UNIVERSITY OF CALIFORNIA, SAN DIEGO

Marine actinomycete diversity, biogeography, and secondary metabolite production.

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Marine Biology

by

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2006

The dissertation of Paul Robert Jensen is approved, and is acceptable in quality and form for publication on microfilm:

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LIST OF ABBREVIATIONS

BSC	biological species concept
CFU	colony forming unit
DNA	deoxyribonucleic acid
ESI-MS	electrospray ionization mass spectrometry
JGI	Joint Genome Institute
LC-MS	Liquid chromatography-mass spectrometry
LGT	lateral gene transfer
MRSA	methicillin resistant *S. aureus*
NMR	nuclear magnetic resonance
PCR	polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SEM	scanning electron microscopy
SPP	species
SSU	small subunit

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ABSTRACT OF THE DISSERTATION

Marine actinomycete diversity, biogeography, and secondary metabolite production.

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Actinomycetes are the single most important source of bioactive microbial products yet discovered. Although these bacteria are best known from soils, they can also be recovered from marine samples if the appropriate growth conditions are provided. The goals of this research were to gain a better understanding of marine actinomycete diversity, population genetics, and secondary metabolite production. The results have been interpreted using modern theories of prokaryotic genomics, speciation, and population biology. The research detailed herein is focused on marine actinomycetes belonging to the genus *Salinispora* as species within this taxon are proving to be model organisms with which to address fundamental questions about actinomycete biology. Chapter I provides an introduction to marine actinomycetes and the context within which the research described in subsequent chapters was performed. Chapter II presents a detailed study of the actinomycete diversity cultured from marine sediments collected around the island of Guam in the South Pacific. Chapter III explores the biogeographical distributions of the actinomycete genus *Salinispora*. Chapter IV is the result of the surprising discovery that *Salinispora*

species produce secondary metabolites in a predictable, species-specific manner. This chapter includes a description of the patterns observed and the evolutionary implications of this finding. Chapter V provides a summary of the research performed and directions for future research in this field.

CHAPTER I

Introduction to the thesis research

Introduction

The actinomycetes. Actinomycetes are common soil bacteria best known from an environmental perspective for the breakdown of recalcitrant organic materials (Williams et al., 1984). Among prokaryotes, these bacteria possess some of the largest genomes observed (up to 8 million base pairs) and a complex life cycle that can include the formation of a well-developed substrate mycelium followed by differentiation into actinospores, usually in response to nutrient limitation or unfavorable environmental conditions. Actinomycetes generally grow slowly relative to many Gram-negative bacteria and produce structurally diverse, biologically active secondary metabolites that likely perform important but largely undefined ecological functions (Chalis and Hopwood, 2003). These secondary metabolites have proven effective at treating a variety of human diseases and include some of the most important antibiotics discovered to date (Okami and Hotta, 1988).

The term actinomycete is a common name that is most appropriately applied to bacteria belonging to the Order Actinomycetales (E. Stackebrant and A. Ward, pers. comm.). This Order falls within the recently redefined Class Actinobacteria (Stackebrant et al., 1997) and, as a result, all Actinobacteria are not actinomycetes. This is of particular relevance to discussions of marine actinomycetes, as Actinobacteria are frequently observed when culture-independent techniques are applied to studies of marine bacterioplankton (eg., Giovannoni and Ulrich, 2005), yet, to the best of this author's knowledge, actinomycetes have not been observed among these communities (see future directions, Chapter V).

To further complicate the picture, the application of molecular techniques to bacterial systematics has revealed that actinomycetes form a monophyletic group that includes many species that do not possess the typical mycelial growth forms commonly associated with these bacteria. Thus, some actinomycetes are not readily recognizable as such based on colony morphology alone and therefore have been largely overlooked in past, morphology-based studies of actinomycete diversity. These "non-traditional" actinomycetes are non-the-less of interest, from both an ecological and biomedical perspective, as their diversity and ability to produce antibiotics has not been studied. Non-traditional actinomycetes also possess relatively small genomes (eg., *Mycobacterium* spp. range from 2.8-4.4 million base pairs, Casjens, 1998), possibly a reflection of the fact that they do not undergo the complex developmental stages typical of filamentous forms. Smaller genomes may also be an indication that they do not possess pathways encoding secondary metabolite production, a hypothesis currently under investigation by another graduate student in the laboratory. For the purpose of the research described herein, discussions of actinomycetes are restricted to "traditional" forms, ie., those producing tough, leathery colonies on agar media, and a well-developed and branching vegetative mycelium that may or may not fragment with age. This includes common taxa, such as *Streptomyces* spp., the single most prolific source of microbial antibiotics yet discovered (Berdy, 2005), and new marine genera such as *Salinispora* (Maldonado et al., 2005).

Marine actinomycetes. It has long been recognized that actinomycetes can be recovered from marine samples (Weyland, 1969). However, they are recovered in low abundance relative to other marine bacteria and to the populations typically observed

from soils. Given that actinomycetes are common in soils and produce resistance spores that are undoubtedly introduced in large numbers into near-shore marine environments, it is not surprising that questions have been raised about the origin and metabolic activity of marine-derived strains (Goodfellow and Haynes, 1984). These questions are not without merit, as it is highly likely that some, as of yet unknown percentage of the strains recovered from marine samples are of terrestrial origin and metabolically dormant until plated on a suitable growth medium by an unsuspecting microbiologist. Even the ability to grow on a seawater-based medium is not an indication that a specific strain was metabolically active in the marine environment, as non-marine actinomycetes are known to be salt tolerant (Okazaki and Okami, 1975). This ability is merely an indication that a strain has the potential to grow in the marine environment given the appropriate environmental conditions.

The proposal that some marine-derived strains were recently introduced from land is supported by molecular sequence data, as it is not uncommon for cultivars from both environments to possess 100% 16S rDNA sequence identity, especially when working with samples collected near-shore (pers. obs.). It is also clear however that some strains cultivated from marine samples are phylogenetically distinct from all previously sequenced strains and thus have the potential to represent new marine taxa. This potential is strengthened in cases where phylogenetically unique strains are not only capable of growth on media prepared with seawater but require seawater for growth, thus providing clear evidence of a marine adaptation that results from metabolic activity in the sea.

Although many questions about actinomycetes in the sea remain unanswered, a new understanding of this field is beginning to emerge. This understanding began in 1984 with the taxonomic description of the first marine actinomycete taxon *Rhodococcus marinonascens* (Helmke and Weyland, 1984). Although *R. marinonascens* does not require seawater for growth, a physiological trait traditionally associated with Gram-negative, marine bacteria (Macleod, 1965), it provided the first formal recognition that marine actinomycete taxa reside in the sea. The genus *Rhodococcus*, however, falls into the "non-traditional" actinomycete category, due to its unicellular life style, and it wasn't until 1991 that the first evidence supporting the existence of filamentous, marine-adapted actinomycete populations was reported (Jensen et al., 1991, Appendix A). These actinomycetes were cultured from sediments collected around the Bahamas and were the first marine-derived strains observed that possessed a demonstrable requirement of seawater (more specifically sodium) for growth. Although little more was done with these actinomycetes at the time, they were subsequently re-investigated approximately 10 years later (Mincer et al., 2002) and formally described as the genus *Salinispora*, comprised of the two species *S. tropica* and *S. arenicola* (Maldonado et al., 2005). *Salinispora* species remain the only actinomycetes described to date that require seawater for growth and are proving to be a source of useful information about actinomycete ecology, evolution, natural product production, and adaptations to life in the sea. Future studies on marine actinomycetes will be greatly facilitated by the *S. arenicola* and *S. tropica* genomes, currently being sequenced at the Joint Genome Institute (JGI), US Department of Energy.

Despite a rocky start to the acceptance of actinomycetes as a component of the autochthanous marine microbiota, it is now abundantly clear that marine actinomycetes exist and warrant continued study as a part of the marine bacterial community. Evidence that interest in these bacteria is on the rise can be found in a recent issue in the journal Antonie van Leeuwenhoek (to which this author contributed, Jensen et al., 2005, Appendix C) devoted solely to marine actinomycetes. This interest is undoubtedly linked to reports that marine actinomycetes are proving to be a productive resource for the discovery of new medicines (Bernan et al., 2004).

Little is known about the distributions of actinomycetes in the sea. There is currently little evidence that they occur in seawater (although this certainly warrants additional study), while they are consistently recovered from marine sediments. Despite the regularity with which actinomycetes are cultured from marine sediments, their spatial distributions and biogeography remain largely unstudied. A recent exception was a report on the diversity of bacteria in subsurface sediments in which Actinobacteria were cultured from core sections 50-100 cm below the surface (Köpke et al., 2005). Although in this case it is not clear if the cultured Actinobacteria include actinomycetes, it is none-the-less a step in the right direction. These types of studies can be used to indirectly assess *in situ* metabolic activity, as it may be possible to recognize actinomycetes that were recently introduced from land by their distributional patterns. Progress was made in this direction when it was demonstrated that certain taxa (ie., *Streptomyces* sp.) are more common in near-shore sediments while others increase in abundance as distances from shore increase (Jensen et al., 1991, Appendix A). This information was used to suggest that certain taxa are washed

in from land while others are metabolically active members of the sediment bacterial community. This is not to rule out the possibility that some common soil actinomycetes are metabolically active in the sea, as Moran and co-workers demonstrated that streptomycetes occur in sediments as mycelia, not merely as spores (Moran et al., 1995).

Bacterial diversity. The application of molecular techniques to the field of microbial ecology has provided unprecedented opportunities to address questions about bacterial diversity. Not only have these techniques made it possible to determine that bacterial diversity is far greater than previously perceived, but they have been applied extensively to marine samples and, as a result, seawater communities are now among the most intensively studied microbial habitats on the planet (Giovannoni, 2004). What is most clear from culture-independent studies of marine bacterial diversity is that major groups of bacteria exist for which representative strains have yet to be cultured (Rappé and Giovannoni, 2003). Although of great value to our understanding of bacterial diversity, this fact creates a dilemma as, with few exceptions, a strain must be cultured before a formal taxonomic description can be made. In the absence of cultured strains, we are left with impressive phylogenetic trees, and growing sequence databases, but little information about the ecology or physiology of the organisms that occur in nature.

Clearly it is of fundamental importance to develop improved methods for the cultivation of marine bacteria. Fortunately, great strides have been made in this regard, including the cultivation of the ubiquitous SAR11 clade of bacterioplankton (Rappé et al., 2003), for which the name *Pelagibacter unbiquitous* has been proposed.

Other improvements include the development of high throughput cultivation techniques (Zengler et al., 2002), which have the potential to increase the rate at which new species are brought into culture. Despite these advances, the effort devoted to cultivation has been disproportionately small in comparison to that devoted to culture-independent studies, and as a result we are faced with a growing scientific discipline that is forced to draw ecological conclusions based on extrapolations from gene sequences as opposed to empirical data derived from laboratory experimentation with living microorganisms. Cultivation remains an important, microbiological research technique that, in combination with molecular approaches, remains the most practical method by which to study microbial ecology.

Molecular microbial ecology has opened many doors to the world of microbial diversity (Pace, 1997). This is especially true when considering phylogenetic analyses based on 16S rDNA gene sequence data. Molecular techniques, however, although widely applied, are subject to significant and often overlooked biases (eg., Kobayashi et al., 1999). For example, it can be mistakenly perceived that culture-independent techniques always provide a more accurate picture of bacterial diversity than culture dependant methods. Although this may be correct in many or even most cases, there are dramatic examples where culture independent techniques are unable to recognize dominant members of a bacterial community (eg., Polz et al., 1999). This type of bias is of particular concern when considering Gram-positive bacteria, whose cells walls are relatively difficult to lyse due to a thick peptidoglycan layer. In addition, many Gram-positive species spend a portion of their life cycle in a spore stage that is particularly recalcitrant to standard DNA extraction procedures. These bacteria have

undoubtedly been under-represented in most studies of marine bacterial diversity, as special efforts are required to obtain their DNA from environmental samples.

Actinomycetes clearly represent a group of bacteria that can be difficult to detect when applying culture independent techniques to environmental samples. Even when culture-based methods demonstrate that actinomycetes occur in sediment samples at abundances of 10^3 CFU/ml, extraordinary efforts can be required to detect these bacteria in those same samples using culture independent techniques (Mincer et al., 2005, Appendix B). These experiences made it clear that culturing can be the preferred method when studying certain bacterial taxa. This conclusion is supported by previous culture-independent studies of the marine actinomycete *Salinispora*, as no new species-level diversity was detected in comparison to that which could be cultured. In addition, the presence of this genus could only be detected using culture-independent techniques in 3 of 12 samples, and only following the application of a more sensitive, semi-nested PCR technique, despite the ease with which strains could be cultured from all of the samples analyzed (Mincer et al., 2005).

A final bias associated with the application of culture-independent techniques that warrants discussion (although there are more) is PCR and sequencing error. If a strain is in culture, it is a simple procedure to check for sequencing and PCR error, as these procedures can be repeated. In the case of culture-independent studies, there is no simple way to determine if a nucleotide change that is observed one time in one clone is a real substitution or an error. Once again, in the case of cultured *Salinispora*, strains, the re-analysis of an early set of sequence data led to the detection of 18 single nucleotide changes that occurred only once and in highly conserved regions of the 16S

rRNA gene. A second round of PCR and sequencing revealed that all 18 of these nucleotide substitutions were due to error and, once corrected, revealed that the intraspecific diversity within *Salinispora* spp. was considerably less than originally believed. Thus, unless careful corrections are made for these types of errors (eg., Acinas et al., 2004), it is likely that a considerable amount of the micro-diversity observed in environmental clones libraries is due to experimental artifact.

Speciation in the Prokaryotes. Classifying life into species, the primary unit of diversity, has long been a challenging but fundamental scientific endeavor. One widely applied species definition developed by Mayr (1963) is the biological species concept (BSC). This concept states that a species can be delineated as a group of interbreeding populations, and that divergence within these populations is constrained by genetic exchange. This constraint is a cohesive force that unifies individual species into the sets of tightly clustered traits that form the basis of all classification schemes. Species cohesion is maintained by recurrent interbreeding, which tends to homogenize populations at all genetic loci. In the absence of this genetic mixing, these same populations would become free to diverge without limits (Cohan, 2002), as is observed when animal populations become geographically isolated. Although the BSC has worked well for sexually reproducing organisms, it fails to adequately describe bacterial species, as prokaryotic reproduction is an asexual process.

Given that the BSC doesn't accommodate asexual reproduction, how can the species concept be applied to bacteria? First, it must be clarified that non-reproductive genetic exchange occurs among bacteria in the form of lateral gene transfer (LGT), however this event is relatively rare and insufficient to constrain diversification. What

then is the cohesive force that maintains species structure in bacteria? Templeton (1989) has argued that natural selection accomplishes this task by purging all genetic diversity from an asexual population. This process, also known as "period selection", re-sets within species genetic diversity to zero at all loci. Thus, an adaptive mutation that improves fitness will become fixed within a population resulting in the loss of not only the less fit (non-mutated) wild-type allele but also the genetic diversity at all other loci.

Cohan used the concept of periodic selection to develop a model of bacterial speciation that groups bacteria into ecologically distinct populations that can be recognized, at the molecular level, as clusters of closely related gene sequences (Cohan, 2002). In this model, a species is formed when an asexual lineage evolves to the point where it can occupy a new ecological niche. This new lineage, or ecotype, is no longer affected by adaptive mutations (ie., period selection) within its former population as it is no longer competing for spatial or temporal resources with that population. Thus, the diversity of asexual species (as well as sexually reproducing species) is constrained by forces of cohesion and, once speciation has occurred, it is understood that these species are irreversibly separate from one another and subject to distinct evolutionary fates (Cohan, 2002).

There is currently little empirical data to support the ecotype model of bacterial diversification. It is clear that most marine bacteria fall into closely related clusters that can be recognized by gene sequence analysis (eg., Acinas et al., 2004). However, in most cases, the bacteria from which the sequences were derived have not been cultured and therefore the ecological factors that differentiate individual ecotypes have

seldom been identified. One of the best examples in which this distinction has been made is in the marine cyanobacterium *Prochlorococcus* (Moore et al., 1998). In this study, co-occurring *Prochlorococcus* strains, possessing different light-dependant physiologies, were cultured from the same water sample. These distinct ecotypes could be differentiated by 16S rRNA gene sequence analysis leading the authors to conclude that the microdiversity typically observed in marine bacterial communities can be the result of co-existing but physiologically distinct populations.

Analysis of the complete genome sequences of the high and low-light adapted *Prochlorococcus* ecotypes has provided dramatic new insight into how these strains are differentiated (Rocar et al., 2003). Most significantly, the genomes differ by ca. 0.5 Mb and are highly dissimilar in GC content. The high light adapted strain, which has the smaller of the two genomes, has undergone numerous gene deletions, lacking 923 genes that are present in the low-light adapted strain. These results provide strong support for Cohan's theory that ecotypes can be differentiated based on sequence data and that individual ecotypes have the fundamental properties of species, as the two strains differ at the genomic level by > 30%. As genome sequencing becomes more readily available, and culture methods continue to improve, it will be interesting to see how these developments affect the classification of individual bacterial species. This author, for one, agrees with Cohan's proposal that most currently named bacterial species would be more appropriately described as groups of related species. If this proves to be correct, it will have a dramatic effect on global estimates of bacterial species diversity.

Overview of the thesis. The format of this thesis has been influenced by the fact that much of the research presented has been published or submitted for publication. The introductory material presented in Chapter I was intended to cover topics that are relevant to the thesis without being redundant with the introductory sections of any of the other chapters. This chapter also addresses some of the challenges associated with molecular studies of bacterial diversity. Chapter II describes the results of a culture-dependant study of actinomycete diversity in marine sediments. The samples were collected and processed during an expedition to the island of Guam in the South Pacific and the chapter consists of a reprint of the resulting publication as it appeared in Environmental Microbiology. This study was spurred by our recent discovery of the marine actinomycete genus *Salinispora*, and the major goals of the research were to test new cultivation techniques in an attempt to discover additional new diversity within this genus and to determine if other marine actinomycete taxa could be cultured from marine sediments.

Chapter III addresses the biogeographical distributions of the genus *Salinispora*. Taking advantage of a culture collection that contains thousands of *Salinispora* strains derived from global collection sites sampled over a 15-year time frame, this study represents a detailed examination of the phylogenetic diversity of a biomedically important group of actinomycetes. The results have been submitted to Environmental Microbiology for consideration for publication. While studying the secondary metabolites produced by various *Salinispora* strains, a clear pattern began to emerge. Chapter IV describes these patterns and presents an evolutionary interpretation of how secondary metabolite production may affect speciation in

actinomycetes. This chapter has been submitted to the Proceedings of the National Academy of Sciences for publication.

Three papers with relevance to this thesis have been included as appendixes. Appendix A is the 1991 publication in which I describe the original cultivation of *Salinispora* strains. At the time, I proposed that these bacteria represented a new species in the genus *Micromonospora*, as phylogenetic methods to delineate these two genera were not readily available. This paper provides the historical framework from which the entire thesis was developed as well as the results of the only sodium requirement experiments that have been performed to date with these bacteria. Appendix B is a manuscript that resulted from a portion of Tracy Mincer's thesis research. This manuscript was our first attempt to fully characterize the culture independent diversity within the genus *Salinispora* and to compare that diversity to what we had observed using culture-dependent methods. This paper provides important introductory material as well as strong support for the biogeographical patterns presented in chapter III. Finally, Appendix C provides a brief review of the secondary metabolites that we have discovered from marine actinomycetes and the first suggestion that there may be a correlation between phylogeny and secondary metabolite production in the genus *Salinispora*. This publication is part of an entire issue of the journal Antonie van Leeuwenhoek devoted to the subject of marine actinomycetes. All of these appendixes are included in reprint format.

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CHAPTER II

Culturable marine actinomycete diversity from tropical Pacific Ocean sediments.

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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.

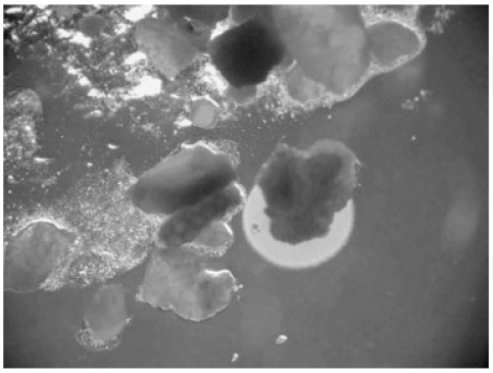

Fig. 1. Light micrograph (64×) of an actinomycete colony growing away from a sand grain.

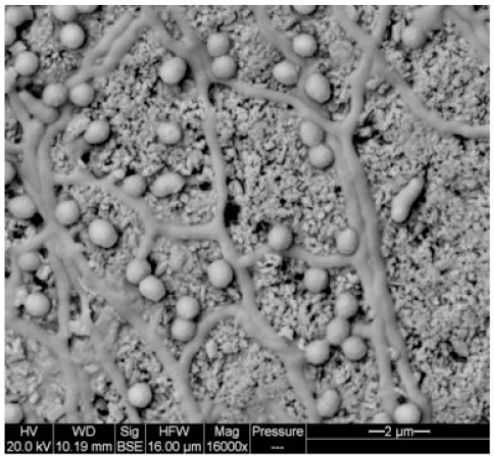

Fig. 2. Microscopic examination (SEM) (16 000×) of an actinomycete growing on a sand grain. Branching filaments, a diagnostic characteristic of many actinomycetes, and spores are clearly evident. HV, high voltage; WD, working distance, Sig, signal; HFW, horizontal field width; Mag, magnification.

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

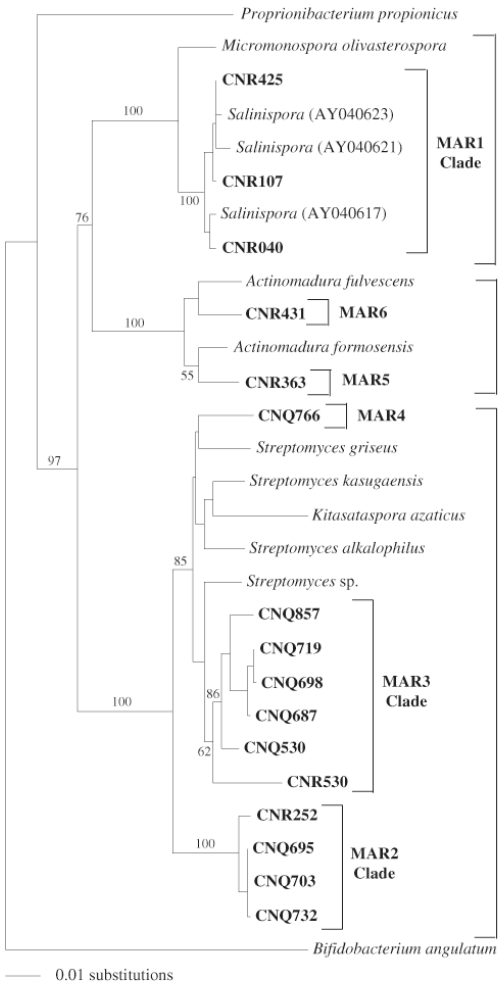

Fig. 3. Phylogenetic relationships among nearly complete (1476 nucleotide positions) SSU rRNA gene sequences of cultured marine actinomycetes (in bold) and closely related sequences obtained from an NCBI BLAST (BLASTN) search. MAR1–MAR6 are tentative designations for new marine actinomycete phylotypes. 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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CHAPTER III

Biogeography of the marine actinomycete *Salinispora*.

Abstract

Marine actinomycetes belonging to the genus *Salinispora* were cultured from marine sediments collected at six geographically distinct locations. Detailed phylogenetic analyses of both 16S rRNA and *gyrB* gene sequences reveal that this genus is comprised of three distinct but closely related clades corresponding to the species *S. tropica*, *S. arenicola*, and a third species for which the name "*S. pacifica*" is proposed. *S. arenicola* was cultured from all locations sampled and provides clear evidence for the cosmopolitan distribution of an individual bacterial species. The co-occurrence of *S. arenicola* with *S. tropica* and *S. pacifica* suggests that ecotype differentiation as opposed to geographical isolation is driving speciation within the genus. All *Salinispora* strains cultured to date share greater than 99% sequence identity and thus comprise what has been described as a microdiverse ribotype cluster. The description of this cluster as a new genus, containing multiple species, affects global estimates of bacterial species diversity and provides evidence that fine-scale 16S rRNA gene sequence analysis can be used to delineate among closely related species. The remarkable lack of interspecific 16S rRNA sequence diversity indicates recent speciation with the genus while non-synonymous/synonymous nucleotide substitution patterns indicate recent selection on the *gyrB* gene in *S. tropica*.

