of from one to eight of the following agar media. All media were prepared with 1 l of natural seawater and contained the anti-fungal agents cycloheximide (100 μg ml^{-1}) and, when listed, nystatin (50 μg ml^{-1}).

Medium 1 (AMM). Eighteen grams of agar, 10 g of starch, 4 g of yeast extract, 2 g of peptone.

Medium 2 (NPS). Eight grams of noble (purified) agar, 100 ml of NPS extract, rifampicin (5 μg ml^{-1}). Nutrient-poor sediment extract was prepared by washing (extracting) 900 ml (wet volume) of sand collected from a high-energy beach with 500 ml of seawater. The water (extract) was decanted and stored at 4°C before use.

Medium 3 (NRS). Eight grams of noble (purified) agar, 100 ml of NRS extract, rifampicin (5 μg ml^{-1}). Nutrient-rich sediment extract was prepared as above using 300 ml (wet volume) of sediment collected at low tide from a mangrove channel.

Medium 4 (SHG). Eight grams of noble (purified) agar, 100 mg of humic acids sodium salt, 500 mg of galactose, nystatin (50 μg ml^{-1}), 10 ml of trace metal solution (0.43 g of $Na_2B_4O_7$, 0.25 g of $FeSO_4$, 0.18 g of $MnCl_2$, 0.004 g of $CoCl_2$, 0.003 g of Na_2MoO_4, 0.004 g of ZnCl, 1 l of deionized water).

Medium 5 (SMC). Eight grams of noble (purified) agar, 500 mg of manitol, 100 mg of casamino acids, nystatin (50 μg ml^{-1}).

Medium 6 (SMP). Eight grams of noble (purified) agar, 500 mg of mannitol, 100 mg of peptone, rifampicin (5 μg ml^{-1}).

Medium 7 (SNC). Eighteen grams of agar, novobiocin (25 μg ml^{-1}).

Medium 8 (SPC). Eighteen grams of agar, polymixin B sulfate (5 μg ml^{-1}).

Medium 9 (SRC). Eighteen grams of agar, rifampicin (5 μg ml^{-1}).

Medium 10 (SSC). Eight grams of noble (purified) agar, 500 mg of soluble seaweed (*Ascophyllum nodosum*, Cropmaster http://www.uas-cropmaster.com/index1.htm), 100 mg of casamino acids, nystatin (50 μg ml^{-1}).

Medium 11 (STC). Eighteen grams of agar, 2 ml of Tween 80.

Medium 12 (SMY). Eight grams of noble (purified) agar, 500 mg of mannitol, 100 mg of yeast extract.

Actinomycete quantification and isolation

Inoculated Petri dishes were incubated at room temperature (*c.* 28°C) and monitored periodically over 3 months for actinomycete growth. Actinomycetes were quantified on each plate by eye and with the aid of a Leica MZ6 stereomicroscope (10–64×). Actinomycetes were recognized by the presence of filamentous hyphae, a characteristic that was just within the range of detection at the highest magnification used, and/or by the formation of tough, leathery colonies that adhered to the agar surface. Thus, only mycelium-forming bacteria belonging to the order Actinomycetales were included in this study. Colonies were tentatively assigned to the genera *Salinispora*/*Micromonospora* if, for larger colonies, they produced orange pigment, black spores that darkened the colony surface, and lacked areal hyphae. Smaller colonies, viewed microscopically, could be ascribed to the *Salinispora*/*Micromonospora* group if they possessed finely branched, scattered hyphae that formed a moderately developed substrate mycelium. Hundreds of these colonies were successively transferred onto new media until pure cultures were obtained and a distinction between the genera *Salinispora* and *Micromonospora* could be made by sequence analysis and by testing for the requirement of seawater for growth (see below). With experience, it became possible to rapidly and accurately assign very small colonies (0.5 mm in diameter) to the *Salinispora*/*Micromonospora* group based on low magnification (64×) evaluation. No new actinomycete colonies were observed after 3 months of monitoring. For every plate that yielded actinomycete colonies, the total number of colonies observed was counted and representatives of all morphotypes were obtained in pure culture by repeated transfer from a single colony. All pure strains were grown in liquid culture (medium 1 without agar) and cryopreserved at –80°C in 10% glycerol.