Introduction

Bacterial biogeography remains an unresolved issue in microbiology (Fenchel, 2003). Due to their small size, high abundance, and ease of dispersal, the prevailing

hypothesis in the field is that free-living bacteria are not subject to geographical isolation and, without this constraint, should exhibit a cosmopolitan distribution (reviewed by Staley and Gosink, 1999). While it is widely accepted that bacterial genera are widely distributed in their respective habitats (Hedlund and Staley, 2004), there is currently little empirical support for the "everything is everywhere" paradigm when applied at the species level. Without a better understanding of the extent to which geographical isolation affects the population structure of individual bacterial species, it will not be possible to effectively estimate global species richness or to understand the forces driving speciation among bacteria.

Little emphasis has been given to the study of microbial biogeography (Cho and Tiedje, 2000), and as a result the "everything is everywhere" paradigm has not been rigorously tested. The most outspoken support for microbial cosmopolitanism comes from studies of microeukaryotes (Findlay, 2002) however this support is based largely on the analysis of protozoan morphospecies. As might be expected, evidence for cosmopolitanism among environmental prokaryotes includes taxa with robust survival strategies, such as the spore-forming genus *Bacillus*, for which it has been shown that migration rates are sufficiently high to prevent geographical isolation (Roberts and Cohan, 1995). Additional evidence comes from a study of fluorescent *Pseudomonas* strains where cosmopolitanism was evident by the analysis of 16S rDNA and, to a lesser extent, 16S-23S intergenic spacer regions (Cho and Tiedje, 2000). Evidence for endemism was documented at the infraspecific level among the same *Pseudomonas* strains when higher resolution genomic fingerprinting methods were applied. Additional evidence for endemism is found among prokaryotes

inhabiting extreme environments where the barriers to surviving dispersal are high. This includes gas vacuolated sea ice bacteria (Staley and Gosink, 1999), the thermophilic archeon *Sulfolobus* (Whitaker et al., 2003), and the thermophilic cyanobacterium *Synecococcus* (Papke et al., 2003).

Any discussion of species-level bacterial biogeography is affected by uncertainty surrounding the species concept for bacteria (Cohan, 2002; Gevers et al., 2005). Recently, it has been proposed that molecular sequence data can be used to define natural units of bacterial diversity termed ecotypes (Cohan, 2002). These units can be recognized as clusters of sequences that share greater similarity to each other than to related sequences and are proposed to represent ecologically distinct populations (Cohan, 2002). Ecotype assignment can require a level of phylogenetic resolution that is difficult to achieve using the 16S rRNA gene (Fox et al., 1992; Palys 1997; Staley and Gosink, 1999) and easily obscured by PCR and sequencing errors (Acinas et al., 2004). This has led to an increased reliance on protein coding genes and, more recently, multi-locus sequence analysis (MLSA) for the resolution of intrageneric relationships (Gevers et al., 2005). In several cases, it has been demonstrated that named species are comprised of multiple, distinct ecotypes (Palys et al., 2000), leading to the suggestion that the bacterial species generally recognized today are in fact comprised of multiple ecotypes each possessing the dynamic properties of individual species (Cohan, 2002).

We recently reported the discovery of the actinomycete genus *Salinispora*, which is widely distributed in tropical and sub-tropical marine sediments (Mincer et al., 2002, Maldonado et al., 2005). To date, two species have been formally described

(*S. arenicola* and *S. tropica*), and a third, for which the name "*S. pacifica*" is proposed, has been cultured. *Salinispora* belongs to the Micromonosporaceae and is the first actinomycete genus known to require seawater for growth. Since these bacteria produce resistant spores and have been cultured from worldwide locations, they represent model organisms to test hypotheses about bacterial biogeography and the processes that drive speciation. In this paper, the phylogenetic relationships of 152 strains were assessed using 16S rRNA and *gyrB* gene sequences. The results provide compelling evidence that individual bacterial species can exhibit a cosmopolitan distribution and that speciation within the genus *Salinispora* is not due to geographical isolation.

Results

Salinispora strains were cultivated from all six tropical/sub-tropical locations sampled. These locations included multiple collection sites within the Bahamas, where they were originally discovered (Jensen et al., 1991), the U.S. Virgin Islands, the Red Sea, the Sea of Cortez, Palau, and Guam (Fig. 1). In addition, strains were recently reported from the sponge *Pseudoceratum clavata* collected from the Great Barrier Reef (Kim et al., 2005) and the ascidian *Polysyncraton lithostrotum* collected from Fiji (He et al., 2001), providing the first Southern hemisphere sites from which *Salinispora* strains have been recovered. Despite extensive effort, we have yet to cultivate *Salinispora* strains from temperate Pacific Ocean sediments collected off La Jolla, California. They also do not appear to be among the numerous

Micromonospora strains recovered from North Sea sediments (E. Helmke, personal communication). Detailed phylogenetic analyses of 46 *Salinispora* strains clearly reveal that the genus, as we know it today, is comprised of three distinct but closely related phylotypes (Figs. 2 and 3). These three phylotypes correspond to the recently described species *S. arenicola* and *S. tropica* (Maldonado et al., 2005) and, based on < 60% interspecies DNA-DNA hybridization (performed by the DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig), a third species for which the name "*Salinispora pacifica*" is proposed.

Biogeographical distribution. The three *Salinispora* phylotypes vary in their biogeographical distributions (Figs. 1-3). *S. arenicola* has a cosmopolitan distribution having been recovered from all six of the locations sampled. It is also consistently the most abundant phylotype observed, representing 86% of the 152 strains examined in this study. *S. tropica* has the most restricted distribution having thus far only been detected from the Bahamas, where it has been consistently recovered over a 15 year period and represents 7 of the 19 strains examined. Surprisingly, this phylotype was not recovered from the U.S. Virgin Islands, despite the examination of 20 strains from this site and its proximity to the Bahamas. "*S. pacifica*" has been recovered from Guam, Palau, and the Red Sea, with only one strain being recovered from the later. This phylotype is also considerably less common than *S. arenicola* (3 of 59 strains from Guam, 7 of 23 strains from Palau, 1 of 18 strains from The Red Sea). Although more widely distributed than *S. tropica*, "*S. pacifica*" was absent or remained below the detection limit in the Caribbean and the Sea of Cortez and represents a second species that, at present, appears to be geographically restricted relative to *S. arenicola*.

The *Salinispora* strains recently reported from the Great Barrier Reef (Kim et al., 2005) fall within both the *S. arenicola* and "*S. pacifica*" phylotypes, while the single strain reported from Fiji (He et al., 2001) is identical (based on 16S rRNA gene sequence) to "*S. pacifica*".

SSU rRNA gene diversity. There is a remarkable lack of intraclade diversity within the three *Salinispora* phylotypes. Despite the inclusion of strains isolated over a 15-year period from multiple collections sites throughout the Bahamas, all *S. tropica* strains cultured to date share 100% sequence identity throughout the 1479 base pairs examined (Table 1). This absence of sequence variation could only be detected once careful corrections were made for PCR and sequencing errors, including corrections to previously reported data (Mincer et al., 2002; Jensen et al, 2005). The 34 *S. arenicola* strains examined in detail possessed nearly identical sequences (99.86% similarity) with the only variations arising from strains cultured from the Sea of Cortez, all of which contained one of two possible single nucleotide polymorphisms resulting in the subclades *S. arenicola* "A" (12 strains observed) and *S. arenicola* "B" (5 strains observed, Fig. 2). None of the Sea of Cortez strains were a perfect sequence match with the *S. arenicola* type strain (CNH-643) providing extremely fine scale (one nucleotide) biogeographical resolution of these two Sea of Cortez populations. Despite analyzing partial sequence data for an additional 96 *S. arenicola* strains, including multiple representatives from all locations, no new intraclade sequence diversity was detected. As with *S. arenicola*, "*S. pacifica*" intraclde similarity was 99.86% (2 variable nucleotide positions out of 1479 examined). Both of these

nucleotide variations occurred in strain CNS-055 relative to the proposed type strain (CNS-143) and delineate the "*S.* "*pacifica* A" phylotype.

Interclade diversity among the three *Salinispora* phylotypes was also low and places the entire genus into what has been described as a microdiverse sequence cluster (Acinas et al., 2004). Pairwise similarities (BLAST bl2seq, NCBI) reveal that *S. tropica* and *S. arenicola* share 99.53% 16S rRNA gene sequence identity (Table 1). This is a difference of 7 nucleotides out of 1479 examined. *S. tropica* was found to differ from the *S. arenicola* subclades "A" and "B" by one additional nucleotide (99.46% similarity). The greatest sequence differences occurred between *S. arenicola* and "*S. pacifica*" (11 nucleotides, 99.26%) and between *S. arenicola* subclade "A" or "B" and "*S. pacifica*" (12 nucleotides, 99.19% similarity). The most similar species were *S. tropica* and "*S. pacifica*" which differed by only 6 nucleotides (99.59% similarity). Despite the high level of sequence identity, *S. tropica* and *S. arenicola* have been classified as distinct species (Maldonado et al., 2005), while the classification of "*S. pacifica*" as a third species is supported by genomic DNA-DNA hybridization experiments (Wayne et al., 1987) in which the proposed type strain was <60% similar to *S. tropica* and *S. arenicola* (data provided by the DSMZ). *Salinispora* species share 96.50%-96.60% similarity with *M. chalcea*, the type strain for the genus *Micromonospora*, and 97.28%-97.56% similarity with *M. rosaria*, the most closely related *Micromonospora* species.

The majority of the *Salinispora* sequence diversity thus far detected occurs in appropriately variable regions of the SSU rRNA gene and, with the exception of CNS-055, in multiple strains, providing strong evidence that these changes are not due to

PCR or sequencing errors. Nine of 15 variable nucleotide positions (138-232) occur in the V2 variable region (Rijik et al., 1992) with all but one of these (position 183) occurring in non-conserved helixes (Table 2). Of the remaining substitutions, only the G⇔A hairpin loop transition (position 262) occurs in a conserved region (90-98% among all bacteria). Eight of the helix-associated changes are transitions that result in wobble (G:U) base parings (5 G:C to G:U pyrimidine changes, 3 A:U to G:U purine changes) and as such show no positional co-variation. Two of the three helix-associated transversions (G⇔U, nts 188 and 202) are positional co-variants in helix 10 of the V2 region, thus maintaining Watson-Crick pairing. The remaining helix-associated transversion (U⇔G, position 637) results in the heteropurine G:A base pair. As already mentioned, the isolation of 12 strains with this *S. arenicola* "A" nucleotide substitution pattern confirms the validity of this subclade. The four remaining substitutions occur in a single base bulge or in conserved loop structures and thus are not affected by base pairing.

***gyrB* phylogeny.** The phylogenetic tree based on nearly complete *gyrB* DNA sequences (1164 nucleotides) re-affirms the monophyletic nature of the *Salinispora* clade and its separation from other genera within the Micromonosporaceae (Fig. 3). The three *Salinispora* phylotypes, corresponding to *S. tropica*, *S. arenicola*, and "*S. pacifica*" are clearly delineated providing additional phylogenetic support for the separation of these taxa. There were no variations among any of the 46 strains in terms of species-specific 16S rDNA and *gyrB* cladding patterns, although there is a difference in the branching patterns in the two trees. The basal position of phylotype A (CNS-055) in the "*S. pacifica*" clade is maintained in both the 16S rDNA and *gyrB*

trees using both neighbor joining and parsimony (not shown) treeing methods. The *S. arenicola* subclades "A" and "B" are maintained in the *gyrB* tree with the exception of strain CNH-962.

As with the 16S rRNA gene sequence data, there was a remarkably high level of sequence similarity within the three *Salinispora gyrB* phylotypes (Table 1), with *S. tropica* strains sharing 99.57% sequence identity, *S. arenicola* strains sharing 96.13% sequence identity, and "*S. pacifica*" strains sharing 97.16% sequence identity, with 26 of the 33 variable positions being attributed to the "*S. pacifica*" A phylotype. The interspecies similarity was greatest between *S. tropica* and "*S. pacifica*" (95.10%) and least between *S. arenicola* and the other two phylotypes (92.87%). The closest Blastn *gyrB* sequence match for all three of the *Salinispora* phylotypes was *Micromonospora rosaria* (BAA89737) for which the sequence identity was 89-90%. No additional biogeographical patterns could be resolved based on the sequencing of this protein-coding gene.

The *Salinispora gyrB* sequence data was translated into 388 amino acids and representatives of each species aligned with *M rosaria* and *E. coli* K12 (Table 3). As expected, both the intra and interspecies amino acid similarities are high (98.45-99.23% and 96.39-98.71%, respectively). The intraspecific non-synonymous (d_N) to synonymous (d_S) ratios were approximately equal for "*S. pacifica*" and *S. arenicola* while for *S. tropica* the ratio increased by a factor of ten providing evidence of positive selection on this protein (Page and Holmes, 1998). *Salinispora* species share

90-92% amino acid sequence identity with *M rosaria*, the closest BLASTp (NCBI) match.

Effects of temperature on growth. In previous studies, we have observed that *Salinispora* strains are capable of growth at 10°C but not at 4°C. To test the effects of exposure to 4°C on *Salinispora* growth and viability, seven strains were maintained at 4°C for 2, 4, 6, or 8 weeks then incubated at 25°C for 2 months. All strains incubated at 4°C for 2 or 4 weeks showed no reduction in growth relative to controls upon transfer to 25°C. After six weeks at 4°C however, all strains exhibited reduced growth at 25°C with two strains (CNS-103 and CNR-114) remaining reduced even after two months at this temperature. After 8 weeks at 4°C, these same two strains lost viability while the remaining 5 strains all displayed a reduction in growth relative to controls. Both of the strains that lost viability belong to the "*S. pacifica*" phylotype.

Discussion

The extent to which individual bacterial species are globally distributed in all environments capable of supporting their growth remains a fundamental question in microbiology. Although this question is mired in uncertainty over how to apply the species concept to bacteria (Rosselló-Mora and Amann, 2001), the analysis of molecular sequence data is providing new insight into the biogeographical distributions of specific bacterial types. Recent examples of sequence-based analyses have included clear evidence for species-level endemism among bacteria inhabiting

extreme environments (Staley and Gosink, 1999; Papke et al., 2003). At the infraspecific level, endemism has also been documented among free-living bacteria (Cho and Tiedje, 2000). Based on these results, it is becoming increasingly clear that all bacteria are not cosmopolitan in distribution and that the ability to detect bacterial endemism is a function of the bacterial populations studied and the resolution of the analytical techniques applied.

The detailed phylogenetic characterization of the actinomycete genus *Salinispora* provides clear evidence that an individual bacterial species can be globally distributed among environments in which its growth requirements are meet. This evidence comes from *S. arenicola,* which to the best of our knowledge represents the first free-living bacterial species within which multiple strains possessing 100% 16S rRNA gene sequence identity have been cultured from worldwide locations. Analysis of *Salinispora gyrB* gene sequences further supports the cosmopolitan distribution of *S. arenicola* as no new biogeographical patterns were revealed despite the added phylogenetic resolution provided by this protein-coding gene (Kasai et al., 2000). At this time, it cannot be determined if the relative regional endemism detected for "*S. pacifica*" and *S. tropica* is due to insufficient sampling, reduced fitness, less effective dispersal, recent speciation, or limited niche availability. Of these, insufficient sampling seems least likely, as culture-independent studies of sediments collected in the Bahamas did not reveal the presence of "*S. pacifica*" or any previously uncultured species (Mincer et al., 2005).

There is a remarkable lack of 16S rDNA sequence diversity within the genus *Salinispora* suggesting relatively recent divergence among the three species. The co-

occurrence of *S. arenicola* with *S. tropica* and "*S. pacifica*" indicates ecotype selection as the force driving speciation, as opposed to genetic drift due to geographical isolation. That genetic drift is not driving speciation is further supported by low global species diversity (despite the examination of more than 150 strains collected over 14 years from six worldwide locations), rapid coalescence in the phylogenetic trees (Figs. 2 and 3), and the lack of correlation between genetic and geographic distances (ie., *S. tropica* and "*S. pacifica*" are not the most dissimilar by 16S or *gyrB* gene sequence comparisons). One potential example of genetic drift due to geographical isolation occurs at the infraspecific level among *S. arenicola* strains cultured from the Sea of Cortez (phylotypes "A" and "B"), which differ at one of two nucleotide positions (Table 2) from all other *S. arenicola* strains. This drift remains apparent (with the exception of CNH-962), yet subtle, in the *gyrB* sequence data where it is restricted to synonymous nucleotide changes.

Members of the genus *Salinispora* share >99% sequence identity and thus exemplify what has been described as a microdiverse ribotype cluster (Acinas et al., 2004). Molecular analyses of environmental samples reveal that most bacterioplankton fall into such clusters, however it has not been possible to determine if they represent ecologically distinct populations (Acinas et al., 2004). The classification of the *Salinispora* sequence cluster as a new actinomycete genus provides culture-based evidence that a microdiverse sequence cluster can represent a clearly defined unit of bacterial diversity. The characterization of three species within this genus further demonstrates that sequence clusters can be more complex than previously believed, with taxonomic significance extending to consensus groups that

share >99% sequence identity. Although it can not be determined if the lack of 16S rRNA gene sequence diversity within individual *Salinispora* species is maintained by periodic selection or the result of recent ecological differentiation, it has been possible to use fine-scale phylogenetic analyses to resolve closely related species within a single ribotype cluster thus affecting 16S rDNA-based estimates of global species diversity.

The negative effects of exposure to 4°C on growth and survival may explain why we have not successfully cultured *Salinispora* strains from temperate waters off San Diego. To date, the deepest sediment we have examined was collected at 1100 m off the Bahamas, and *Salinispora* strains were successfully recovered from this sample (Mincer et al., 2005). Given that the vast majority of the world's ocean temperatures at 1500 m are below 4°C (Fig. 1), it will be important to determine if *Salinispora* strains can be recovered from samples collected at greater depths and, if so, how they are related to the strains recovered from near-shore sediments.

Ocean sediments cover 70% of the earth's surface, yet little is known about the bacterial diversity within this vast environment. The present study provides evidence that an individual bacterial species within the spore-forming actinomycete genus *Salinispora* is globally distributed in tropical and sub-tropical ocean sediments and that speciation within this genus is driven by ecotype selection not geographical isolation. Although the ecological characteristics that distinguish these species are presently unknown, the results provide evidence that commonly applied OTU (Operational Taxonomic Unit) criteria (eg., 97% sequence identity) may underestimate global species diversity. These results support the occurrence of species-level

cosmopolitanism among free-living bacteria that possess robust survival strategies and the concept that many currently named species consist of multiple units of diversity that possess the fundamental properties of individual species (Cohan, 2002).

Experimental Procedures

Strain isolation. Marine sediments were collected from six distinct geographical locations (the Bahamas, the U.S. Virgin Islands, Guam, the Sea of Cortez, the Republic of Palau, and the Red Sea) and processed for the cultivation of actinomycetes using previously described methods (Jensen et al., 1991; 2005; Mincer et al., 2002; 2005). In general, these methods consisted of pre-treatment, usually either drying in a laminar flow hood or diluting in seawater and heating, prior to inoculation onto various types of agar media prepared with natural seawater and selective antibiotics. *Salinispora* strains were initially recognized on primary isolation plates by colony morphology and the taxonomic assignment of pure cultures subsequently confirmed by partial 16S rRNA gene sequence analysis.

One hundred and fifty-two strains from various locations and sample depths were examined. These strains consisted of 19 from the Bahamas, 20 from the U.S. Virgin Islands, 56 from Guam, 17 from the Sea of Cortez, 23 from Palau, and 17 from the Red Sea. Of these strains, 36 were selected for nearly complete 16S rRNA gene sequence analysis. These strains were obtained from independent sediment samples and include multiple representatives of each phylotype (based on partial 16S rRNA gene sequence analysis) observed from each collection site and year. An additional 10

strains (5 from the Bahamas, 3 from Guam, 1 from the Red Sea, and 1 from the Sea of Cortez) from previous studies (Mincer et al., 2002; Jensen et al., 2005) were also included in the analyses.

DNA extraction, 16S rRNA gene amplification and sequencing. Genomic DNA template was prepared as previously described (Mincer et al., 2002) using a method modified from Marmur (1961). Nearly complete 16S rRNA genes were PCR amplified in 50 μl reactions using from 10 to 50 ng of genomic DNA template, 0.5 μM of the forward FC27 (5'to 3' AGAGTTTGATCCTGGCTCAG) and reverse RC1492 (5' to 3' TACGGCTACCTTGTTACGACTT) primers, 100 μM (each) dATP, dCTP, dGTP, and dTTP, 2.5U taq polymerase (New England Biolabs), and 1x PCR buffer. The PCR condition were 94°C for 5 min followed by 35 cycles of 94°C for 45 sec, 50°C for 45 sec, 72°C for 45 sec, followed by 72°C for 7 min. The PCR products were purified using a Qiagen QIAquick PCR clean-up kit following the manufacturers protocols (Qiagen, Chatsworth, CA), quantified, and submitted for sequencing to the UCSD Cancer Center DNA Sequencing Shared Resource (3100 Genetic Analyzer, PE-Applied Biosystems, USA). Partial sequences were obtained using the forward primer FC27 while nearly complete gene sequences were obtained for top and bottom strands using the additional forward primers F514 (5'-3' GTGCCAGCAGCCGCGGTAA) and F1114 (5'-3' GCAACGAGCGCAACCC) and the reverse primers R530 (5'-3' CCGCGGCTGCTGGCACGTA), R936 (5'-3' GTGCGGGCCCCCGTCAATT) and RC1492.

***gyrB* gene amplification and sequencing.** Two sets of primers were designed to amplify partially overlapping, double stranded contigs encompassing the nearly

complete *gyrB* gene (1164 bps). These primer sets were 1) F33NT and R662T (5'to 3' TGTAAAACGACGGCCAGTgtctccggcggyctgcaccg and CAGGAAACAGCTATGACCcctcgtgggtrccgccctc) and 2) F611T and R1300T (5'to 3' TGTAAAACGACGGCCAGTcgartcstayggcgagtcggtctacacc and CAGGAAACAGCTATGACCcagcacsaycttgtggtascgcagctt). M13 forward and reverse sequencing tags (capitalized) were added to the 5' ends. PCR reactions were performed in a total volume of 50 μl that contained for primer set 1, 50-100 ng DNA template, 1.0 μM each F33NT and R662T, 200 μM (each) dATP, dCTP, dGTP, and dTTP, 5.0U AmpliTaq Gold (Applied Biosystems), 1x $MgCl_2$, 1x PCR buffer. For primer set 2, all reagent concentrations were halved except for the template and DNA polymerase. PCR products were purified and sequenced as described above using M13 forward and reverse primers. The PCR conditions were as follows: 95°C for 10 min followed by 35 cycles of 94°C for 1 min, 65°C for 1 min (primer set 1) or 68°C for 1 min (primer set 2), 72°C for 1 min, followed by 72°C for 7 min.

Phylogenetic analyses. 16S rRNA gene sequence contigs were assembled and checked for accurate base calling using Sequencher (ver. 4.5, Gene Codes Corp., Ann Arbor, MI), aligned using Clustal X, and imported into MacClade (ver. 4.07, Sinauer Assoc., Sunderland, MA) for manual alignment and masking. Single nucleotide changes that were observed in only one strain and occurred in areas of ≥ 98% conservation among all bacteria (Cannone et al., 2002) were confirmed by performing a new PCR reaction. Secondary structure analyses were performed using the ARB software package (Ludwig et al., 2004). Neighbor-joining, parsimony, and bootstrap analyses were performed using PAUP (ver. 4.0b10, Sinauer Assoc., Sunderland, MA).

gyrB DNA sequences were aligned, translated, and analyzed using MacClade. Sequence similarities were calculated using various NCBI (National Center for Biotechnology Information Basic Alignment Search Tool (BLAST) functions. Sequence data have been deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html) under accession numbers AY040617-AY040623, AY464533-AY464534, DQ224159-DQ224165, DQ092624 for 16S rRNA genes and DQ228678-DQ228693 for *gyrB* genes.

Effects of temperature on growth. Seven strains were tested for the effects of storage at 4°C on growth and viability. The strains tested were CNB-440, CNR-699 (*S. tropica*), CNR-114, CNS-103, CNS-143 ("*S. pacifica*"), CNB-527, CNR-425 (*S. arenicola*). Strains were started in 25 ml liquid cultures (A1 medium, 1.0% starch, 0.4% peptone, 0.2% yeast extract, 100% seawater) then inoculated by dilution streaking onto five replicate A1 agar plates. One replicate for each strain was immediately incubated at 25°C (positive control). All of the positive controls reached maximum visible growth within two weeks at 25°C. The remaining replicate plates were incubated at 4°C with one replicate per strain being transferred to 25°C after 2, 4, 6, and 8 weeks at reduced temperature. Following transfer to 25°C, plates were monitored for 2 months and growth recorded as equal, reduced, or no growth relative to controls.

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The text of Chapter III, in full, is a reprint of the material as it appears in Jensen, P.R, and Mafnas, C. (submitted). Biogeography of the marine actinomycete *Salinispora*. Environ. Microbiol. The dissertation author was the primary author and directed and supervised the research, which forms the basis for this chapter.

Table 1. *Salinispora* intra- and interspecific genetic similarity (number of strains in parentheses after species identifier). St = *S. tropica*, Sa = *S. arenicola*, Sp = "*S. pacifica*". 16S similarities generated from 1479 nucleotide positions, *gyrB* DNA similarities generated from 1164 nucleotide positions, *gyrB* amino acid similarities generated from 388 positions. Number of invariant positions in parentheses after percent similarities. d_N = *gyrB* non-synonymous nucleotide substitution, d_S = *gyrB* synonymous nucleotide substitution. Interspecific comparisons made using the type strains for each species.

Species	16S (rDNA)	gyrB (DNA)	gyrB (aa)	d_N	d_S	d_N/d_S
St (6)	100% (1479)	99.57% (1159)	99.23% (385)	3	2	1.5
Sa (34)	99.86% (1477)	96.13% (1119)	98.45% (382)	6	39	0.15
Sp (6)	99.86% (1477)	97.16% (1131)	99.23% (385)	5	30	0.17
St:Sp	99.59% (1473)	95.10% (1107)	98.71% (383)	5	52	0.10
Sa:Sp	99.26% (1468)	92.87% (1081)	96.65% (375)	13	70	0.19
St:Sa	99.53% (1472)	92.87% (1081)	96.39% (374)	14	69	0.20

Table 2. *Salinispora* phylotype-specific nucleotides. St = *S. tropica*, Sa = *S. arenicola,* Sp = "*S. pacifica*", * = positional co-variants, H = helix, B = bulge, HL = hairpin loop, IL = internal loop, ML = multistem loop, TS = transition, TV = transversion.

	Nucleotide position (*E. coli* numbering)														
	138	183	186	187	188	192	202	219	232	262	546	613	637	837	1251
St	C	G	U	G	G	C	U	U	G	G	A	C	U	A	A
Sa	C	G	C	A	U	U	G	U	G	G	A	C	U	G	G
Sa"A"	C	G	C	A	U	U	G	U	G	G	A	C	G	G	G
Sa"B"	C	G	C	A	U	U	G	U	G	G	A	U	U	G	G
Sp	U	G	U	G	G	C	U	C	A	A	G	C	U	G	A
SP"A"	C	U	U	G	G	C	U	C	A	A	G	C	U	G	A
Location	H	B	H	H	H	H	H	H	H	HL	ML	H	H	H	IL
Base	G:C	-	G:C	A:U	G:U	G:C	G:U	G:C	A:U	-	-	G:C	U:A	A:U	-
pairing	G:U	-	G:U	G:U	U:G	G:U	U:U	G:U	G:U	-	-	G:U	G:A	G:U	-
Change	TS	TV	TS	TS	TV*	TS	TV*	TS	TS	TS	TS	TS	TV	TS	TS

Table 3. Amino acid alignments of gyrB sequence data from *S. pacifica*, *S. tropica*, and *S. arenicola*.

```
M. rosaria (BAA89737)  110VSGGLHGVGVSVVNALSTRMAVEIHKAGFVWRQQYTNSKP-SPLEKGETT
"S. pacifica" (CNS143) 110......R.................Q.D.YF...S..D...-T......P.
S. tropica (CNB440)    110......R.................Q.D.YL...S..D...-T......S.
S. arenicola (CNH643)  110......R.................Q.D.YF...S..D...-.......P.
E. coli K12 (P06982)   110.................QKLELV.QRE.KIH..I.EHGV.QA..AVTGE.
M. rosaria (BAA89737)  160DRTGSAVSFWPDPDVFETV-DLDFQTIYRRLQEMAFLNRGLTIHLLDERV
"S. pacifica" (CNS143) 160.A...........TV....-S.............................
S. tropica (CNB440)    160.A...........RV....-A.............................
S. arenicola (CNH643)  160.A...........AV....-...............................
E. coli K12 (P06982)   160EK..TM.R...SLET.TN.TEFEYEILAK..R.LS..N-SGVSIR.RDKR
M. rosaria (BAA89737)  209AEDEDGKQREVTFCYKGGIADFVRHLNASKNPIHKSVVEFGAEEEGMSVE
"S. pacifica" (CNS143) 209D.GD.S.M.......Q..............TS..................
S. tropica (CNB440)    209D.GD...M.......Q..............TS..................
S. arenicola (CNH643)  209.A.....M.....................T...................
E. coli K12 (P06982)   209----...--.DH.H.E...KA..EY..KN.T...PNIFY.ST.KD.IG..
M. rosaria (BAA89737)  253IAMQWNESYGESVYTFANTINTHEGGTHEEGFRAALTGIVNRYG-ADKKL
"S. pacifica" (CNS143) 253..................N..............S..........-.E.R.
S. tropica (CNB440)    253..................N..............S..........-.E.R.
S. arenicola (CNH643)  253..................N.........................-T..R.
E. coli K12 (P06982)   253VAL...DGFQ.NI.C.T.N.PQRD....LA.....M.RTL.A.MDKEGYS
M. rosaria (BAA89737)  303.KGDEKLSGEDIREGLAAIISVKLANPQFEGQTKTKLGNTPVKSFVQRVC
"S. pacifica" (CNS143) 303..S.............................................
S. tropica (CNB440)    303..S.............................................
S. arenicola (CNH643)  303..S.....................T.......................
E. coli K12 (P06982)   303K.AKVSAT.D.A....I.VV...VPD.K.SS...D..VSSE...A.EQQM
M. rosaria (BAA89737)  353NEWLVDWLDRNPAEAKIIITKASQAARARIAAQQARKLARRKSLLESGSM
"S. pacifica" (CNS143) 353.................V............V...................
Table 3. Continued
S. tropica (CNB440)    353.................V............V...................
S. arenicola (CNH643)  353.................V............V...................
E. coli K12 (P06982)   353..L.AEY.LE..TD...VVG.IID.....E..RR..EMT...GA.DLAGL
M. rosaria (BAA89737)  403PGKLADCQSTDPRESEVFIVEGDSAGGSAKQGRDPRTQAILPIRGKILNV
"S. pacifica" (CNS143) 403..............C..............................
S. tropica (CNB440)    403..............C..............................
S. arenicola (CNH643)  403..............C..............................
E. coli K12 (P06982)   403........ER..AL..LYL..............NRKN.....LK......
M. rosaria (BAA89737)  453EKARIDRVLKNNEVQALITALGTGIH-DDFDIEKLRYHKIVL
"S. pacifica" (CNS143) 453.........................-.............
S. tropica (CNB440)    453.........................-.............
S. arenicola (CNH643)  453.........................-...........X..
E. coli K12 (P06982)   453....F.KM.SSQ..AT......C..GR.EYNPD.....S.II
```

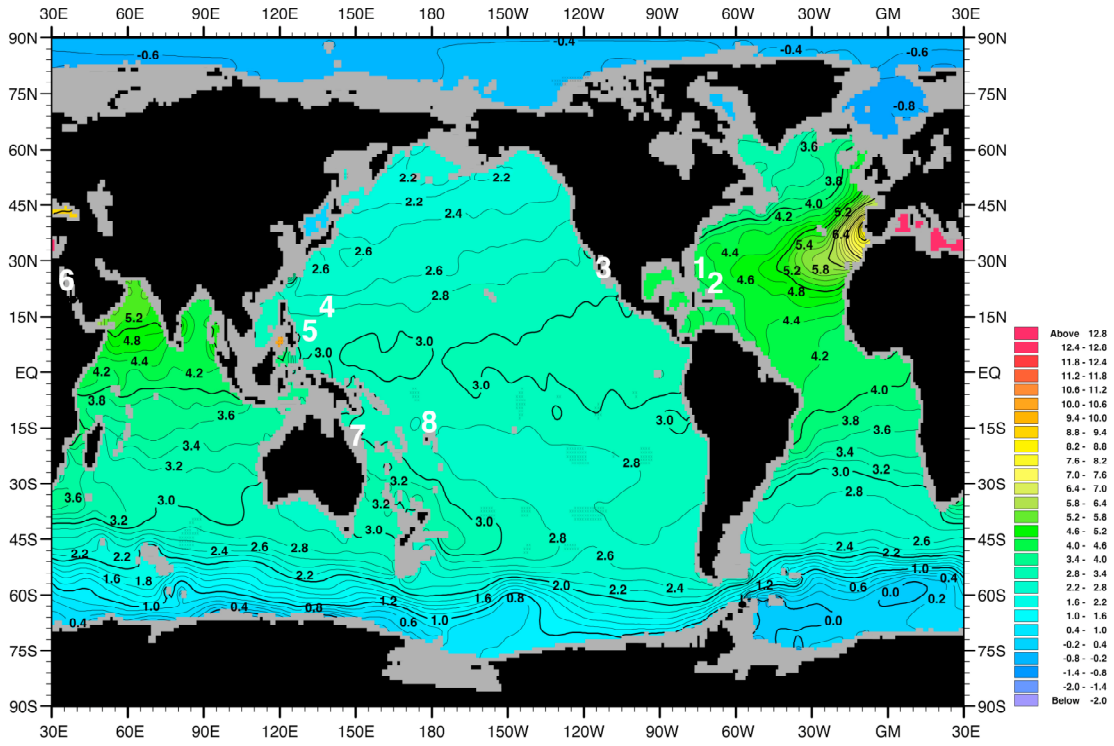

Figure 1. Annual mean ocean temperatures (°C) at 1500 m (Stephens et al., 2002) with sites from which *Salinispora* strains have been cultivated indicated. 1 = the Bahamas, 2 = the U.S. Virgin Islands, 3 = the Sea of Cortez, 4 = Guam, 5 = Palau, 6 = the Red Sea, 7 = the Great Barrier Reef (Kim et al., 2005), 8 = Fiji (data provided by V. Bernan and R. Raju). *S. arenicola* has been cultivated from all 8 sites. *S. tropica* has only been recovered from site 1 while "*S. pacifica*" has been recovered from sites 4-8.

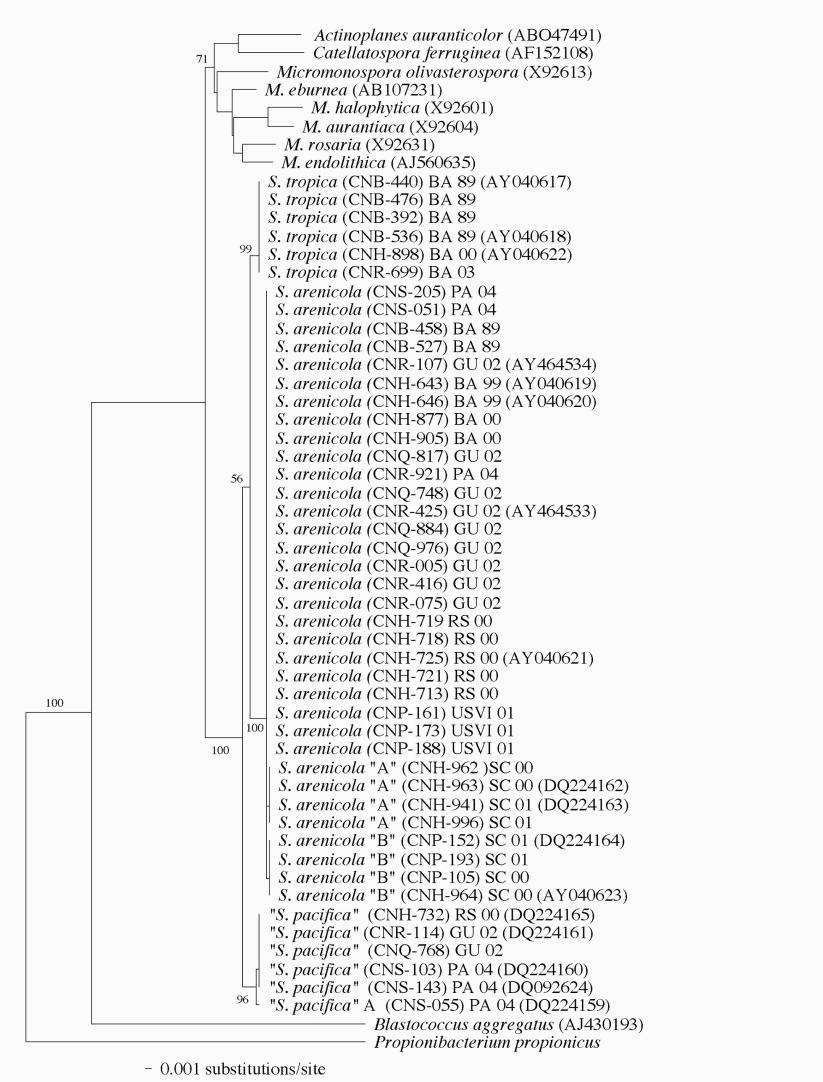

Figure 2. Neighbor-joining phylogenetic tree created from 46 nearly complete (1449 nucleotides) 16S rRNA gene sequences from *Salinispora* strains cultured from worldwide locations. The three major *Salinispora* phylotypes, consisting of the two formally described species *S. tropica* and *S. arenicola* and the proposed species "*S. pacifica*", are clearly delineated. Type strains representing the five *Micromonospora* species most closely related to *Salinispora*, along with *M. halophytic*, are included. Species names are followed by strain number, strain source (BA = Bahamas, RS = Red Sea, GU = Guam, PA = Palau, VI = Virgin Islands, SC = Sea of Cortez), year of collection (89 = 1989, etc,) and accession number (for representative sequences). *P. propionicus* and *B. aggregatus* were used as outgroups.

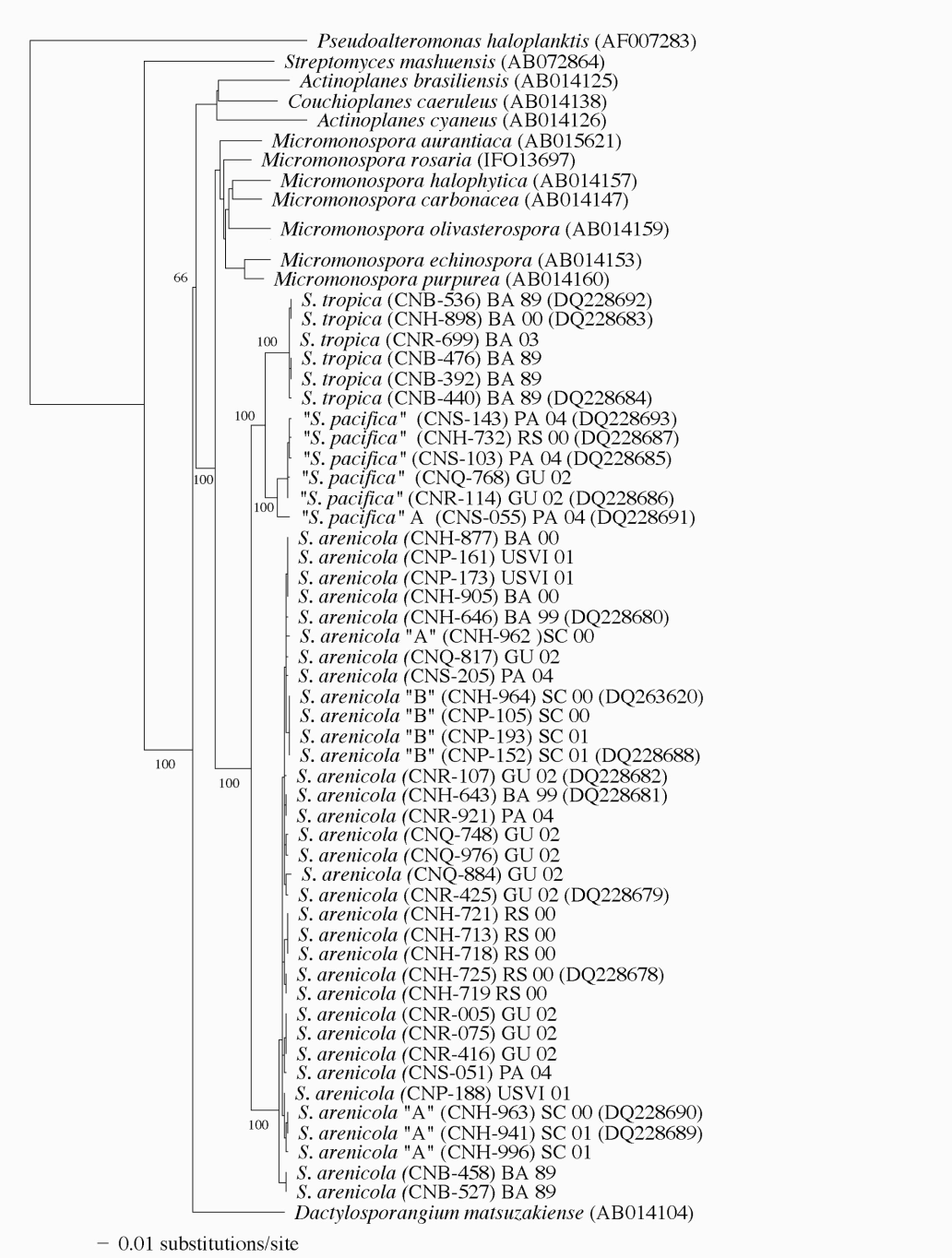

Figure 3. Neighbor joining phylogenetic tree created from 46 nearly complete (1164 nucleotides) *gyrB* gene sequences from *Salinispora* strains cultured from worldwide locations. Labeling is similar to Fig. 2. *P. haloplanktis* was used as an outgroup.

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CHAPTER IV

Evolutionary implications of secondary metabolite production.

Abstract

Fine-scale phylogenetic analyses of environmental libraries have revealed that bacterial populations can be delineated as clusters of closely related gene sequences, the ecological significance of which remain largely unknown (Acinas, et al. 2004). Here we report an invariant correlation between secondary metabolite production and phylogenetically distinct but closely related groups of marine actinomycetes belonging to the genus *Salinispora*. These patterns were observed on a global scale and indicate that secondary metabolite production can be a species-specific, phenotypic trait associated with a widely distributed bacterial population. Correlations between actinomycete phylotype and chemotype provide an effective, diversity-based approach to natural product discovery and contradict the conventional wisdom that secondary metabolite production is strain specific. The structural diversity of the metabolites observed, coupled with gene probing and phylogenetic analyses, implicate lateral gene transfer as the source of the biosynthetic pathways responsible for compound production. Correspondence between pathway acquisition and phylogenetic divergence, in addition to species-specific compound production, supports the hypothesis that secondary metabolite production is a previously unrecognized selective force driving actinomycete diversification. These results provide important new evidence for the adaptive significance of secondary metabolism and a rare example in which clearly defined biochemical distinctions have been made among closely related bacterial populations.

Introduction

Lateral gene transfer (LGT) is widely recognized to have played an integral role in the evolution of bacterial genomes (Doolittle, 1999; Ochman et al., 2000). This mechanism of genetic exchange provides an extremely effective strategy by which bacteria can rapidly exploit new resources (Ochman et al., 2005) and has been proposed as the selective force behind the physical clustering of genes within bacterial genomes (Lawrence, 1997). While it is increasingly clear that most bacterial genomes include large numbers of acquired genes (Koonin et al., 2001), not all genes are equally subject to this process. As detailed in the complexity hypothesis (Jain et al., 1999), LGT is largely restricted to contingency genes, such as those involved with metabolism or virulence, and is generally not associated with informational genes, such as RNAs, which are typically members of large, complex systems.

Streptomycetes possess a single linear chromosome (Bently and Parkhill, 2004) consisting of a conserved core flanked by two non-conserved arms (Bently et al., 2002). The arms of the chromosome contain largely acquired DNA and are the location of most contingency genes, including those that code for non-essential functions such as secondary metabolite production (Bently et al., 2002). Evidence that genes involved with actinomycete secondary metabolism are subject to LGT has been inferred from incongruent phylogenies (Koonin et al., 2001), the occurrence of biosynthetic pathways on plasmids (Kinashi et al., 1987), their association with mobile genetic elements (Omura et al., 2001), and sequence analysis (Egan et al., 2001).

If the biosynthetic pathways encoding secondary metabolite production move freely and with little evolutionary affect among actinomycetes, there should be no species specificity associated with the products of these pathways. This was the historical paradigm adopted by the pharmaceutical industry in response to the observation that strains within the same species frequently produce different secondary metabolites. This paradigm, which was developed using traditional taxonomic methods, necessitates the screening of large numbers of strains for every new structural class of molecules discovered, and suffered from the unavoidable isolation of many redundant compounds. The inefficiency associated with the frequent re-discovery of known, biologically active secondary metabolites contributed to the decision by the pharmaceutical industry at large to move away from microbial natural products drug discovery in favor of alternative (and ultimately less productive) discovery platforms (Koehn and Carter, 2005), despite the wealth of drug quality molecules derived using the former approach (Bérdy, 2005).

Modern evolutionary theory predicts that bacterial taxa are natural units of diversity that can be recognized as clusters of closely related gene sequences (Cohan, 2002). The genetic diversity within these taxa is constrained by periodic selection, a cohesive force that selectively sweeps within population diversity from all genetic loci while having no effect on co-occurring ecotypes (Palys et al., 1997). We have been studying a unique group of marine actinomycetes that conform to the ecotype model of bacterial diversity. Phylogenetic analyses of these bacteria reveal three distinct but closely related clades corresponding to the species *Salinispora arenicola*, *S. tropica* (Maldonado et al., 2005; Mincer et al., 2002; 2005), and a third species for which the

name "*S. pacifica*" has been proposed (fig. 1). We recently reported the cultivation from world-wide locations of *S. arenicola* strains that share 100% 16S rDNA sequence identity thus providing new evidence for the cosmopolitan distribution of an individual bacterial species (Jensen and Mafnas, submitted). The co-occurrence of *Salinispora* species was used as evidence that diversification within the genus is the result of ecological differentiation not geographical isolation. What remains unclear, however, is the ecological basis upon which the three species are differentiated.

Salinispora strains are a prolific source of structurally diverse secondary metabolites including salinosporamide A (Feling et al., 2003), a potent proteasome inhibitor that is rapidly advancing towards clinical trials for the treatment of multiple myeloma. Detailed chemical analyses of 46 *Salinispora* strains reveal clear metabolic distinctions among the three species in the form of unprecedented, species-specific secondary metabolite production. Evidence that the pathways encoding secondary metabolite production were acquired by LGT suggests that the ability to produce a specific set of molecules fosters ecological differentiation and that pathway acquisition is a here-to-for unrecognized force driving actinomycete diversification.

Results

Salinispora species have been cultured from worldwide tropical and sub-tropical locations (Jensen and Mafnas, submitted) thus providing a unique opportunity to address the relationships between actinomycete population structure and secondary metabolite production. Time course LC-MS analyses were performed on 46 strains

that originated from six global collection sites including multiple representatives of all phylotypes cultured from each site and collection year. The results of >300 analyses reveal a high degree of structural diversity (fig. 2) and an unprecedented correlation between phylotype and secondary metabolite production. To the best of our knowledge, this is the first evidence that fine-scale phylogenetic analyses can be used to differentiate among strains that produce different secondary metabolites.