Seawater requirements

All of the actinomycetes isolated were tested for the requirement of seawater for growth. Frozen stocks were inoculated onto the surface of an agar medium (usually medium 1) and, once sufficient growth had occurred, either a sterile cotton swab or wire loop was used to transfer cell material onto a new plate of the same medium prepared with seawater and a plate prepared with purified water (Fisher Scientific, Optima grade). Growth was monitored on both plates visually and with the aid of a stereomicroscope for up to 4 weeks. If no growth was observed on the plate prepared with purified water, that strain was determined to require seawater for growth.

DNA extraction, polymerase chain reaction amplification and phylogenetic analyses

Seawater-requiring actinomycetes and select strains that grew poorly in the absence of seawater were divided into groups based on colony size, morphology, pigmentation, spore appearance and the presence or absence of aerial hyphae. Representatives of each group were selected for partial small subunit (SSU) rRNA gene sequence analysis. An additional 45 strains that morphologically resembled *Salinispora* and were isolated using a range of cultivation techniques from diverse samples were also included to help ensure that the full range of cultured *Salinispora* diversity was assessed. Five additional strains that had been placed in the *Salinispora*/*Micromonospora* group but did not require seawater for growth were selected for sequencing to confirm their affiliation with the genus *Micromonospora*.

Genomic DNA template was prepared as previously described (Mincer *et al*., 2002) following a method modified from Marmur (1961). The SSU rRNA gene was polymerase chain reaction (PCR) amplified using the primers FC27 (5′-AGAGTTTGATCCTGGCTCAG-3′) and RC1492 (5′-TACGGCTACCTTGTTACGACTT-3′) and the products purified using a Qiagen QIAquick PCR clean-up kit following the manufacturers protocols (Qiagen, Chatsworth, CA). Polymerase chain reaction products were quantified and submitted to the UCSD Cancer Center DNA Sequencing Shared Resource for partial sequencing (3100 Genetic Analyzer, PE-Applied Biosystems, USA) using the primer FC27. Partial SSU rRNA gene sequences (*c.* 0.6 kb) were aligned using the Ribosomal Database Project (RDPII) Phylip interface (Michigan State University, East Lansing, Michigan, release number 8.1; Cole *et al*., 2003). Aligned sequences and related sequences obtained from an NCBI BLAST (BLASTN) search were imported into MacClade (version 4.03; Maddison and Maddison, 2001) and further aligned by hand. Neighbour-joining trees were created using PAUP (version 4.0b10; Swofford, 2002) and phylogenetically diverse strains selected for nearly full SSU rRNA gene sequencing of both top and bottom strands using the additional forward primers F514 (5′-GTGCCAGCAGCCGCGGTAA-3′) and F1114 (5′-GCAACGAGCGCAACCC-3′) and the reverse primers R530 (5′-CCGCGGCTGCTGGCACGTA-3′) and R936 (5′-GTGCGGGCCCCCGTCAATT-3′).

Upper and lower strand contigs were assembled in MacClade and base calling ambiguities resolved by reviewing the sequencing chromatograms in Editview (version 1.0.1, Applied Biosystems, Foster City, CA). The resulting *c.* 1.5 kb sequences, along with related sequences obtained from an NCBI BLAST (BLASTN) search, were imported into CLUSTAL X (version 1.8; Thompson *et al*., 1997) where multiple alignments were performed using the default alignment parameters. Aligned sequences were imported into MacClade where manual refinements were made and ambiguous nucleotides masked resulting in the inclusion of 1476 nucleotide positions in the phylogenetic analyses. Phylogenetic neighbour-joining and maximum parsimony analyses were performed using PAUP (4.0b10, Sinauer Associates, Sunderland, MA). Similarity values were generated using the RDPII Phylip interface distance matrix function following the Kimura 2-parameter method.

Nucleotide sequence accession numbers

The nucleotide sequence data reported in this study have been deposited in GenBank under Accession No. AY464533–AY464548.

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