Regardless of the geographical origin of the *Salinispora* strains, secondary metabolite production followed a well-defined pattern. Of the three species, *S. tropica* has thus far only been cultured from the Bahamas and the six strains examined all share 100% 16S rDNA sequence identity. These six strains, which were obtained in culture during three expeditions spanning 14 years, all produced the same two sets of secondary metabolites (fig. 2). These molecules are represented by the proteasome inhibitor salinosporamide A (**1,** Feling et al., 2003), which contains a rare bicyclic beta-lactone gamma-lactam ring system, and sporolide A (**2**), an unprecedented, polyketide-derived macrolide (Buchanan et al., 2005) with unknown biological activity. No other secondary metabolites were identified from this species.

Unlike *S. tropica*, *S. arenicola* has a cosmopolitan distribution in tropical and subtropical sediments (Jensen and Mafnas, submitted). A total of 30 *S. arenicola* strains from six geographically distinct locations were examined and they all produced compounds in the well-studied rifamycin (**3**, antibiotic) and staurosporine (**4**, protein kinase inhibitor) classes, as well as the new, bicyclic compound salinoketal (**5**), which shares some biosynthetic features with rifamycin (**3**). These three compounds (**3-5**) were produced by all 30 of the strains examined and represent the common *S.*

arenicola chemotype. Like *S. tropica*, the *S. arenicola* strains share 100% 16S rDNA sequence identity with the exception of phylotypes "A" and "B", both cultured from the Sea of Cortez, which each differ from all other *S. arenicola* strains at one of two nucleotide positions (fig. 1). The secondary metabolites produced by phylotypes "A" and "B" did not differ from the common *S. arenicola* chemotype.

In addition to the production of compounds **3-5**, four *S. arenicola* strains cultured from Guam (CNR-005, CNR-416, CNR-075, and CNQ-884) also produced the unique polyketide derived 804-macrolide (**6**). These four strains, all cultured from different sediment samples, cannot be distinguished phylogenetically based on 16S or *gyrB* (data not shown) sequence analysis. A second deviation from the common *S. arenicola* secondary metabolite profile was observed in strains CNS-205 (Palau) and CNR-425 (Guam). These strains produced the cyclic peptide cyclomarin A (**7**), previously reported by our laboratory from a marine-derived *Streptomyces* species (Renner et al., 1999), in addition to compounds **3-5**. Like the 804-macrolide producing strains, these two strains are phylogenetically indistinguishable within the *S. arenicola* clade. Thus, while all *S. arenicola* strains produced compounds **3-5**, we identified 6 cases in which strains produced one additional secondary metabolite (**6** or **7**), and none of these strains could be distinguished phylogenetically based on 16S or *gyrB* gene sequences.

"*S. pacifica*" is the third species thus far detected and, although it was not as frequently encountered or as widely distributed as *S. arenicola*, it possesses the greatest phylogenetic diversity (fig. 1). The most commonly encountered "*S. pacifica*" phylotype is represented by five strains that share 100% 16S rDNA sequence identity.

These strains, which were isolated from Guam, Palau, and the Red Sea, all produced cyanosporoside A (**8**), a novel cyclopenta[*a*]indene glycoside with a rare cyano functionality and a new 3-keto-pyanohexose sugar (Oh et al., in press). Interestingly, two additional "*S. pacifica*" phylotypes, each represented by only one strain, have recently been identified. Both of these strains possessed unique secondary metabolite profiles with phylotype "A" (strain CNS-055) producing a new polyene macrolide, the structure of which is currently under investigation, and phylotype "B" (strain CNS-237) producing a series of unique pyrones characterized by salinispyrone A (**9**). Neither of these strains produced cyanosporoside A (**8**). Although phylotypes "A" and "B" differ from "*S. pacifica*" by only 2 and 3 nucleotides, respectively, no overlap has been detected among the secondary metabolites produced by the three chemotypes. Efforts are currently underway to obtain additional "*S. pacifica*" strains in culture to better understand the relationships between chemotype and phylotype within this relatively diverse species.

The secondary metabolites isolated to date from *Salinispora* species have varied biological activities (table 1). These compounds however have only been tested in biomedically relevant assays and therefore their activities in nature remain unknown. The fact that only two of the compounds (**3** and **5**) possess antibiotic properties suggests that antibiosis may not be a common ecological function of *Salinispora* secondary metabolites. Further tests with appropriate microorganisms would be required however before such a conclusion could be reached.

Based on the analyses performed to date, five unique *Salinispora* chemotypes have been identified, one each from *S. tropica* and *S. arenicola*, and three from "*S.*

pacifica". All five of these chemotypes can be clearly distinguished by 16S rDNA sequence analysis. In addition, all phylotypes that differ by more than one nucleotide produced unrelated secondary metabolites. The dramatic structural differences and lack of overlap in the secondary metabolites produced by the five closely related *Salinispora* phylotypes suggests that the biosynthetic pathways responsible for their production were not inherited vertically from a common *Salinispora* ancestor. The observation that three of the compounds (cyclomarin A, **7**, rifamycin, **3**, and staurosporine, **4**) have been reported from other actinomycete families (Renner et al., 1999; Floss and Yu, 2005; Omura et al., 1995, respectively) further supports this suggestion. To investigate the role of LGT in the acquisition of biosynthetic pathways in *Salinispora* species, and to determine if the secondary metabolites produced in culture by one *Salinispora* species are predictive of the genetic capacity of that species, we probed all three species for the presence of the rifamycin biosynthetic pathway, the product of which is consistently observed in *S. arenicola*. The gene selected to represent this pathway was AHBA (3-amino 5-hydroxy benzoic acid) synthase (*rifK*), which catalyzes the last step in the biosynthesis of AHBA, the unusual starter unit in the rifamycin (**3**) mixed PKS/NRPS pathway (Floss and Yu, 2005).

Using *rifK*-specific primers, seven *S. arenicola* (rifamycin-producing) strains all yielded PCR products of the expected size (440 bp) and with a high level of homology (90% amino acid identity) to the AHBA synthase gene from the rifamycin producer *Amycolatopsis mediterranei* (fig. 3). No PCR products were observed from two *S. tropica* or two "*S. pacifica*" (non-rifamycin producing) strains. These results provide evidence that *S. arenicola* is the only species that possesses the rifamycin

pathway and that the LC-MS results for this compound were indicative of *Salinospora* genotype as opposed to phenotypic responses to culture conditions. Phylogenetic analysis of the *S. arenicola* AHBA synthase gene reveals a recently shared evolutionary history with *A. mediterranei*, a member of the Pseudonocardiaceae, and a more distant relationship with *Micromonospora* and *Actinoplanes* homologues, even though the later two genera share membership with *Salinispora* in the Micromonosporaceae (fig. 4). The high level of sequence homology between the *A. mediterranei* and *S. arenicola rifK* genes suggests that the pathway responsible for rifamycin biosynthesis has recently moved by LGT between these two species.

Discussion

We previously reported the cultivation from marine sediments of unusual populations of seawater requiring actinomycetes (Jensen et al., 1991; Mincer et al., 2002) belonging to the genus *Salinispora* (Maldonado et al., 2005). Careful analysis of *Salinispora* secondary metabolite production reveals an unprecedented species specificity that was maintained regardless of the geographical site from which the strains were collected. This unusual observation indicates that a suite of secondary metabolites can represent a consistent phenotype characterizing a globally distributed population and contradicts the conventional wisdom that actinomycete secondary metabolite production is strain specific. These results are also what would be expected if *Salinispora* secondary metabolites provide ecological opportunities that foster ecotype diversification and, ultimately, phylogenetic divergence.

Although consistent secondary metabolite profiles were observed for the three *Salinispora* species, these results must be carefully interpreted in the context of most currently described actinomtycete taxa, as they include considerably more intra-specific diversity than the species analyzed here. If similar correlations occur among other actinomycetes, they would be observed at the infra-specific level following fine-scale phylogenetic analysis. This level of resolution pushes the limits of 16S sequence analysis and may in some cases require the analysis of more rapidly evolving protein-coding genes (Gevers et al., 2005). Despite concerns over the lack of species-level resolution attainable by 16S gene sequencing (Rosselló-Mora and Amann, 2001), it is remarkable that the three *Salinispora* species, which share >99% 16S rDNA sequence identity, could be readily distinguished once careful corrections were made for PCR error (Jensen and Mafnas, submitted). The species-level classification of the three closely related *Salinispora* phylotypes supports the suggestion by Cohan that many currently named species are comprised of multiple ecotypes, each with the attributes of individual species (Cohan, 2002).

It is now widely recognized that bacterial secondary metabolites have important ecological functions and are not merely artifacts of laboratory culture or metabolic waste products (Chalis and Hopwood, 2003; Firn and Jones, 2000). It has not, however, been proposed that these ecological functions may be associated with ecotype diversification or that they may foster speciation. The remarkable species-specificity of *Salinispora* secondary metabolite production, and evidence that the pathways responsible for their production were acquired by LGT, opens the possibility that pathway acquisition represent a previously unrecognized evolutionary force

driving diversification within the actinomycetes. To test this hypothesis, the rate of 1% 16S change per $50x10^6$ years (Ochman et al., 1999) was used to estimate that *S. arenicola* shared a common ancestor with *S. tropica* $11.8x10^6$ years ago (7 substitutions in 1479 nucleotide positions) while *S. arenicola* and "*S. pacifica*" shared a common ancestor $18.6x10^6$ years ago (11 substitutions in 1479 nucleotide positions). Likewise, it can be calculated that *S. arenicola* and *A. mediterranei* shared an ancestral AHBA synthase gene approximately $19.3x10^6$ years ago, based on the estimate of 0.45% change per $1x10^6$ years (Ochman et al., 1999) and 38 synonymous site substitutions in 437 nucleotide positions. Although these evolutionary rates are estimates, they indicate that the acquisition of the rifamycin pathway occurred at approximately the same time as the initiation of 16S phylogenetic divergence within the genus *Salinispora*, thus supporting an association between pathway acquisition and phylogenetic diversification. While the importance of acquired genes to niche invasion has been discussed in relation to pathogens (Hacker and Kaper, 2000), this is the first evidence that the genes involved in secondary metabolism may provide the ecological basis upon which individual populations are differentiated.

An alternative hypothesis is that the pathways responsible for compound production were acquired post-speciation or following the initiation of population diversification. In this scenario, it would not be expected that all strains within a given species would produce a species-specific set of secondary metabolites unless expression of the pathways provided a selective advantage that resulted in their fixation in all populations. Based on the data presented here, the later scenario would have to be the case if this alternative hypothesis is correct. Thus, if pathway

acquisition is not initiating speciation it is none-the-less playing a role in interspecies genomic differentiation. With the exception of minor differences in sole carbon source utilization (Maldonado et al., 2005), the secondary metabolite profiles of each species are the single most significant phenotypic traits that can be used for their differentiation. The genomes *S. arenicola* and *S. tropica* (currently being sequenced at JGI) will provide much new insight into the ecological basis that differentiates these two species.

The remarkable diversity of secondary metabolites produced by individual *Salinispora* species complicates the interpretation of their ecological significance. Given the absolute consistency with which compounds **3-5** were produced by *S. arenicola*, each may have a distinct, ecological function. Alternatively, these compounds may act synergistically, a well-known phenomenon associated with actinomycete secondary metabolism (Chalis and Hopewood, 2003). It is of particular interest that six *S. arenicola* strains produced compounds (**6** or **7)** in addition to the standard chemotype (compounds **3**-**5**). These are the only strains observed that displayed variation within a chemotype. Given that these strains could not be differentiated phylogenetically (fig. 1), it is possible that the pathways encoding the production of these compounds were acquired relatively recently and have not yet become fixed in the population or, if they foster ecotype differentiation, reflected in the 16S sequence data. Alternatively, the products of these pathways may not offer opportunities for niche expansion and therefore, even if they provide a selective advantage, they may not foster phylogenetic divergence. Finally, it is possible that these pathways are present in all *S. arenicola* strains but expressed by only six of 30

under the fermentation conditions applied. These questions will be addressed using data from the *S. arenicola* genome, currently being sequenced at the Joint Genome Institute.

Adaptive evolution within a bacterial lineage may proceed slowly by the sequential accumulation of favorable mutations or rapidly by gene or gene pathway acquisition. The later has clear advantages as a gene or gene pathway that improves fitness can lead to rapid ecotype differentiation (Ochman et al., 2004; Cohan, 2002) while an acquisition with neutral or negative effects will be rapidly lost at little or no cost to the population. The genetic organization of the biosynthetic pathways encoding actinomycete secondary metabolite production has been studied in detail and reveals a generally ordered and tight packaging of genes into multi-operon clusters that include not only structural and regulatory elements but resistance genes that allow the producing strain to avoid auto-toxicity (eg., Pojet et al., 2002). This genetic arrangement is facilitated by lateral gene transfer and provides a fitting corollary to the selfish operon model (Lawrence, 1997). Regardless of the mechanisms by which genes are acquired (transformation, transduction, or conjugation), the data presented here provides evidence that the lateral transmission of pathways involved in secondary metabolite production provides an effective strategy to sample (as opposed to accumulate, Ochman et al., 2000) genes from a common gene pool, the products of which may provide opportunities for immediate access to a new ecological niche (ecotype differentiation) or an effective mechanism to out-compete con-specifics (selective sweep). This concept compliments previous ideas of “a global gene pool" (Maiden et al., 1996), expands our understanding of LGT as a driving force in

bacterial diversification (Ochman et al., 2000; Levin and Bergstrom, 2000), and provides new evidence for the evolutionary significance of secondary metabolism.

Based on recent actinomycete genome sequences (Bently et al., 2002; Omura et al., 2001), it is unlikely that the full biosynthetic potential of the three *Salinispora* species has been expressed. Nor is it surprising that individual actinomycetes produce diverse suites of secondary metabolites. What is surprising about the results presented here is that careful phylogenetic analyses of closely related strains reveal distinct sequence clusters that can be used as indicators of secondary metabolite production. These results contradict the traditional (pre-molecular systematics) paradigm of the antibiotic discovery era in which secondary metabolite profiles were believed to represent a strain-specific phenotype (Zahner, 1979). Furthermore, these results invalidate the widely applied stochastic model of microbial drug discovery that necessitates the screening of large numbers of strains, and in the process the inevitable isolation of large numbers of redundant compounds, for every new metabolite discovered.

Further research is required to determine the extent to which secondary metabolite production is correlated to phylogeny in the actinomycetes. Additional evidence will also be needed to better understand the potential link between the acquisition of pathways encoding secondary metabolite biosynthesis and ecotype diversification. What is clear, however, from analysis of the genus *Salinispora* is that phylogenetic diversity can be used as an effective tool to guide secondary metabolite isolation and, as such, a method to identify strains capable of producing different suites of secondary metabolites. Although it remains unknown how broadly these

findings can be applied to other bacteria, it is clear that a diversity-based discovery strategy, focused on chemically-prolific actinomycetes residing in poorly studied environments such as marine sediments, will yield a high rate of novel natural product discovery and present an improved strategy for the discovery of novel microbial products.

Experimental Procedures

Bacterial strains and genetic analyses. The 46 strains included in this study were obtained in culture as previously described (Jensen et al., 1991; Mincer et al., 2002). They consist of 2-9 representatives of each unique 16S rDNA phylotype observed from six worldwide collection sites (Jensen and Mafnas, submitted) with the exception of "*S. pacifica*" phylotypes "A" and "B" for which only a single strain was examined. Phylogenetic analyses were performed as previously described (Jensen et al., 2005).

A 440 base pair segment of the AHBA synthase (*rifK*) gene was PCR amplified using the forward (MAO1F 5'-3' TTCGAGCGGGAGTTTGCSG) and reverse (MAO1R 5'-3" CSGTCATCAGCTTGCCGTTC) primers designed based on previously reported amino acid sequences (Mao et al., 1999). PCR reactions (50 μl) contained 10-100 ng DNA template, 0.4 μM each MAO1F and MAO1R primer, 200 μM (each) dATP, dCTP, dGTP, and dTTP, 1.25 U AmpliTaq (Applied Biosystems), 5 μl $MgCl_2$, 5 μl 10x PCR buffer, 10% DMSO. The PCR conditions were as follows: 95°C for 12 min followed by 35 cycles of 94°C for 1 min, 59.6°C for 1 min, 72°C for

1 min, followed by 72°C for 7 min. PCR products were purified and sequenced (using the MAO1F and MAO1R primers) as described above. Sequence contigs were assembled using Sequencher (ver. 4.5, Gene Codes Corp., Ann Arbor, MI) and phylogenetic analyses performed using PAUP (ver. 4.0b10, Sinauer Assoc., Sunderland, MA).

Secondary metabolite screening. For comparison purposes, all 46 *Salinispora* strains were cultured in identical fermentation conditions. These consisted of shaking (230 rpm) at 25-27°C in medium 1 (10 g starch, 4 g yeast extract, 2 g peptone, 5 mL $Fe_2(SO_4)\cdot 4H_2O$ at 8g/L, 5 mL KBr at 20 g/L, 1 liter seawater). Aliquots (25 ml) of the fermentation broth were removed, extracted three times with 25 mL EtOAc, the organic layer concentrated to dryness *in vaccuo*, and the residue re-suspended at 10 mg/mL in 50% aqueous methanol. Samples were taken every two days from days 3-21 (for at least one representative of all phylotypes and collection sites) or from days 7-13 (which proved to be the optimal time range to detect all of the compounds).

Chemotype analysis. Ten µl of each extract was analyzed by LC-MS (Agilent 1100) using a linear gradient of 10-80% aqueous acetonitrile over 30 min (Hypersil ODS, 4.6x100 mm column, flow 0.7 mL/min, UV detection 190-800 nm). Mass spectra were collected (scanning 100-2000 AMUs) in the positive mode (ESI voltage 6.0 kV, capillary temperature 200°C, auxiliary and sheath gas pressure 5 units and 70 psi, respectively). Compounds were identified by comparison of molecular weights, UV spectra, and retention times with authentic standards or, in the case of the rifamycins and staurosporines, by comparison with published UV and MS values. The

limits of accurate LC-MS detection were determined by serial dilution of standards to be ca. 5-50 μg compound per liter culture, well below the lowest yield at which any of the compounds were recovered (200 μg/L). Details for compounds **1, 2**, and **8**, which are new structures isolated from *Salinispora* strains, are as previously reported (Buchanan et al., 2005; Feling et al., 2003; Oh et al., in press; respectively). Details for compounds **5**, **6**, and **9**, which are also new structures, will be published elsewhere. Retention times and molecular ions for new *Salinispora* compounds are as follows: salinosporamide A (17.0 min, MH^+ 314, MNa^+ 336), sporolide A (12 min, MH^+ 539, MNa^+ 561), salinoketal (15.8 min, MNa^+ 418), cyanosporaside A (10.1 min, MNa^+ 440), 804-macrolide (19.2 min, MNa^+ 827), salinispyrone A (19.0 min., MNa^+ 315).

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The text of Chapter IV, in full, is a reprint of the material as it appears in Jensen, P.R., Williams, P.G., Oh, D.C., Zeigler, L, and Fenical, W. (submitted). Proc. Natl. Acad. Sci. The dissertation author was the primary author and directed and supervised the research, which forms the basis for this chapter.

Table 1. Secondary metabolites isolated from *Salinispora* spp. and their biological activities.

Compound	Source	Biological activity	Molecular target
(**1**) salinosporamide A	*S. tropica*	anticancer	proteasome
(**2**) sporolide A	*S. tropica*	unknown	unknown
(**3**) rifamycin	*S. arenicola*	antibiotic	RNA polymerase
(**4**) staurosporine	*S. arenicola*	anticancer	protein kinase
(**5**) salinoketal	*S. arenicola*	antibiotic	unknown
(**6**) 804-macrolide	*S. arenicola*	unknown	unknown
(**7**) cyclomarin A	*S. arenicola*	antiinflammatory	unknown
(**8**) cyanosporoside A	"*S. pacifica*"	unknown	unknown
(**9**) salinipyrone A	"*S. pacifica*" B	unknown	unknown

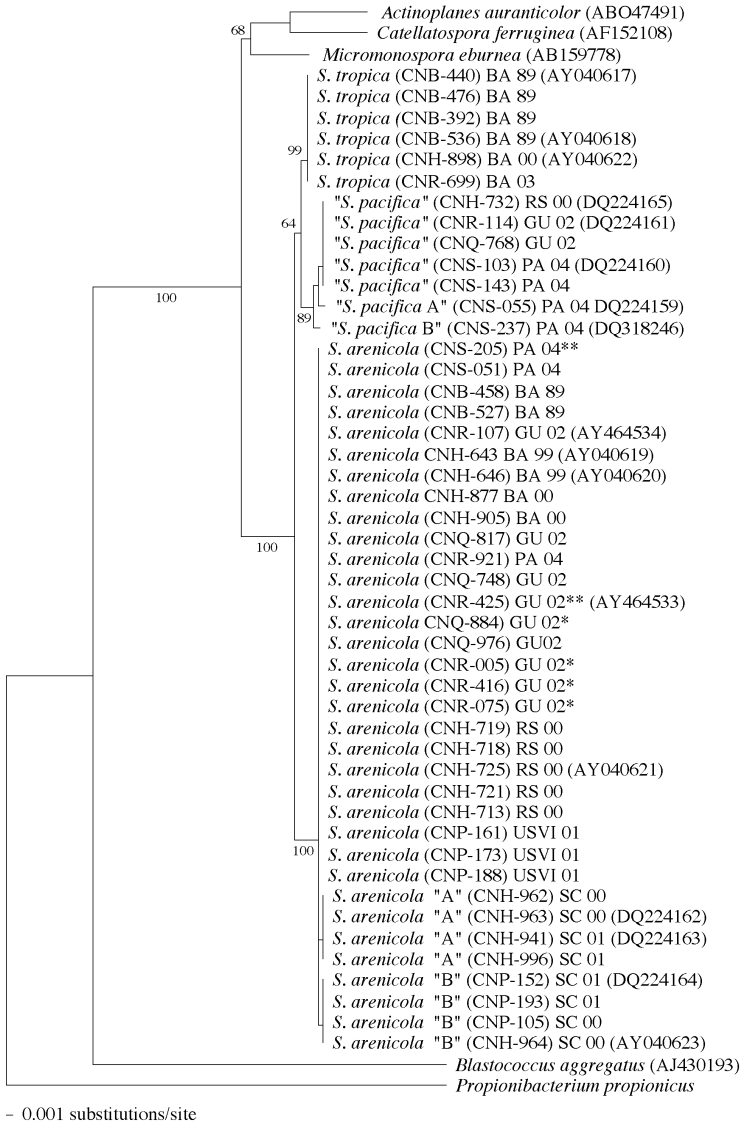

Figure 1. Neighbor-joining phylogenetic tree created from 46 nearly complete (1449 nucleotides) *Salinispora* 16S rRNA gene sequences. The three major *Salinispora* phylotypes, consisting of the two formally described species *S. tropica* and *S. arenicola* and the proposed species "*S. pacifica*", are clearly delineated. Species names are followed by strain number, source (BA = Bahamas, RS = Red Sea, GU = Guam, PA = Palau, USVI = US Virgin Islands, SC = Sea of Cortez), year of collection (89 = 1989, etc,) and accession number (for representative sequences). * produces 804-macrolide (**6**), ** produces cyclomarin A (**7**). *P. propionicus* and *B. aggregatus* were used as outgroups.

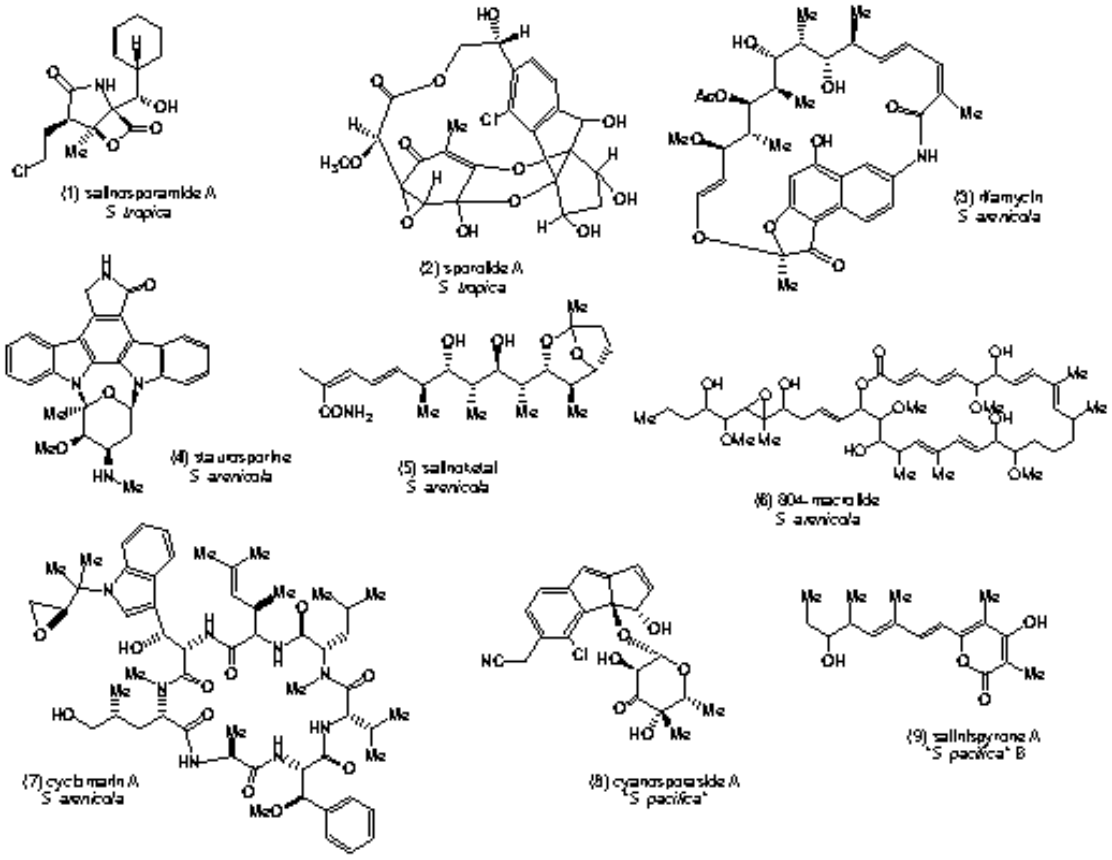

Figure 2. Secondary metabolites produced by *Salinispora* species. Compound names and the producing species are listed under the structures. Compounds **1**, **2**, **5**, **6, 8,** and **9** are new secondary metabolites recently discovered from *Salinispora* species while compounds **3**, **4,** and **7** were previously reported from other actinomycetes (22,23,20, respectively).

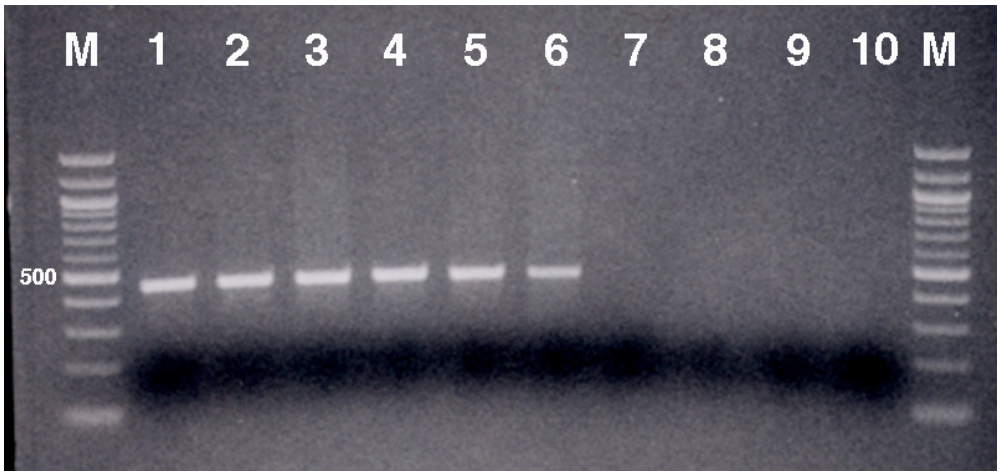

Figure 3. AHBA synthase (*rifK*) PCR amplification. Presence of the AHBA synthase (*rifK*) gene in three *Salinispora* species was determined using PCR primers specific for a 440 bp region of the gene. M = 100 bp marker, lanes 1-6 = *S. arenicola* (strains CNH-643, CNR-562, CNP-161, CNR-107, CNP-193, CNS-051), lanes 7-8 = *S. tropica* (strains CNB-440, CNB-476), lanes 8-9 "*S. pacifica*" (strains CNH-732, CNQ-768).

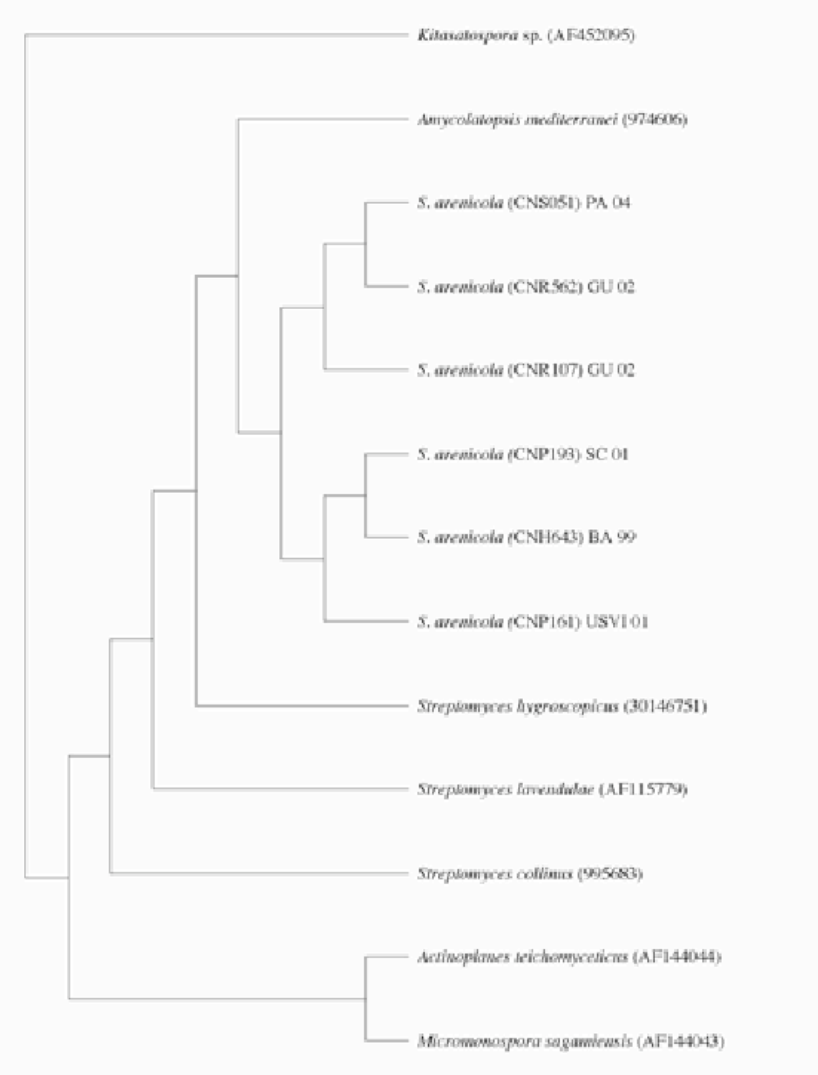

Figure 4. Neighbor-joining cladogram of AHBA synthase gene sequences (based on 264 nucleotides) obtained from *S. arenicola* and NCBI BLAST analyses. The *S. arenicola* (Micromonosporaceae) sequences clade with *A. mediterranei* (Pseudonocardiaceae), not with *Micromonospora* or *Actinoplanes* homologues (also Micromonosporaceae

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CHAPTER V

Summary and future directions

Summary

The research presented in this thesis adds to the growing body of literature describing actinomycetes in the marine environment. What is made clear from this research is that actinomycetes can be readily cultivated from marine samples, however it is relatively uncommon to find strains that require seawater for growth. It is also uncommon to find strains that share <97% 16S rRNA gene sequence identity with strains previously reported from land. Despite the extensive cultivation efforts discussed in chapter 2, the only marine-derived actinomycete taxon observed to date that appears to have genus-level status and within which all strains consistently require seawater for growth is *Salinispora* (Maldonado et al., 2005). Thus, it appears that a requirement of seawater for growth is a rare physiological trait among marine actinomycetes.

It is also noteworthy that the phylogenetically unique marine actinomycetes observed to date are being described as new genera or, more commonly, new species. Thus, marine actinomycetes appear to be more closely related to their terrestrial relatives than some common marine bacterioplankton, which have been distinguished at the Family level, eg., the SAR11 clade. This observation implies that, among bacteria, actinomycetes were either introduced into the sea relatively recently or they are relatively successful at transitioning the land-sea barrier and thus not restricted to either environment for periods of time sufficient for differentiation into higher-level taxa. This later concept would imply that any combination of effective dispersal, lack

of geographical isolation, and rapid adaptation and genomic evolution has reduced the level of diversification that can result from the selective pressures associated with life in the marine environment.

Supporting the rapid and widespread dispersal of actinomycetes in the marine environment is the recovery of clonal (16S rDNA) populations of *S. arenicola* from global collecting sites. As mentioned in chapter 3, I believe this is the first report of its kind for any bacterium and provides clear evidence that some bacterial species exhibit a cosmopolitanism distribution in the marine environment. This finding has important implications for studies of microbial diversity as it implies that sampling from distant but environmentally similar locations will not necessarily increase the likelihood of recovering new species. The key to discovering new diversity will be to explore new ecological niches, regardless of geographical location, and to develop new cultivation techniques in an effort to recover new ecotypes. This concept is equally applicable to the discovery of secondary metabolites as, if the pattern observed for *Salinispora* holds true for other actinomycetes, new ecotypes will yield new suites of secondary metabolites, while similar ecotypes, regardless of where they were recovered, will produce the same metabolites. This finding has profound implications for natural product discovery as it largely eliminates the need to study phylogenetically redundant strains thus streamlining the screening process. Fine-scale phylogenetic analyses could instead be used to identify new phylotypes and representatives of these groups selected for detailed natural product study. Although not widely disseminated, there is evidence that this paradigm applies to fungal secondary metabolite production (Larsen et al., 2005).

Streptomycetes devote a significant portion of their genome to secondary metabolite production. Of the two genomes that have been sequenced to date (Bentley et al., 2002; Omura et al., 2001), this proportion ranges from 5-6%. Clearly, this volume of genetic material, and the structural architecture of the biosynthetic pathways that encode secondary metabolite production, is the result of long and complex evolutionary processes. But are the products of these pathways of sufficient ecological significance to foster ecotype differentiation and ultimately speciation? Chapter 4 directly addresses this question and, once again in the case of *Salinispora*, all evidence supports a role for secondary metabolism in speciation. Since most bacteria do not produce secondary metabolites, this observation may be restricted to the actinomycetes. In fact, it may be restricted to only a few actinomycete genera as only a small handful account for more than 90% of the metabolites discovered to date (Berdy, 2005).

Future Directions

Clearly a great deal of research remains to be performed before we will begin to have a clear understanding of the diversity and ecology of actinomycetes in the sea. In some ways, it is fortunate that these bacteria have important industrial applications, as this may help drive basic research forward, as well as lead to new, useful products, that hopefully include medicines to treat recalcitrant diseases such as cancer and antibiotic resistant infectious disease. Hopefully future research on marine bacteria will include careful studies of actinomycetes in the sea so that we can begin to

learn how they participate in the microbiological processes that are fundamental to all marine ecosystems.

It is clear that some of the highest priority research that remains to be performed includes an assessment of actinomycete abundance and diversity in seawater. One place to start would be with an analysis of the Sargasso Sea dataset, as Actinobacteria are a clear component of the communities sampled, yet how many of these are actinomycetes and what types of actinomycetes they represent has not been determined. A second priority is to look at samples collected further from shore. Ocean sediments cover 70% of the planet's surface yet only a small handful of deep-sea sediments have been analyzed for actinomycete diversity. Certainly, if additional, indigenous marine actinomycetes exist, they will be found in these environments.

It is of particular interest that few marine-derived actinomycetes appear to require seawater for growth. Given that *Salinispora* has this requirement, it should be possible to address the genetic basis of actinomycete sodium dependency and compare this to other types of marine bacteria. One priority will be to determine if marine actinomycetes possess a sodium-dependant quinone reductase as has been reported in Gram-negative marine bacteria (Hayashi et al., 2001). It should also be possible to screen for actinomycetes that possess alternative sodium dependent genes, once these are defined, and use this information as a rapid method to select for marine-adapted strains. A high priority of the *Salinispora* genome analyses will be to identify marine adaptation genes and develop probes for their rapid identification in both samples and strains. We have observed that seawater requirements can be a transient phenotype in

some marine-derived actinomycetes so the molecular basis behind this transition must also be deciphered.

No discussion of actinomycete secondary metabolites would be complete without raising the question of their ecological significance. Although I have proposed that selection acts on strains producing specific suites of secondary metabolites, I have offered no suggestions as to what ecological roles these compounds may play in the natural environment. Assuming that they are expressed in nature (it is unlikely that such complex genetic machinery would be maintained without expression), the most obvious suggestion is that these molecules are used as agents of chemical warfare. The fact that some of the compounds produced by *Salinispora* strains are potent antibiotics supports this suggestion. However, the in situ effects of these compounds are undoubtedly much more complex than simple direct cell killing, and it will take considerable experimentation for their effects to be deciphered. For example, actinomycete secondary metabolites may inhibit bacterial quorum sensing or render sediments unpalatable to eukaryotic mesograzers. These types of effects could provide significant selective advantage but remain undetected when testing for standard, biomedically oriented biological activity. It will be important to develop new bioassays that can be used to explore the ecological roles of these compounds in marine sediments.

Finally, it will be of great interest to determine if the correlations observed between *Salinispora* chemotype and phylotype are maintained in other actinomycete taxa. *Salinispora* has proven to be a model organism with which to address these types of questions, however the evolutionary significance of secondary metabolism

will not be fully appreciated until this model is tested more broadly. This will require the cultivation of large numbers of strains followed by careful phylogenetic and secondary metabolite analyses. The results of these studies may help support recent suggestions that bacterial species, as currently described, more appropriately represent composites of ecologically distinct phylotypes, each with the characteristics of individual species (Cohan, 2002). Thus, the search for new medicines from marine actinomycetes may ultimately add important new information about the diversity of bacteria in the sea and how individual species are distinguished.

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APPENDIX A

Distribution of actinomycetes in near-shore tropical marine sediments.

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Distribution of Actinomycetes in Near-Shore Tropical Marine Sediments

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Actinomycetes were isolated from near-shore marine sediments collected at 15 island locations throughout the Bahamas. A total of 289 actinomycete colonies were observed, and all but 6 could be assigned to the suprageneric groups actinoplanetes and streptomycetes. A bimodal distribution in the actinomycete population in relation to depth was recorded, with the maximum numbers occurring in the shallow and deep sampling sites. This distribution can be accounted for by a rapid decrease in streptomycetes and an increase in actinoplanetes with increasing depth and does not conform to the theory that actinomycetes isolated from marine sources are of terrestrial origin. Sixty-three of the isolated actinomycetes were tested for the effects of seawater on growth. Streptomycete growth in nonsaline media was reduced by 39% compared with that in seawater. The actinoplanetes had a near obligate requirement of seawater for growth, and this is presented as evidence that actinomycetes can be physiologically active in the marine environment. Problems encountered with the enumeration of actinomycetes in marine sediments are also discussed.

It is well documented that actinomycetes can be isolated from marine sediments (see review by Goodfellow and Haynes [4] and, more recently, references 1, 14, 15, and 23), including samples from the deep sea (19). In addition, the descriptions of *Rhodococcus marinonascens* (8), along with other marine species (6), show that certain actinomycetes are indigenous to the marine environment. However, because actinomycetes are more abundant in terrestrial soils relative to marine sediments (5), show varying degrees of salt tolerance (9, 12, 13, 18), and produce spores that are undoubtedly washed in large numbers from shore into the sea, it remains unclear what component of the actinomycetes isolated from marine sources represents an autochthonous marine microflora (4, 6, 13).

In addition to uncertainties about the origin of actinomycetes in marine habitats, it is not known to what extent these bacteria represent a physiologically active component of the marine microbial community. It has been shown that actinomycetes can grow in a seawater-based medium (22) and at increased hydrostatic pressures (7) and that increased numbers of *Micromonospora* occur with increasing depth in deep-sea sediments (21, 23). Yet, due to the overall observation that actinomycetes decrease in number as distances from shore increase (19–21), and a lack of experimental evidence describing the distribution and metabolic activity of these bacteria in marine habitats, it has been concluded that, unless exceptional conditions occur, actinomycetes isolated from marine sources arise from spores or resting propagules (4, 5).

We report here the occurrence of actinomycetes in near-shore tropical marine sediments collected throughout the Bahamas. The observed actinomycetes were grouped taxonomically, and their distributions and the effects of seawater on biomass production were determined for the dominant taxonomic groups. Based on these results, conclusions are made regarding the ability of actinomycetes to grow in the marine environment.

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MATERIALS AND METHODS

Collection of samples. Marine sediments were collected from 15 island locations throughout the Bahamas as part of a research expedition aboard the R/V *Columbus Iselin* (University of Miami) in June 1989. The locations sampled included the islands of Chub Cay (sites 1 to 5), Grand Bahama (site 6), Abaco (site 7 to 9), San Salvador (sites 10 and 15), Aklins (sites 11 to 13), and Hogsty Reef (site 14). These locations included diverse environments ranging from an oceanic atoll (Hogsty Reef), ca. 65 km from the nearest island, with only a sand spit and scrub vegetation above the high-water line, to areas highly influenced by terrestrial runoff, e.g., mangrove habitats.

At each of the 15 locations, divers collected five sediment samples (total, 75 samples), one from each of the depths 0 to 1, 1 to 3, 3 to 6, 6 to 15, and 15 to 33 m, by starting near shore and heading seaward. The habitats encountered at each sampling depth varied and were categorized as sand, mangrove, seagrass, hard bottom, or reef. Sediments were collected in sterile 50-ml tubes and kept refrigerated until shipboard processing later that day.

Sample processing. Sediment samples were vigorously shaken by hand to ensure uniformity and then allowed to settle for ca. 10 min. The overlying water was decanted and used for the determination of pH and salinity. Sediment organic content was determined by drying triplicate subsamples of each sediment (1 to 9 g, dry weight) to a constant weight at 90°C, ashing at 450°C for 2 h, and reweighing. The organic contents were determined as the average difference between dry and ash weights for the triplicate subsamples.

Serial dilutions of the 75 sediment samples were made by aseptically removing 1.0 ml of wet sediment and adding it to 4.0 ml of sterilized filtered seawater (dilution, 2×10^{-1}), mixing, and further diluting 1:10 with sterilized filtered seawater (dilutions, 2×10^{-2} through 2×10^{-4}). The dilution procedure was repeated in triplicate for each sample, resulting in a total of 225 serial dilutions. Sediment dry weight per milliliter was determined for each sample as the average weight of two additional (2×10^{-1}) dilutions prepared with deionized water and oven dried at 90°C for 48 h.

The diluted sediments were inoculated onto two types of

nutrient agar. Medium 1 (M1; 0.25% starch, 0.1% peptone, 0.05% yeast extract, 0.01% glycerophosphate [disodium pentahydrate], 1.6% agar, 75% filtered seawater, 25% deionized water) was selected as a general medium for the enumeration of heterotrophic bacteria. Medium 2 (M2; 1.0% starch, 0.1% casein, 0.01% trace element mix [Marineland Aquarium Products], 1.6% agar, 75% filtered seawater, 25% deionized water) was chosen for the isolation of actinomycetes (6). All media contained 75 μg of filter-sterilized cycloheximide per ml, added aseptically after the media had been autoclaved and cooled, to reduce fungal contamination.

Plates of M1 and M2 were inoculated with 50 μl of dilutions 2×10^{-2} through 2×10^{-4}, and the samples were spread with a sterilized bent glass rod and plate spinner. The 2×10^{-2} and 2×10^{-3} dilutions were then heated to reduce the number of unicellular bacteria in favor of actinomycetes (2) by submersion in a water bath at 50°C for 60 min, and 50 μl of these heat-treated samples was inoculated onto M1 (M1+H).

Bacterial and actinomycete enumeration. Colonies of unicellular bacteria were counted on M1 plates after 14 and 21 days of room temperature (20 to 24°C) incubation. The average colony count at each dilution was calculated with plates with 20 to 250 colonies. CFU per milliliter of wet sediment were calculated for each sample, using the highest dilution that yielded an average of >20 colonies. CFU per gram (dry weight) were calculated based on the dry weight per milliliter of wet sediment.

Actinomycetes were recognized by their characteristic tough, leathery colonies, branched vegetative mycelia, and, when present, aerial mycelia and spore formation. Due to these criteria, only actinomycetes with well-developed and branched hyphae were included in this study. Actinomycete colonies were counted on M1, M2, and M1+H plates after 21, 30, and 45 days of room temperature incubation, and information about colony morphology, including the presence or absence of aerial mycelia, was recorded.

Due to problems associated with the use of serial dilution and plating techniques for the enumeration of actinomycetes (21; this report), only actinomycete colonies on the 2×10^{-2} dilution plates were counted. Because of the low numbers of actinomycetes observed, counts from the triplicate plates were summed. Total actinomycete colonies were calculated for each depth as the individual and collective sums (15 collecting sites) for the M1, M2, and M1+H plates. Actinomycetes were not quantified per unit sediment, and colony counts are of value in a relative sense for the comparison of depth distributions, treatments, etc.

Actinomycete isolation and taxonomic evaluation. Actinomycetes representing all colony morphologies observed from each sample were isolated by repeated transfer on medium M3 (1.0% starch, 0.4% yeast extract, 0.2% peptone, 1.6% agar, 75% filtered seawater, 25% deionized water) until pure strains were obtained as judged by colony morphology. All isolated strains were grown at room temperature in 10 ml of M3 without agar, observed microscopically for the presence of mycelial fragmentation, and frozen with 10% glycerol as a cryoprotectant.

All isolated actinomycetes with aerial mycelia, and representatives of all morphologies with only vegetative mycelia, were analyzed chromatographically for isomeric diaminopimelic acid configurations and for whole-cell sugar composition as described by Schaal (16) with the following modifications: isolates were grown in 4.0% yeast extract–4.0% glucose–75% filtered seawater–25% deionized water, and the cells were hydrolyzed by autoclaving in 1 ml of 6 N HCl at 121°C for 15 min. Chromatographies were performed by using glass-backed cellulose-coated thin-layer chromatography plates (Merck 5716-7). Based on isolate morphology and diaminopimelic acid and whole-cell sugar analyses, all but a few strains could be grouped suprageneric ally as described by Goodfellow (3) and Lechavalier (10). Taxonomic information from the representative isolates was extrapolated to the total actinomycete population observed on the 2×10^{-2} plates.

Effects of seawater on actinomycete growth. Representatives of the dominant suprageneric groups were grown in medium M4 (1% starch, 0.4% yeast extract, 0.2% peptone, 100% filtered seawater) and M4 substituted with deionized water to determine the effects of seawater on growth as determined by biomass production. Starter cultures were generated by inoculating 1.0 ml of frozen culture (M3 plus 10% glycerol) into 10 ml of M3. These cultures were shaken at 150 rpm and room temperature until adequate growth was obtained (3 to 10 days), at which time 5.5 ml was dispensed into 100 ml of both M4 and M4 substituted with deionized water. Because starter cultures were grown in a seawater-based medium (M3), the final concentration of seawater in 100 ml of M4 substituted with deionized water was approximately 4%; therefore, these cultures were provided with trace quantities of salts and minerals.

All 100-ml cultures were shaken at 230 rpm and room temperature for 8 days, after which the entire volumes were filtered onto 47-mm type A/E glass fiber filters (Gelman Sciences), freeze-dried, and weighed. The average dry weights of triplicate 100-ml volumes of uninoculated M4 and M4 substituted with deionized water were calculated and subtracted as medium controls.

RESULTS

A total of 289 actinomycete colonies were observed from the 75 sediment samples collected. From these, 35 colonies with aerial mycelia and 64 colonies with only substrate mycelia were isolated as morphological representatives. All 35 isolates with aerial mycelia and 18 of 64 isolates with only substrate mycelia, including representatives of all morphologies observed, were examined chemotaxonomically. The combined morphological and chemotaxonomic evaluations of the representative isolates were extrapolated to the entire observed population. From this, it was determined that 91 of the 289 observed colonies had both aerial and substrate mycelia, did not fragment when grown in liquid culture, possessed predominantly the LL isomer of diaminopimelic acid, and had no diagnostic whole-cell sugar pattern. Based on these results, 91 of the total observed actinomycetes were assigned to the suprageneric group streptomycetes or the genus *Kitasatosporia* (10). For the purpose of this study, all 91 of these actinomycetes will be considered to belong to the streptomycete group, within which there are five genera, including the genus *Streptomyces* (3).

Of the remaining 198 observed actinomycetes, 192 lacked aerial mycelia, possessed predominantly the *meso* isomer of diaminopimelic acid, and had xylose and arabinose as diagnostic whole-cell sugars. These bacteria commonly displayed orange to red-brown mycelia and, when present, spores that blackened the surface of the colonies. Based on these results, 192 of the observed actinomycetes belong to the suprageneric group actinoplanetes (3). There are six genera within this suprageneric group and, based on morphological characteristics, most of the observed strains appear to belong to the genus *Micromonospora*. In total,

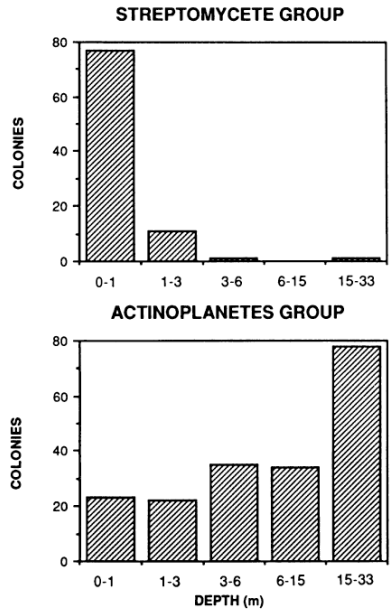

FIG. 1. Distribution versus depth for streptomycetes and actinoplanetes calculated as the total number of colonies observed from 15 locations (2×10^{-2} dilution).

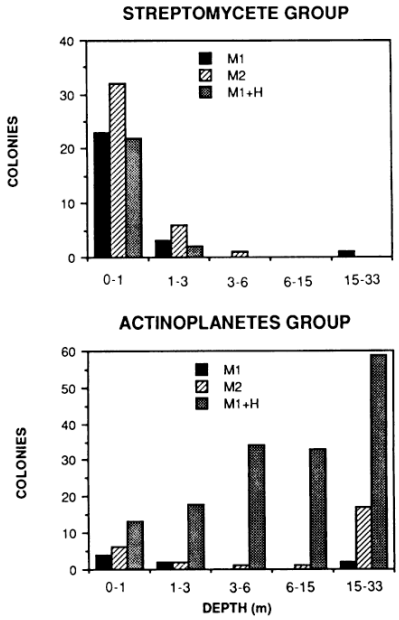

FIG. 2. Distribution versus depth for streptomycetes and actinoplanetes calculated as the total number of colonies observed from 15 locations (2×10^{-2} dilution) for M1, M2, and M1+H.

97% of the observed actinomycete population could be grouped suprageneric ally within the streptomycetes or actinoplanetes. Of the remaining six colonies, one was placed in the suprageneric group nocardioforms, two could not be grouped, and three could not be grown in sufficient quantities for chemotaxonomic evaluation.

The highest numbers of actinomycetes were observed from the 0- to 1- and the 15- to 33-m sampling sites (Fig. 1). This bimodal maxima in the actinomycete population can be accounted for by the taxon-specific distributions of the suprageneric groups. The number of streptomycetes decreased rapidly with increasing water depth, with 86% being recovered from 0 to 1 m and 12% from 1 to 3 m. In all, 98% of the streptomycetes observed were from water $\leq$3 m deep. Conversely, the number of actinoplanetes increased with increasing water depth, with 41% of the 192 observed colonies coming from the deepest sampling site (15 to 33 m). However, unlike the streptomycetes, the actinoplanetes were more evenly distributed throughout the sampling zones, with 12, 11, 18, and 18% of the total isolates coming from 0 to 1, 1 to 3, 3 to 6, and 6 to 15 m, respectively.

The large number of actinomycetes observed from the deep sediments can, for the most part, be accounted for by the high number of actinoplanetes obtained from the heat-treated samples (Fig. 2). If heat treatment as a selective isolation method had not been used, the extent of the deep-water actinomycete population would not have been recognized. Both the heat-treated and non-heat-treated samples resulted in approximately equal numbers of streptomycete colonies, with the numbers of these bacteria decreasing rapidly in all cases with increasing depth.

Actinomycetes were observed and isolated from each of the 15 sampling locations (Table 1) including site 14, an oceanic atoll far removed from terrestrial influences, from which all of the actinomycetes observed belonged to the actinoplanetes group. Of the five types of habitats sampled, none proved to be a rich source of actinomycetes, and the numbers observed from any one habitat were variable.

The salinity, pH, and percent organic content of the sediments became less variable as depth increased (Table 2); however, these parameters appeared to have little effect on the number of actinomycetes observed. High numbers of bacteria were recorded from organic rich sediments, yet there was no correlation between percent organic content and number of actinomycetes or in the ratio of actinomycetes to bacteria as a function of depth as reported by Weyland (21).

Standard serial dilution and plating techniques were used in this study for the purpose of isolating and quantifying actinomycetes. However, the actinomycete counts were not a quantitative function of dilution as would be expected when using these techniques. A similar observation was reported by Weyland (21) for actinomycetes isolated from deep-sea sediments. Of the 225 serial dilutions plated, the incidence of a more dilute sample within one dilution series

TABLE 1. CFU of unicellular bacteria (10^4) reported per gram (dry weight) of sediment and actinomycetes reported as the sum of all colonies observed (M1, M2, M1+H, 2×10^{-2} dilution)[a]

Site	Depth														
	0–1 m			1–3 m			3–6 m			6–15 m			15–33 m		
	Habitat	Bacteria	Actinomycetes	Habitat	Bacteria	Actinomycetes	Habitat	Bacteria	Actinomycetes	Habitat	Bacteria	Actinomycetes	Habitat	Bacteria	Actinomycetes
1	MG	455.7	1	SG	179.9	0	SG	243.9	1	RF	19.8	1	RF	184.1	2
2	MG	560.2	28	SG	23.2	2	HB	60.3	4	RF	14.9	3	RF	70.6	1
3	SD	3.1	34	SG	30.0	0	RF	34.4	4	SD	33.7	3	RF	125.4	1
4	SD	3.3	4	HB	64.3	8	HB	34.3	3	SG	19.3	5	SD	45.8	1
5	MG	403.7	0	HB	886.7	5	HB	281.3	0	SG	6.1	0	RF	203.9	0
6	MG	194.7	0	RF	181.5	1	RF	70.9	1	RF	89.3	2	RF	101.2	6
7	MG	192.5	18	HB	190.8	2	HB	127.9	9	RF	589.3	15	RF	163.2	11
8	MG	547.4	2	HB	79.1	5	HB	65.2	3	RF	141.1	0	RF	91.6	3
9	SD	74.4	7	HB	20.0	1	HB	194.3	2	HB	75.8	1	SD	44.9	8
10	SD	41.7	5	SD	54.3	1	SD	34.9	0	SD	32.2	1	RF	25.2	5
11	SD	1.1	0	SD	40.4	6	HB	95.0	0	RF	23.1	0	RF	50.1	24
12	SD	0.5	0	SD	2.2	1	SD	67.3	7	RF	80.3	0	RF	68.8	0
13	SD	3.2	0	SD	11.6	6	SG	1.8	2	RF	5.7	2	RF	32.2	1
14	SD	1.7	1	HB	12.2	0	RF	7.2	0	RF	142.6	0	RF	125.6	7
15	SD	0.6	2	HB	79.7	0	SD	57.8	0	RF	113.3	0	RF	65.6	8

[a] Habitats were categorized as mangrove (MG), sand (SD), seagrass (SG), hard bottom (HB), or reef (RF).

yielding an equal or greater number of actinomycetes than a less dilute sample in that same series was high. For the purpose of this report, we have called this observation a nonquantitative dilution. There were 14 nonquantitative dilutions on M1 of 27 serial dilutions that yielded actinomycetes; for M2, 17 of 39 actinomycete-yielding serial dilutions were nonquantitative, and for M1 following heat treatment, 49 of 129 were nonquantitative.

Nonquantitative dilutions were not observed for unicellular bacteria or streptomycetes. They were only observed for the actinoplanetes, occurring on both media and for the heat-treated samples, and usually on plates with few total bacteria. For example, a sample diluted to 2×10^{-3} and plated would yield 20 colonies of unicellular bacteria and one actinoplanete. This same sample further diluted to 2×10^{-4} would yield two unicellular bacteria and four actinoplanetes. In many cases, the apparent inhibition of the actinoplanetes on the plates inoculated with the more concentrated samples was not caused by the overgrowth of unicellular bacteria, which were few in number and formed well-isolated, individual colonies.

Because of unexplained irregularities in the serially diluted actinoplanete colony counts and the low numbers of total actinomycetes observed, actinomycetes were counted only at the 2×10^{-2} dilution and were not quantified per unit sediment. Of the three isolation methods used, M1 following heat treatment produced a total of 185 actinomycete colonies, with an average of 2.2 colonies on 85 plates. Medium 2 produced a total of 69 actinomycete colonies, with an average of 2.5 colonies on 28 plates; and M1 yielded 35 colonies, with an average of 2.5 colonies on 14 plates.

TABLE 2. Range and mean (x) values for salinity, pH, and sediment organic content

Depth (m)	Salinity (‰)	x	pH	x	Organic content (%)	x
0–1	36–46	38.8	7.4–8.3	7.9	0.3–5.9	2.5
1–3	36–39	37.4	7.5–8.1	7.9	1.6–5.9	2.8
3–6	36–38	37.1	7.8–8.1	7.9	1.4–5.2	2.4
6–15	36–38	36.5	7.7–8.2	7.9	1.6–2.4	2.3
15–33	36–37	36.4	7.7–8.0	7.9	1.6–2.4	2.1

The effect of seawater on biomass production was determined for 32 of the streptomycete isolates and 31 of the actinoplanetes. Of the streptomycetes tested, biomass production in all but five of the isolates was reduced when streptomycetes were grown in deionized water (Fig. 3). The average decrease in biomass for the 32 isolates was 39%. The actinoplanetes (with the exception of isolate 394) grew poorly or not at all in deionized water (Fig. 4), producing on average 92% more biomass when grown in seawater. The dry weights recorded for the actinoplanetes grown in deionized water can, for the most part, be accounted for by the biomass of the initial inoculum.

DISCUSSION

The isolation of actinomycetes from marine sediments is well documented, yet it remains unclear what component of these bacteria represents an indigenous marine microflora. This question persists, in part, because there is little published information describing the distribution, growth, and ecological role of actinomycetes in marine habitats. In addition, because actinomycetes represent a small component of the total bacterial population in marine sediments (4, 5), their role in the marine environment is difficult to assess.

It has been proposed that most actinomycetes isolated from marine sources are of terrestrial origin and reside in the sea as spores or resting propagules (4, 5). Goodfellow and Haynes (4) support this proposition by showing that actinomycetes isolated from North Atlantic sediments, including streptomycetes, and *Micromonospora* and *Rhodococcus* species show no specific seawater requirements, growing equally well on media prepared with either distilled water or seawater. In addition, because actinomycetes are common soil bacteria, produce resistant spores, and are known to be salt tolerant (9, 12, 13, 18), it is likely that they are washed in large numbers from shore into the sea, where some portions remain viable. This concept is supported by the observations that actinomycetes are less common in marine sediments

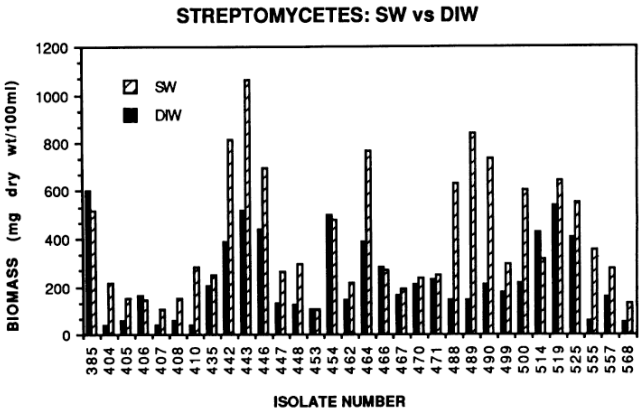

FIG. 3. Dry weight biomass for streptomycetes grown in a seawater-based medium (SW) and the same medium substituted with deionized water (DIW).

relative to terrestrial soils (5) and that they are encountered less frequently as distances from shore increase (19–21).

Contrary to these results, it has been shown by Weyland (22) that nocardioform actinomycetes isolated from marine sediments possess features of bacteria indigenous to the sea and that, with respect to seawater tolerance, *Rhodococcus* species behave like typical marine bacteria (23). We believe that various taxonomic groups of actinomycetes, when isolated from marine sources, differ in their degree of adaptation to the marine environment, and we conclude, based on the results presented here, that certain actinomycetes found in near-shore tropical marine sediments are well-adapted and functional members of the marine microbial community.

Nearly all of the actinomycetes observed in this study could be assigned to the suprageneric groups streptomycetes and actinoplanetes. The streptomycetes decreased in number with increasing distance from shore and were not observed from site 14, the area farthest removed from terrestrial influence. Although the streptomycetes were capable of growth in the absence of seawater, better growth was observed in media containing seawater. This is in agreement with the results of Weyland (22) for streptomycetes isolated from deep-sea sediments.

Based on the streptomycete distribution and their ability to grow in seawater, we speculate that these bacteria are mainly of terrestrial origin but under appropriate conditions

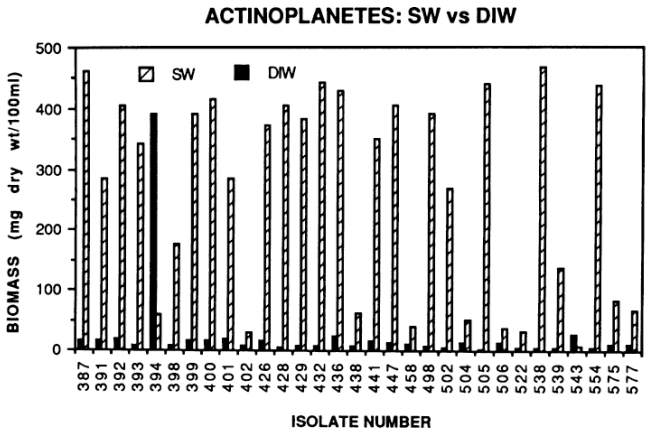

FIG. 4. Dry weight biomass for actinoplanetes grown in a seawater-based medium (SW) and the same medium substituted with deionized water (DIW).

can grow in the marine environment. This conclusion is supported by the finding that terrestrial streptomycetes can adapt to salt-supplemented media by stepwise exposure to increasing concentrations of sodium chloride (13). It is curious, however, that the streptomycete distribution was limited to a narrow range of sediments collected between 0 and 3 m. Although variable spore precipitation rates have been reported for actinomycetes (11), it is difficult to explain this distribution by the limits of dispersal alone. It is likely, considering that streptomycetes grow well in seawater, that other as yet unknown factors affect the occurrence of these actinomycetes in marine sediments.

The actinoplanetes were found in highest numbers in the deepest sediments sampled. This distribution is difficult to explain if these bacteria are of terrestrial origin. The actinoplanetes were most commonly observed from the heat-treated samples, and based on morphology, most of these appear to belong to the genus *Micromonospora*. This genus is more heat resistant than other actinomycetes (2, 17), and higher numbers of *Micromonospora* in deeper water are consistent with the results of Weyland (21) and Weyland and Helmke (23) for sediments collected between 0 to 200 and >2,000 m.

The finding that the actinoplanetes grow poorly or not at all in the absence of seawater suggests that these bacteria are adapted to the marine environment, an accomplishment that would be difficult to achieve without metabolic activity. This near obligate requirement of seawater for growth contradicts the results of Weyland (22), who reported that *Micromonospora* from deep-sea sediments grew better in the absence of seawater than in 100% seawater. Based on a comparison of these results, it appears that actinoplanetes isolated from near-shore tropical habitats are better adapted to grow in the marine environment than strains isolated from deep-sea sediments.

It should be emphasized that the actinomycetes reported in this study represent only a portion of the total population. Because all isolation methods are selective for certain groups of bacteria, and because of the criteria used for the recognition of actinomycete colonies, certain marine genera that produce fragmenting mycelia, e.g., *Rhodococcus marinonascens* (8), along with other nocardioform actinomycetes, may have been excluded. Certainly if members of the suprageneric group actinobacteria (3), e.g., *Micrococcus*, *Arthrobacter*, etc., were present in the marine environments sampled, they were not included in this study.

Another potential cause for the underestimation of actinomycetes is that spores may have been suspended during the sediment mixing process. If these spores did not settle within the 10 min allocated, they would have been discarded with the supernatant. Conversely, because samples were plated on two media prior to heat treatment and again on one medium following heat treatment, actinomycetes able to grow under all of these conditions would be better represented than those that could not. Although all of these factors may have influenced the results to some extent, it is emphasized that the numbers reported here are not an attempt to define the total actinomycete population but rather to show how the distributions of certain subgroups of this population change with increasing depth as observed by using consistent methodologies for all samples.

A number of problems regarding the enumeration of actinomycetes were encountered during the course of this study. The first such problem is caused by the relatively low numbers and slow growth of actinomycetes in relation to many common unicellular bacteria (2). Neither of the methods used for the isolation of actinomycetes in this study yielded sufficient numbers of colonies for the extrapolation of plate counts to CFU per unit sediment. Of the methods used, mild heat treatment of samples prior to plating was best for the growth of actinomycetes and was especially effective for the actinoplanetes, while having no obvious effect on the streptomycete population.

An additional problem was that the actinoplanete counts were not quantitatively correlated when serial dilution and plating techniques were used. In our experience, we commonly observe that actinomycetes appear to be inhibited from forming colonies on plates that are crowded with unicellular bacteria. However, the nonquantitative dilution as reported here often occurred on plates with few total bacteria and was not necessarily due to overgrowth of the agar surface by unicellular bacteria, but rather some other mechanism that remains to be determined.

The implications of the nonquantitative dilution can be considered in terms of the concept that actinomycetes represent only a small fraction of the bacterial population in marine sediments (5). In the most dilute samples, where the inverted dilution was often observed, the actinomycete numbers, although low, approached or in some cases exceeded the numbers of unicellular bacteria. From this, it can be speculated that the actinomycete population is greater than that predicted based on the use of standard colony counting methods and that their numbers have been underestimated because colony development is inhibited on plates used for the enumeration of unicellular bacteria. Evidence has not been presented to support this conclusion, and it remains possible that other factors are involved. For example, fragmentation of actinomycete hyphae during vortex mixing could result in multiple colonies arising from what would have been a single colony. This theory may have some validity as we only observed the nonquantitative dilution for the actinoplanetes, and it has been shown that *Micromonospora* strains are present in soils and lake mud as a mixture of spores and mycelia, while streptomycetes are largely spores (17).

It is clear that questions concerning the true numbers of actinomycetes in marine sediments and the best methods for their quantification remain unanswered. Based on the problems reported in this study and elsewhere in the literature, we caution the use of serial dilution and plating for the quantification of actinomycetes in marine sediments. However, if consistent methodologies are used, this technique can provide valuable information concerning relative actinomycete distributions.

We conclude that actinomycetes have taxon-specific distributions in near-shore tropical marine sediments. These distributions, and the extent that seawater is required for growth, cannot be explained by the generalized theory that actinomycetes isolated from marine sources are of terrestrial origin and physiologically inactive in the marine environment. The streptomycetes observed in this study were severely limited to shallow sediments and are probably of terrestrial origin. However, these bacteria grew better when seawater was present in the medium, and therefore it can be assumed that they are capable of growth under the appropriate conditions in the marine environment. Clearly, the factor limiting the distribution of streptomycetes is not their inability to grow in seawater. The actinoplanetes appeared in highest numbers in the deepest waters sampled and had a near obligate requirement of seawater for growth. These results lead us to believe that the actinoplanetes are physiologically adapted to the marine environment and metabol-

ically active members of the marine microbial community. Based on these conclusions, it appears that the role of actinomycetes in the marine environment is more complex than previously believed.

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Acknowledgements

The text of Appendix A, in full, is a reprint of the material as it appears in Jensen, P.R., Dwight, R., and Fenical, F. 1991. Distribution of actinomycetes in near-shore tropical marine sediments. Applied and Environmental Microbiology, 57, 1102-1108. The dissertation author was the lead author of this paper and directed and supervised the research, which forms the basis for this appendix.

APPENDIX B

Culture-dependent and culture-independent diversity within the obligate marine actinomycete genus *Salinispora.*

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Culture-Dependent and Culture-Independent Diversity within the Obligate Marine Actinomycete Genus *Salinispora*

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Salinispora **is the first obligate marine genus within the order *Actinomycetales* and a productive source of biologically active secondary metabolites. Despite a worldwide, tropical or subtropical distribution in marine sediments, only two *Salinispora* species have thus far been cultivated, suggesting limited species-level diversity. To further explore *Salinispora* diversity and distributions, the phylogenetic diversity of more than 350 strains isolated from sediments collected around the Bahamas was examined, including strains cultured using new enrichment methods. A culture-independent method, using a *Salinispora*-specific seminested PCR technique, was used to detect *Salinispora* from environmental DNA and estimate diversity. Overall, the 16S rRNA gene sequence diversity of cultured strains agreed well with that detected in the environmental clone libraries. Despite extensive effort, no new species level diversity was detected, and 97% of the 105 strains examined by restriction fragment length polymorphism belonged to one phylotype (*S. arenicola*). New intraspecific diversity was detected in the libraries, including an abundant new phylotype that has yet to be cultured, and a new depth record of 1,100 m was established for the genus. PCR-introduced error, primarily from *Taq* polymerase, significantly increased clone library sequence diversity and, if not masked from the analyses, would have led to an overestimation of total diversity. An environmental DNA extraction method specific for vegetative cells provided evidence for active actinomycete growth in marine sediments while indicating that a majority of sediment samples contained predominantly *Salinispora* spores at concentrations that could not be detected in environmental clone libraries. Challenges involved with the direct sequence-based detection of spore-forming microorganisms in environmental samples are discussed.**

Bacteria belonging to the order *Actinomycetales*, commonly referred to as actinomycetes, perform significant biogeochemical roles in terrestrial soils and are highly valued for their unparalleled ability to produce biologically active secondary metabolites. These bacteria account for ca. 45% of the bioactive microbial metabolites discovered (3) and have played a central role in the development of the modern pharmaceutical industry. The immense biotechnological utility of actinomycetes has led to exhaustive surveys of cultivars from terrestrial habitats and an associated increase in the numbers of known compounds being rediscovered due to a high rate of redundancy in the strains isolated. Given recent advances in our understanding of marine actinomycetes (7, 13, 24; see also a recent issue of Antonie van Leeuwenhoek, vol. 87, 2005, devoted to the subject), strains isolated from the marine environment represent a relatively unexplored frontier for the discovery of new actinomycete biodiversity and a resource for novel secondary metabolites.

Recently, we reported the discovery of the first obligate marine actinomycete genus for which the name "*Salinospora*" was originally proposed (19) and subsequently revised to *Salinispora* (17). This new taxon belongs to the family *Micromonosporaceae* and is the source of novel secondary metabolites including salinosporamide A, a potent anticancer agent specifically targeting the 20S subunit of the mammalian proteasome (10). The discovery of this taxon provides clear evidence for the existence of autochthonous populations of marine actinomycetes, and the compounds being discovered from them indicate that cultured strains are an important resource for novel secondary metabolites.

Cultivation-based surveys have shown that *Salinispora* spp. occur at abundances of up to 10^4 CFU/ml of sediment and can be isolated from worldwide locations, including the Caribbean Sea, the Sea of Cortez, the Red Sea, the tropical Atlantic Ocean off The Bahamas, the tropical Pacific Ocean off Guam (13, 19), and from a sponge collected from the Great Barrier reef in Australia (15). Despite significant effort, we have yet to cultivate *Salinispora* strains from temperate Pacific or Arctic sediments, suggesting that their distribution may be restricted to tropical and subtropical latitudes. Polyphasic taxonomic studies of isolates obtained from various locations indicate that the cultivated diversity to date is restricted to the two species *S. tropica* and *S. arenicola* (17), while a third phylotype cultured from Palau is being examined to determine its taxonomic status. This low level of species diversity, as well as the historical difficulties associated with the cultivation of marine bacteria, raises the possibility that the full extent of *Salinispora* species diversity has yet to be realized.

The present study was designed to further explore *Salinispora* distributions and species diversity in marine sediments using selective cultivation methods together with cultivation-independent techniques. Our results revealed limited species-level diversity in sediments collected around the Bahamas, the

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TABLE 1. Sediment samples and *Salinispora*-specific PCR amplification results

Sample[a]	Depth (m)	Location	Environmental DNA[b,c]	Enrichment culture DNA[b,d]
BA00-11	30	Little San Salvador	+	NT
BA00-12	22	Little San Salvador	−	NT
BA00-13	10	Little San Salvador	−	NT
BA00-14	1	Little San Salvador	−	NT
BA02-35	1,025	Little San Salvador	−	+
BA02-36	1,100	Little San Salvador	+	+
BA02-47	845	Long Island	−	+
BA02-50	7	Long Island	−	+
BA02-60	18	Stirrup Cay	−	+
BA03-03	20	Sweetings Cay	−	+
BA03-58	714	Little San Salvador	−	+
BA03-63	2	Little San Salvador	−	+
BA03-114	765	Grand Bahama	−	+

[a] All samples were collected from the Bahamas (BA) in the years 2000 (BA00), 2002 (BA02), or 2003 (BA03).

[b] In the nested PCR, all DNA samples yielded a primary amplification product using the primer set F270, RC1492. "+" and "−" symbols indicate secondary amplification results using the *Salinispora*-specific primer set F468, RC1492.

[c] Identical amplification results were obtained using both mechanical and chemical lysis methods for DNA extraction. Clone libraries were prepared from the two samples that yielded positive, nested PCR amplification products.

[d] Enrichment culture DNA was obtained using a chemical lysis method. NT, not tested. All enrichment cultures tested yielded a *Salinispora*-specific PCR product.

cultivation of *Salinispora* from a record depth (1,100 m), and evidence that these bacteria are actively growing in some sediment samples while existing predominantly as spores in others. Special challenges involving cultivation-independent studies of spore-forming microorganisms are discussed.

MATERIALS AND METHODS

Sediment collection and processing. During research expeditions in 2000, 2002, and 2003, a total of more than 200 marine sediment samples were collected around the islands of The Bahamas. Individual sediment samples were homogenized and portions used immediately for cultivation experiments with the remainder frozen at −20°C (−80°C upon return to the laboratory) for subsequent DNA extraction and enrichment cultivation. Sediment samples ranged from fine carbonate muds to coral rubble and were collected using SCUBA or a modified, surface deployed sampler (model #214WA110; Kahlsico, El Cajon, CA) to depths of 1,100 m. Sediment samples yielding 2×10^3 to 5×10^3 *Salinispora* CFU/ml, determined by using previously described methods (17), were considered for subsequent DNA extraction. From this sample pool, 13 sediment samples representing various geographical locations, depths, and sampling dates (Table 1) were chosen for additional cultivation-dependent and cultivation-independent experiments.

Selective actinomycete cultivation. All sediment samples were processed in the field as soon as possible after collection by using desiccation and heat shock as selective cultivation methods (19). These methods were designed to reduce the numbers of gram-negative bacteria and to enrich for slow-growing, spore-forming actinomycetes. Treated samples were then inoculated onto medium M1 (1% starch, 0.4% yeast extract, 0.2% peptone, natural seawater, and 2% agar) or M5 (natural seawater and 2% agar) and incubated for 4 to 8 weeks at room temperature. Novobiocin (10 μg/ml) or rifampin (5 μg/ml) was added to reduce the number of unicellular bacteria. The antifungal agent cycloheximide (100 μg/ml) was added to all isolation media. Actinomycete colonies were recognized by the presence of branching, vegetative filaments and the formation of tough, leathery colonies that adhered to the agar surface. Hence, only mycelium-forming bacteria belonging to the order *Actinomycetales* were included in the present study. Morphologically diverse actinomycetes possessing *Salinispora*-like features (19) were repeatedly transferred on solid media until pure cultures were obtained. All pure strains were grown in M1 broth and cryopreserved at −80°C in 10% glycerol.

In addition to direct plating, enrichment methods were used in an attempt to cultivate new *Salinispora* diversity. These methods were designed based on *Salinispora* antibiotic resistances and the ability of actinomycetes to degrade recalcitrant carbon sources. Enrichment cultures were also used for PCR-based experiments designed to distinguish between the occurrence of *Salinispora* as spores and vegetative filaments in marine sediments. Enrichment cultures were prepared in 20-ml vials by adding 1.5 g of wet sediment (homogenized and previously frozen) to either 10 ml of seawater enriched with crude chitin (0.1% [wt/vol]), 10 ml of sediment extract (SE) autoclaved supernatant from a 0.5% [wt/vol] sediment-seawater solution), or 10 ml of medium $M1_{low}$ (0.2% starch, 0.08% yeast extract, 0.04% peptone, seawater). Each of the three enrichment conditions was supplemented with either 5 μg of rifampin/ml or 25 μg of novobiocin/ml (final concentrations). Similar enrichments were prepared in the field with 0.5 g sediment and one of the following antibiotics (final concentrations): kanamycin (20 μg/ml), novobiocin (10 μg/ml), vancomycin (5 μg/ml), gentamicin (2 μg/ml), or tetracycline (4 μg/ml). All enrichment cultures were incubated for 4 to 15 weeks at room temperature and observed at ×10 to ×64 magnification using a stereomicroscope. Bacteria that formed visible mycelia were harvested directly from the enrichment cultures by using a sterile pipette, serially washed three times in 10 ml of sterile seawater, and plated on medium M5. Colonies that morphologically resembled the genus *Salinispora* (19) were repeatedly transferred onto new media until pure cultures were obtained.

Taxonomic and phylogenetic assignment to the genus *Salinispora* was verified by the requirement of seawater for growth, successful PCR amplification from a genomic DNA template using *Salinispora*-specific 16S rRNA gene primers (F468 and RC1492, Table 2), restriction fragment length polymorphism (RFLP) analysis of PCR products and, in some cases, sequence analysis. Almost complete 16S rRNA gene sequences from cultivated isolates were obtained by using the forward primers FC27, F514, and F1114 and the reverse primers R530, R936, and RC1492 (Table 2). All DNA sequencing samples were submitted to the UCSD Cancer Center DNA Sequencing Shared Resource (3100 Genetic Analyzer; PE/Applied Biosystems). Upper- and lower-strand sequence contigs were assembled in MacClade version 4.03 (Sinauer Associates, Inc., Sunderland, MA) and base calling ambiguities resolved by reviewing the sequencing chromatograms in Edit View (version 1.0.1; Applied Biosystems, Foster City, CA). 16S rRNA gene sequences were aligned by using the Ribosomal Database Project (RDPII) Phylip interface (6). Related sequences obtained from an NCBI BLAST (blastn) search were aligned by using MacClade. Phylogenetic analyses were performed by using distance and parsimony criteria implemented in PAUP version 4.0b10

TABLE 2. Oligonucleotide primers used in this study

Primer[a]	Sequence (5′-3′)	Specificity	Source or reference
FC27*	AGAGTTTGATCCTGGCTCAG	High G+C gram positive	18
RC1492*	TACGGCTACCTTGTTACGACTT	High G+C gram positive	18
F270*	ATGAGCCCGCGGCCTA	*Actinomycetales*	This study
F468*	AGCAGGGACGAAGCGTTT	*Salinispora*	This study
F514	GTGCCAGCAGCCGCGGTAA	*Actinomycetales*	12
F1114	GCAACGAGCGCAACCC	*Actinomycetales*	12
R530	CCGCGGCTGCTGGCACGTA	*Actinomycetales*	12
R936	GTGCGGGCCCCCGTCAATT	*Actinomycetales*	12

[a] All primers are named after their respective 16S rRNA gene priming site (*E. coli* numbering). *, HPLC purified.

(Sinauer Associates). Bootstrapping was accomplished with 1,000 replicates by using heuristic search methods. Secondary structures of *Salinispora* 16S rRNA gene sequences (Fig. 1) were assigned by using *Streptomyces coelicolor* as a reference (5). When portions of the primary sequence differed significantly from that of *S. coelicolor*, short sections (50 to 100 nucleotides [nt]) were analyzed for the best-predicted secondary structure by using the Mfold program (30) with default settings.

Environmental DNA extraction. Two lysis methods (mechanical and chemical) were used to obtain environmental DNA and differentiate between the occurrence of actinomycetes as spores and vegetative cells in a given sample. Mechanical lysis was performed as follows: marine sediment samples (0.5 g) were placed in bead-beating FastDNA spin kit tubes for soil DNA extraction (Q-biogene, Carlsbad, CA) and macerated by using a FastPrep 120 bead-mill according to the manufacturer's protocol except that two cycles, rather than one, at a setting of 5.5 were performed with cooling on ice between intervals. Triplicate crude environmental DNA preparations from each sample were pooled and purified by using Chroma Spin TE 100 columns (Becton Dickinson Biosciences, Palo Alto, CA.) according to the manufacturer's protocol. Overall, this combination of extraction and purification yielded environmental DNA of high purity and molecular weight (2 to >20 kb). Purified samples were concentrated to 1 ng/ml in TE buffer (50 mM Tris, 10 mM EDTA [pH 8.0]) using Microcon YM-100 filters (Amicon, Beverley, MA) and stored at −20°C. Control experiments were performed to ensure that the mechanical lysis method was capable of extracting DNA from both *S. tropica* and *S. arenicola* spore preparations (detailed below). Spore lysis efficiency was determined by phase-contrast microscopy (×1,250), and DNA quality was monitored by gel electrophoresis.

A chemical lysis method to extract environmental DNA from vegetative cells, using conditions determined not to lyse spores, was adapted from previous methods (8). Briefly, triplicate 0.5-g aliquots of homogenized wet sediment from either frozen environmental samples or enrichment cultures were processed separately in 2-ml polyethylene tubes. Sediments were suspended in TE buffer, 1 mg of lysozyme/ml, and 0.2 mg of RNase/ml (at final concentrations) and incubated for 1 h at 37°C. sodium dodecyl sulfate was added to a final concentration of 1% (wt/vol), and samples were incubated for 1 h at 65°C. To clear the lysate of detergent and debris, 200 μl of chloroform and 200 μl of saturated potassium acetate were added, and the tubes were vortexed vigorously for 1 min and centrifuged at 14,000 × *g*. The aqueous top layer was transferred to a clean tube, and crude nucleic acids were precipitated with 1 volume isopropanol and resuspended in 50 μl of TE buffer. Triplicate crude DNA samples were pooled and purified by using Chroma Spin columns and concentrated by using Microcon filters as detailed above.

To ensure that the chemical lysis method did not disrupt *Salinispora* spores, the following control experiments were performed. Spores were harvested by using a sterile swab from *Salinispora* strains grown on M1 plates for 4 to 8 weeks. The spores were suspended in TE buffer and syringe filtered through a sterile cotton plug to remove contaminating vegetative filaments. Purified spores were then concentrated by brief centrifugation, quantified by using a hemocytometer, adjusted to 10^6 per ml, and then processed by using the chemical lysis method. Using a Pico Green DNA detection method, it was determined that no significant spore DNA was liberated compared to an untreated spore preparation. Pico Green assays were performed by using the microliter plate protocol supplied by the manufacturer (Molecular Probes, Eugene, OR) and measured by using a Typhoon 8600 laser scanning system (Amersham Pharmacia Biotech, Piscataway, NJ). Similar control experiments performed using the mechanical lysis method were estimated to have lysis efficiencies of 50 to 70% and yielded good quality DNA from spore suspensions.

Seminested PCR and environmental library construction and analysis. A seminested PCR method was developed to specifically amplify *Salinispora* 16S rRNA gene sequences from environmental DNA samples. The primary amplification using the forward primer F270, biased toward members of the order *Actinomycetales*, and the high G+C reverse bacterial primer RC1492 (Table 2) yielded a 1,250-bp product. Reactions contained the following final concentrations: 1× PCR buffer (Perkin-Elmer, Wellesley, MA), 2.5 mM $MgCl_2$, 200 μM concentrations of each deoxynucleotide triphosphates, 0.05 U of AmpliTaq Gold (Perkin-Elmer)/μl, 0.1 μg of bovine serum albumin (Promega, Madison WI)/μl, 0.5 μM concentrations of each primer, and 100 ng of environmental DNA template per 25-μl reaction. Reaction mixtures were thermocycled as follows: there was an initial denaturation of 94°C for 10 min, followed by 23 cycles of denaturation at 94°C (15 s), annealing at 55°C (15 s), and extension at 72°C (1 min). Care was taken to assure that the primary PCR did not reach saturation by quantifying reaction products using the Pico Green assay. It was determined that 23 cycles yielded sufficient product for secondary amplification and was within the linear amplification range of the PCR. All primary reactions intended for library construction were performed in triplicate, and the products were combined, purified by using a QiaQuick PCR clean-up column, and quantified by the Pico Green method.

Using 10 ng of the primary amplification reaction as a template, a secondary amplification, yielding a 1,040-bp product, was carried out in triplicate with the *Salinispora*-specific forward primer F468 and reverse primer RC1492 as described above (Table 2). Reaction mixtures were thermocycled as follows: an initial denaturation of 94°C for 10 min was followed by 20 cycles of denaturation 94°C (15 s), annealing 65°C (15 s), and extension at 72°C (1 min). Again, to avoid saturation bias, products were quantified by using the Pico Green assay. Negative controls were performed by using 30 thermocycles to aid in the electrophoretic detection of possible contamination.

Clone libraries were constructed from PCR products by using the TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Individual clones were transferred to deep-well, 96-well microtiter plates (each well containing 0.5 ml of LB broth and 50 μg of kanamycin/ml) and grown overnight at 37°C with shaking. Individual plasmid inserts were amplified by direct PCR of clone cell cultures with standard M13 primers and protocols outlined in the Invitrogen TA cloning kit. Amplification products were analyzed by RFLP after digestion with the restriction endonuclease HaeIII (New England Biolabs, Beverly, MA) and analyzed by electrophoresis on a 3% agarose gel containing 0.25 μg of ethidium bromide/ml. Individual clones containing seminested PCR amplified inserts were sequenced by using quantified plasmid template DNA and the M13F and M13R primers by standard methods.

Seminested PCR control library. To determine *Taq* polymerase misincorporation rates or other errors introduced by the seminested amplification process, a control library was constructed from a PCR-amplified plasmid DNA template containing the insert of the almost complete 16S rRNA gene from *S. tropica* isolate CNB-440. The initial plasmid was constructed by using the PCR-amplified 16S rRNA gene (FC27 and RC1492 primers) from strain CNB-440 and the TA cloning kit. Plasmid DNA from a single clone was quantified and sequenced by standard methods. A total of 2 fg (ca. 500 copies) of this plasmid was then processed by using the seminested PCR amplification and library construction methods detailed above. Nine clones were chosen from this library, and the inserts were completely sequenced, aligned with the original clone sequence, and analyzed for errors.

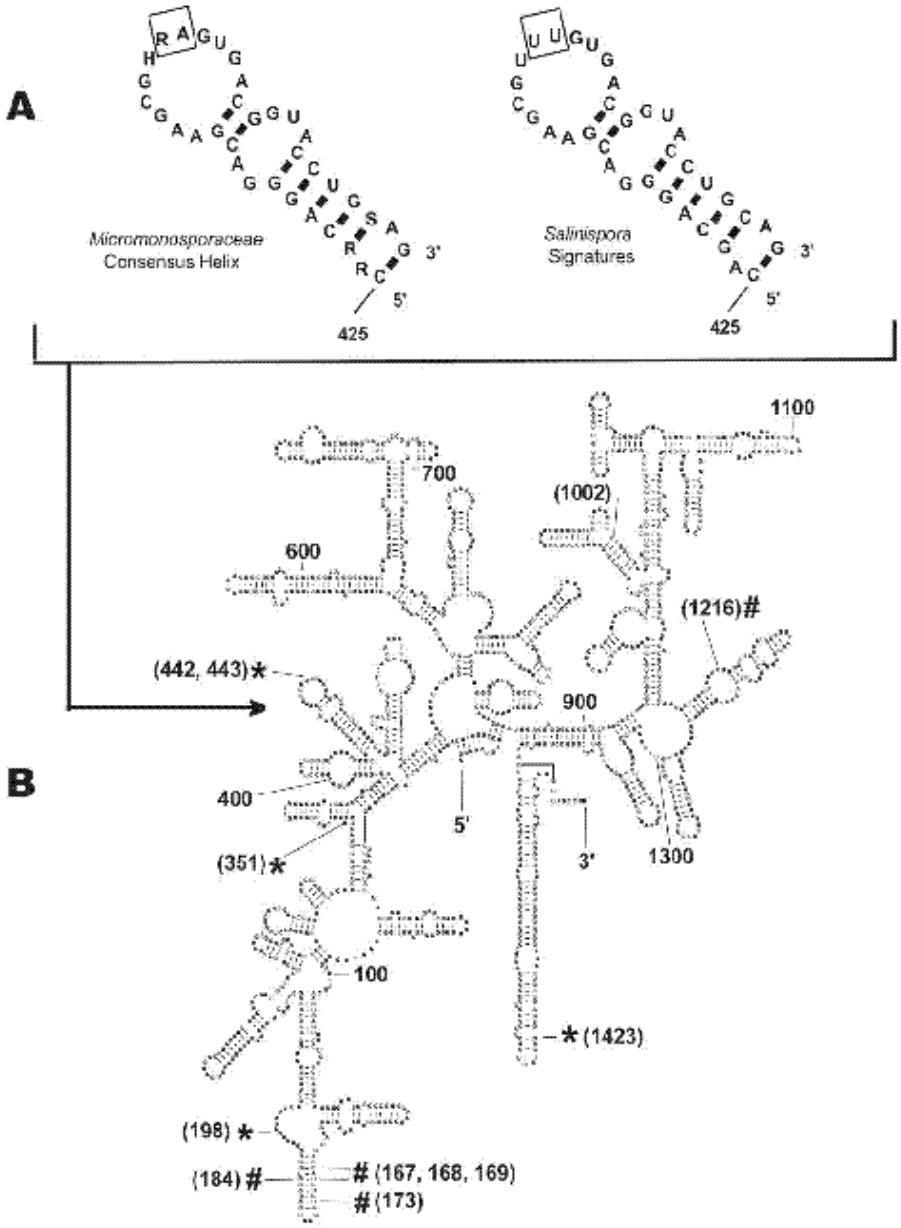
***Salinispora* RFLP analysis.** To rapidly assess *Salinispora* diversity, a selective amplification and RFLP screening method was used. Genomic DNA from cultivated isolates was amplified using the *Salinispora* diagnostic primer set F468, RC1492 as outlined above except that 35 cycles were performed. Amplification products were subsequently digested with the restriction endonuclease HaeIII and analyzed by electrophoresis as indicated above. For the purpose of direct detection and characterization of *Salinispora* in sediment enrichment cultures, template DNA was amplified by the seminested PCR method with 35 thermocycles in the secondary amplification in order to generate sufficient product for subsequent RFLP analysis.

Intragenomic 16S rRNA gene heterogeneity. The occurrence of 16S rRNA gene sequence heterogeneity within individual cultivated isolates was assessed by the construction and analysis of clone libraries from nine *Salinispora* strains. Almost complete 16S rRNA gene sequences (primer set FC27-RC1492) were amplified from each strain using PCR reagent concentrations as described above and 1 μg of genomic DNA template, an initial denaturation of 94°C (10 min), followed by 25 cycles of denaturation 94°C (30 s), annealing 55°C (30 s), and extension at 72°C (1 min). PCR products were cloned and 20 clones were screened from each library using HaeIII RFLP analysis. Select clones from each library were sequenced by using standard M13F and M13R primers, specific for the cloning vector, and primers F514 and R936, specific for the inserts (Table 2).

Chitin hydrolysis assays. The chitin hydrolysis assay medium consisted of 10 g of colloidal chitin prepared by the method of Makkar and Cross (16), 1 liter of natural seawater, and 20 g of agar. Actinomycete cultures were streaked onto colloidal chitin medium, incubated at 28°C for 2 to 4 weeks, and scored based on the clearing zone diameter: strong (>5 mm), moderate (2 to 5 mm), weak (<2 mm), or no detectable hydrolysis.

RESULTS

Cultured *Salinispora* diversity. A total of 366 *Salinispora* strains were cultured from marine sediments collected around the islands of The Bahamas during research expeditions in 2000, 2002, and 2003, including a sample collected at 1,100 m (HA02-36) that exceeded the previous depth record of 570 m


A
Micromonosporaceae
Consensus Helix
425
Salinispora
Signatures
425
B
1100
700
(1002)
600
(1216)#
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(351)*
100
*(1423)
(198) *
(184) #
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(173)

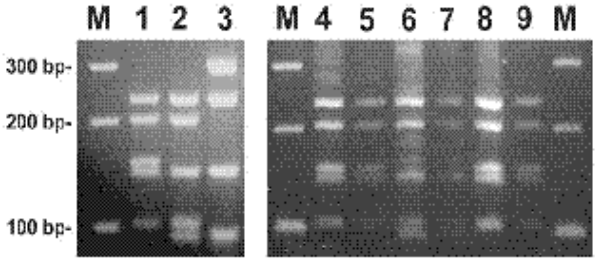

FIG. 2. Ethidium bromide-stained 3% agarose gel comparing HaeIII restriction endonuclease digests of *Salinispora* seminested PCR amplification products. Lanes: M, 100-bp marker; 1, *S. tropica* cultivated isolate; 2, *S. arenicola* cultivated isolate; 3, uncultivated phylotype III from sediment BA02-36 environmental library; 4, sample BA02-35 enrichment (sediment extract and rifampin) showing the *S. tropica* RFLP pattern; 5, sample BA02-35 enrichment (sediment extract and novobiocin) showing the *S. tropica* RFLP pattern; 6, sample BA02-36 enrichment (sediment extract and rifampin) showing the predominant *S. arenicola* RFLP pattern; 7, sample BA02-36 enrichment (sediment extract and novobiocin) showing the *S. arenicola* RFLP pattern; 8, sample BA03-05 enrichment (sediment extract and rifampin) showing the *S. tropica* RFLP pattern; 9, sample BA03-03 enrichment (sediment extract and novobiocin) showing the *S. tropica* RFLP pattern.

(13) for the recovery of this genus. Of these, 105 strains, representing all observed variations in pigmentation, colony size, shape, texture, and level of sporulation, were selected for further study, including strains obtained from various depths and habitats. Seventy-five of these strains originated from the 13 samples listed in Table 1, all of which yielded 2×10^3 to 5×10^3 *Salinispora* CFU/ml. The PCR-amplified 16S rRNA genes of all 105 strains were analyzed by RFLP using HaeIII restriction endonuclease digestion (Fig. 2), which proved to be an accurate method to delineate between *S. tropica* and *S. arenicola*. The RFLP patterns of 102 strains corresponded to *S. arenicola*, whereas only three strains, originating from samples BA02-35 and BA03-03, displayed the characteristic *S. tropica* phylotype. Phylogenetic analysis of the almost complete 16S rRNA gene sequences from nine of these isolates corroborated the RFLP findings that no new species-level diversity had been cultured from the Bahamas. The phylogenetic relationships of nine cultured strains, including strains CNS-143 (Palau), CNH-964 (Sea of Cortez), and CNR-425 (Guam), are shown in Fig. 3A and represent the extant cultured diversity of the genus to date relative to other genera in the *Micromonosporaceae*. Strain CNS-143 is currently being evaluated to determine whether it represents a new species.

Enrichment cultures frequently produced visible aggregates of filamentous bacteria that upon microscopic examination were recognized as actinomycetes by the presence of branching filaments. These actinomycete blooms could be harvested with a sterile pipette and, upon transfer to solid media, yielded an additional 60 actinomycete strains. Based on morphological and RFLP analyses, seven of these strains were identified as *S. tropica* and 31 were identified as *S. arenicola*. The remaining isolates were more closely related to *Micromonospora* or *Streptomyces* spp., including a new taxon within the *Streptomycetaceae* (subject of another study). These non-*Salinispora* actinomycetes were not observed using the original cultivation techniques (heat shock or drying methods), supporting the use of enrichment culture for the recovery of new actinomycete taxa.

The enrichment conditions that yielded *Salinispora* cultivars ($M1_{low}$ and SE) typically led to the formation of visible actinomycete blooms at the sediment-seawater interface, suggesting that these organisms may have a growth preference in the upper sediment layer. In contrast to many of the $M1_{low}$ and SE enrichments, only one chitin enrichment yielded *Salinispora* cultivars, whereas all others yielded actinomycetes that were more closely related to *Micromonospora* or *Streptomyces* spp. Of the 30 *Salinispora* cultures tested for chitin hydrolysis, only 5 displayed moderate to weak chitinolytic activity, indicating that the ability to hydrolyze chitin is not a consistent trait among *Salinispora* strains.

Culture-independent *Salinispora* diversity. In preliminary studies, analysis of over 100 clones from two libraries constructed from environmental DNA amplified using the general bacterial primer set FC27-RC1492 or the actinomycete primer set F270-RC1492 revealed no sequences affiliated with the genus *Salinispora*. Since *Salinispora* cultivars had been obtained from the sediment samples from which the DNA was extracted, a *Salinispora*-specific, seminested PCR method was developed to improve the resolution with which this group could be detected. Thirteen environmental DNA samples were processed by using the seminested PCR method (Table 1), and all yielded an initial PCR product with the actinomycete-biased

FIG. 1. Predicted secondary structures of *Salinispora* 16S rRNA. (A) Detail of the variable loop containing *Salinispora*-specific signature nucleotides (U, U) at positions 442 to 443 (467 to 468 by *E. coli* numbering) used to design the F468 primer. (B) Consensus secondary structure of *S. arenicola* strain CNH-721 (GenBank accession number AY878316) drawn using *Streptomyces coelicolor* as a reference (5). Helix loop structures in hypervariable regions were confirmed by using the Mfold program (29). Genus (*)- and species (#)-specific signatures are identified. Position 1002 is the site of the C→T transition observed in the uncultured *S. arenicola* phylotype III.

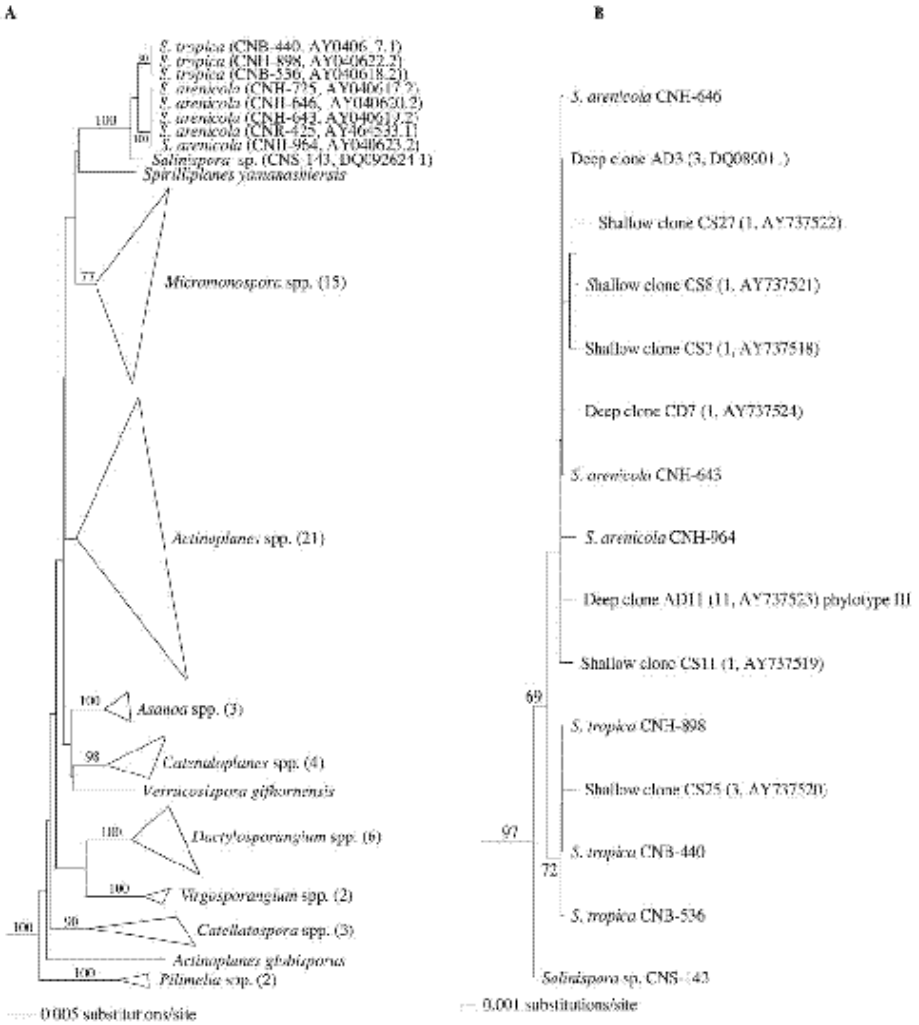

FIG. 3. (A) Neighbor-joining phylogenetic dendrogram constructed with nearly full 16S rRNA gene sequences (1,407 unambiguous nucleotides) illustrating cultured *Salinispora* species diversity including strains isolated from the Red Sea (CNH-727), the Sea of Cortez (CNH-964), Palau (CNS-143), and Guam (CNR-425) in comparison to all formally described genera within the *Micromonosporaceae* (List of Bacterial Names with Standing in Nomenclature [http://www.bacterio.cict.fr/]) for which sequence data was available from the NCBI Web site. Strain CNS-143 was cultured as part of a separate study and is currently being examined to determine whether it represents a third *Salinispora* species. Genera other than *Salinispora* that contain multiple species are represented with a triangle with the number of species included in parentheses. *Propionibacterium propionicus*, *Streptosporangium corrugatum*, and *Streptomyces lividans* were used as outgroups. In both panels A and B, bootstrap values were calculated from 1,000 resamplings and are shown at their respective nodes for values ≥60%. (B) Neighbor-joining phylogenetic dendrogram obtained after seminested PCR amplification of the 16S rRNA gene, using 997 unambiguous nucleotides, illustrating cultured and culture-independent *Salinispora* diversity. Clones were derived from deep (BA02-36) and shallow (BA00-11) samples with the number of identical clones and accession numbers noted in parentheses. Strain CNS-143 was isolated from Palau and may represent a new species (subject of a separate study). *Micromonospora olivasterospora* and *Ductylosporangium aurantiacum* (not shown) were used as outgroups.

primer set F270-RC1492 (Table 2). However, only two samples (BA00-11 and BA02-36) yielded secondary amplification products using the *Salinispora*-specific primer set F468-RC1492. Clone libraries were constructed from both of these PCR products (Table 1).

Forty-eight clones from the library created from the shallow (30 m) sediment (BA00-11) were analyzed by RFLP after HaeIII restriction endonuclease digestion. Five cutting patterns or phylotypes were observed with three occurring only once. These three rare phylotypes, upon careful sequence analysis and comparison with *Salinispora* and other members of the *Micromonosporaceae*, were found to contain bases that differed within highly conserved regions of the 16S rRNA gene, suggesting *Taq* polymerase misincorporation events were responsible. These nucleotide substitutions were masked from subsequent analyses. The remaining 45 clones displayed the typical cutting patterns of *S. tropica* (eight clones, six sequenced) and *S. arenicola* (37 clones, 14 sequenced). The clone sequences were aligned and representatives incorporated into a phylogenetic tree where they are denoted as "shallow clones" (Fig. 3B). As predicted, all of these clones fell into the *S. arenicola* or *S. tropica* phylotypes, differing by one to three nucleotides from previously cultured strains. In one case (CS25), three clones with the identical sequence were observed providing strong evidence that the single nucleotide substitution that distinguishes them from all cultured *S. tropica* represents new intraspecies diversity.

A second environmental library was created from the deep (1,100 m) sediment sample BA02-36 (Table 1). Fifty-four clones were analyzed by RFLP and seven phylotypes were observed of which four occurred only once. As was the case with the shallow library, the rare cutting patterns could be attributed to polymerase misincorporation events that disrupted or created HaeIII restriction sites. Two phylotypes constituted the majority of the remaining clones with five displaying the typical *S. arenicola* cutting pattern (four clones sequenced). These *S. arenicola* clones were either identical in sequence to previously cultured strains (i.e., deep clone AD3) or different by one nucleotide (e.g., CD7). Forty-three clones from the deep sample, however, displayed a unique cutting pattern designated phylotype III (Fig. 2, lane 3). Sequence analysis of 11 phylotype III clones showed one consistent nucleotide difference from cultivated *S. arenicola* (Fig. 1B). Although subtle, phylotype III represents additional new and as-yet-uncultivated, intraspecific *Salinispora* diversity. Despite these sequence differences, all of the clones examined fall within the previously described *S. arenicola* and *S. tropica* phylotypes (Fig. 3B). Two non-*Salinispora* clones observed from the deep library shared low (92%) identity (blastn) over a 937-bp region with an uncultured soil *Verrucomicrobium*.

PCR error analysis. A library constructed from a PCR-amplified plasmid DNA template was used to estimate the frequency of sequence error introduced by the seminested PCR method. Sequence analysis of nine clones from this control library displayed an overall observed error frequency (f) of 24 of 9,459 bases analyzed or 0.25% (i.e., two or three aberrant bases per clone sequence). This corresponds to a misincorporation frequency per template doubling (m) of 1.2×10^{-4} per bp per cycle, using the formula $2(fld) = m$, where d [43 cycles) is the theoretical doubling (23). These base substitutions appeared to be interspersed randomly throughout the amplified gene sequences and not localized in "hot spots." Base transitions G↔A or C↔T accounted for ca. 80% of the overall substitutions observed, a finding which agrees with the *Taq* polymerase transition substitution frequency determined by others (4, 14).

Detection of *Salinispora* spores and vegetative cells. All of the 13 sediment samples in Table 1 yielded 2×10^2 to 5×10^2 *Salinispora* CFU/ml. Using the chemical lysis method, only 2 of these 13 sediment samples yielded a secondary *Salinispora*-specific amplification product. Since this DNA extraction method does not lyse spores, it can be concluded that *Salinispora* were present as vegetative filaments in these two samples. It is also implied that the 11 samples that did not yield a secondary amplification product harbored predominantly *Salinispora* spores. To test this hypothesis, a mechanical lysis method capable of extracting DNA from spores was applied to all 13 samples; however, once again, only the same two samples yielded a secondary amplification product. Given that the CFU/ml for each sample were approximately the same, it appears that spore concentrations of 2×10^1 to 5×10^1/ml are below the detection limits of the mechanical lysis method. Of the 13 sediments, 9 were subjected to enrichment culture, after which all 9 yielded a secondary amplification product using DNA obtained by the chemical lysis method, indicating that spores had germinated in response to these culture conditions.

PCR and RFLP analysis of enrichment cultures. DNA extracted from the SE and $M1_{low}$ enrichment cultures all yielded a nested PCR product with typical *Salinispora* RFLP cutting patterns. Even in cases where growth was not evident by microscopic inspection of the cultures, secondary amplification products were obtained by using the chemical lysis method, indicating that *Salinispora* were actively growing under these conditions. Even though phylotype III was detected in the clone library generated from sample BA02-36, it was not observed in any cultured strains or detected in any of the enrichment cultures inoculated with sediment from this sample and thus members of this phylotype have yet to be cultured.

Typical RFLP results in response to different antibiotic treatments are illustrated in Fig. 2, lanes 4 to 9. Enrichment cultures from sediments BA02-35 (lanes 4 to 5) and BA03-03 (lanes 8 to 9) were the only samples that displayed a clear *S. tropica* RFLP pattern. These are the same two samples from which *S. tropica* was directly cultured. All of the remaining samples subjected to enrichment cultivation displayed the *S. arenicola* RFLP pattern (lanes 6 to 7), while phylotype III (lane 3) was not observed in any of the enrichment cultures. The presence of *S. arenicola* or *S. tropica* RFLP patterns appeared to be specific to the sample and not dependent on antibiotic treatment. The rare occurrence of the *S. tropica* phylotype in the enrichment cultures is consistent with what was detected by direct plating and from environmental clone libraries supporting the observation that this species is relatively uncommon in the samples analyzed.

Intragenomic 16S rRNA gene sequence heterogeneity. Intragenomic heterogeneity among copies of the 16S rRNA gene is well documented (2), has been shown to occur among members of the actinomycetes (29), and was suspected to be the source of the as-yet-uncultured *Salinispora* diversity observed in the two environmental clone libraries. To test this possibil-

ity, clone libraries were constructed from PCR-amplified 16S rRNA genes from six *S. arenicola* and three *S. tropica* cultures. RFLP analyses of 20 clones from each library did not detect the presence of intragenomic heterogeneity or any of the new phylotypes. Further sequencing of two to three clones from each of the nine *Salinispora* libraries displayed no fine-scale heterogeneity. Overall, this suggests that the observed diversity in the environmental libraries is not due to intragenomic allelic diversity of the 16S rRNA gene.

DISCUSSION

The genus *Salinispora* is a pan-tropical, marine actinomycete that has proven to be a productive source of biologically active secondary metabolites (10). Despite a widespread distribution in marine sediments, polyphasic taxonomic analyses of cultivated isolates have led thus far to the recognition of only two species (17). To further investigate *Salinispora* species diversity, new cultivation techniques, along with cultivation-independent methods, were tested to assess the extant diversity and depth distributions of this commercially important marine actinomycete taxon.

The initial cultivation methods used to isolate *Salinispora* strains relied upon the resistance of spores to heat shock and desiccation (19). Since these relatively harsh treatments may have contributed to the limited cultured diversity observed within the genus, new enrichment cultivation techniques and selective antibiotic treatments were tested. Despite the molecular characterization of 143 *Salinispora* strains cultured from sediment samples collected throughout the Bahamas, including 38 isolated from enrichment cultures, no new species level diversity was detected. There was, however, new but subtle intraspecific diversity, within both the *S. arenicola* and *S. tropica* clades, detected from the environmental libraries, including an abundant new phylotype (III) from a library created from a sample collected from 1,100 m. Despite extensive effort, strains possessing these sequence variations were not cultured.

Culture-independent molecular methods have led to a vastly improved understanding of microbial diversity in the world's oceans (9). It is interesting that in the present study both culture-dependent and culture-independent analyses revealed the same level of *Salinispora* species diversity. This is unusual since most culture-independent studies yield diversity estimates that are in striking contrast to what can be cultured from the same samples (26). Given that both methods detected the presence of only two *Salinispora* species in the sediments collected from the Bahamas (a potential third species has been cultured from Palau), the results emphasize that cultivation efforts can be highly successful when a specific taxon is targeted. This was recently demonstrated on a larger scale with the cultivation of members of the ubiquitous marine bacterioplankton clade SAR11 (21). Thus, it is becoming increasingly clear that when appropriate cultivation methods are applied marine bacteria are far more amenable to cultivation than previously believed.

The ability of *Salinispora* to form spores complicated the culture-independent detection of this taxon in the samples studied. PCR amplification of environmental DNA, extracted by using a chemical lysis method that does not extract DNA from spores, led to the detection of *Salinispora* in only 2 of 13 sediments. This result was obtained despite the occurrence of approximately equal numbers of *Salinispora* CFU/ml (2×10^3 to 5×10^3) in all of the samples. Based on these results, it is concluded that *Salinispora* were present as vegetative filaments in only two of the samples, providing additional evidence that actinomycetes are capable of growth in the marine environment (12, 20) and that actively growing actinomycetes may be uncommon and unevenly distributed relative to spores. Although it is not known what triggers spore germination in marine sediments, all sediment enrichment cultures extracted using the chemical lysis method (Table 1) yielded *Salinispora*-specific secondary amplification products, indicating that appropriate nutrient conditions are a factor and that, without prior enrichment, spore-forming actinomycetes may be overlooked in molecular analyses of marine bacterial diversity.

The mechanical lysis method was found to be effective at recovering DNA from spore preparations in control experiments; however, this DNA extraction method did not improve our ability to detect *Salinispora* in the sediments studied. Although at first surprising, it is proposed that spore abundances of 2×10^3 to 5×10^3 CFU/ml remain below the detection limits of this method, an observation that is in agreement with other workers who found that DNA extraction efficiency falls precipitously when spore abundances are $\leq 10^5$/ml of sediment (8). Similar, confounding results have been reported for actinomycetes in terrestrial soils where cultivation-based studies have shown *Streptomyces* spp. to be cosmopolitan and dominant, whereas PCR-based molecular analyses have underestimated or failed to detect this important group of microorganisms (18). These results again emphasize the limitations of molecular techniques when working with spore-forming bacteria that spend a majority of their life cycle in a lysis-resistant resting stage interspersed with sporadic but ecologically important periods of growth.

Nested PCR methods have proven effective for monitoring uncultured *Actinobacteria* in environmental samples (22) and, in the present study, highly specific for the amplification of *Salinispora* DNA. The signature nucleotides (U, U) at positions 442 to 443 (Fig. 1A, positions 467 to 468, *E. coli* numbering) were incorporated into the *Salinispora*-specific primer F468, which yielded *Salinispora* 16S rRNA gene sequences in all but 2 of 102 clones analyzed. The two aberrant clones showed closest homology to members of the *Verrucomicrobium*. It is likely that the *Actinomycetales* biased primer set F270-RC1492, which amplified unacceptable levels of *Verrucomicrobium* and *Acidimicrobium* 16S rRNA gene sequences in pilot studies, amplified a significant amount of *Verrucomicrobium* DNA in the primary amplification step of the nested PCR thus making cross-amplification events more likely in the secondary amplification. Although it is possible that new *Salinispora* species were missed in the present study, the signature nucleotides targeted by the F468 primer are a defining phylogenetic feature of the genus as currently defined (19) and have been observed in all *Salinispora* strains analyzed to date.

Although RFLP provided a rapid method to identify *Salinispora* phylotypes from clone libraries, careful inspection of sequence data revealed that, of the 10 phylotypes originally observed in the two clone libraries, 7 occurred only once and ultimately could be attributed to PCR error. This determination was made if a consensus for a variable position could not

be established in multiple clones and if the nucleotide change did not occur in a region of appropriate variability based upon aligned 16S rRNA gene sequence data and secondary structure. Suspect bases, predominantly transitions, that did not meet these criterion were masked from the phylogenetic analyses, resulting in an overall reduction in branch lengths and in the number of new phylotypes predicted by RFLP from eight to one. These changes did not affect the overall tree topology of Fig. 3B. The 11 phylotype III clones sequenced from the deep library all shared a C→T transition at position 1002 (Fig. 1B). Although this base change is a transition, it appears in an area of moderate to high variability among all bacteria (27) and represents the only new *Salinispora* phylotype that could be identified by RFLP from the present study.

Environmental clone libraries have recently been shown to overestimate diversity unless special precautions are taken to avoid PCR-induced artifacts (1). This appears to be of special concern when *Taq* polymerase is used (4, 14, 23). Given the suspect *Salinispora* diversity originally detected in the environmental clone libraries, a control library was constructed, using plasmid DNA template from a well-characterized strain (CNB-440), to assess misincorporation error in the seminested PCR protocol. The overall error frequency per base pair per template doubling (m) was calculated to be 1.2×10^{-4}, falling toward the high end of previously reported values for *Taq* DNA polymerase (4, 14, 23). Misincorporation rates for the shallow and deep libraries were estimated to be 1.5×10^{-4} and 1.7×10^{-4} per bp per cycle, respectively, again within previously determined values. A large number of transitions, as observed in the control library, is known to be a characteristic of *Taq* polymerase (4, 14, 23), further suggesting that misincorporation events were the major source of error in our environmental libraries. Since neither the primary nor secondary PCR amplifications reached saturation, heteroduplex formation, a known source of clone library error (25), is less likely to explain the error in this particular case.

The obligate marine distribution of the genus *Salinispora* could be a factor in the limited 16S rRNA gene sequence diversity detected. Compared to their terrestrial relatives *Micromonospora*, *Dactylosporangium*, and *Actinoplanes*, *Salinispora* spp. inhabit a less variable environment in terms of temperature, salinity, and hydration and thus possibly fewer selective forces driving speciation. Given that habitat heterogeneity has been posited as one of the major determinants of biological diversity (11), the relatively low level of *Salinispora* species diversity is what would be predicted from a stable marine environment or limited niche suitability. Although difficult to test experimentally, environmental stability may help explain the relatively low level of species diversity detected in this marine actinomycete genus (Fig. 3A). Another factor for lower 16S rRNA gene sequence diversity could be that *Salinispora* have experienced fewer lateral gene transfer events. Evidence exists that *Dactylosporangium* and *Micromonospora* spp. have undergone recent lateral transfers of short gene segments characterized by nonrandom variations outside of hypervariable regions of the 16S rRNA gene that can be identified by variable tree topologies for different regions of the 16S rRNA gene (28). These proposed transfer events can increase the 16S rRNA gene sequence diversity within a given group and are known to cause intragenomic heterogeneity. However, the tree topologies generated from partial and full 16S rRNA gene sequences were always maintained, and intragenomic heterogeneity was not observed in control clone libraries generated from nine cultured *Salinispora* strains.

S. tropica has proven to be an important source of bioactive secondary metabolites (10), yet this species is rarely cultured relative to *S. arenicola* with only ca. 20 strains isolated to date. *S. tropica* was detected by RFLP in only one of the two environmental clone libraries generated (sample BA00-11), where it represented 8 of 45 clones belonging to the *Salinispora* clade, and in only two of the samples subjected to enrichment culture (BA03-03 and BA02-35), thus corroborating the relative rarity of this species. Since the secondary metabolite profiles of these two species differ significantly (subject of another study), it would be interesting to determine whether the ability to produce various secondary metabolites affects fitness.

Although members of the marine actinomycete genus *Salinispora* were readily cultured at 2×10^3 to 5×10^3 CFU/ml from all 13 marine sediment samples analyzed in the present study, only 2 of these samples showed a positive result when a targeted, seminested PCR technique was applied. These results emphasize the extra effort that must be made when culture-independent techniques are used to assess the diversity of spore-forming marine bacteria, which undoubtedly remains widely underestimated, as well as the utility of selective cultivation techniques for bacteria that spend a large portion of their life cycle in a resting stage. Although some actinomycetes may occur in marine sediments primarily as spores and these may be distributed to great depths by ocean currents, evidence is presented for actinomycete growth in marine sediments even at the deepest sites sampled (1,100 m). Based on our current understanding of the genus, it is likely that the two approaches: enrichment cultivation and targeted, seminested PCR have revealed the majority of *Salinispora* species-level diversity within the sediment samples studied. Continued improvements in our ability to culture marine actinomycetes, in combination with sampling new habitats, are essential for a more complete assessment of their diversity and potential as a resource for biotechnology.

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The text of Appendix B, in full, is a reprint of the material as it appears in Mincer, T.J., Fenical, W., and Jensen, P.R. 2005. Cultture-dependent and culture-independent diversity within the obligate marine actinomycete genus *Salinispora*. Applied and Environmental Microbiology, 71, 7019-7028. The dissertation author was a co-author of this paper and directed and supervised the research, which forms the basis for this appendix.

APPENDIX C

Marine actinomycete diversity and natural product discovery.

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Marine actinomycete diversity and natural product discovery

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Abstract

Microbial natural products remain an important resource for drug discovery yet the microorganisms inhabiting the world's oceans have largely been overlooked in this regard. The recent discovery of novel secondary metabolites from taxonomically unique populations of marine actinomycetes suggests that these bacteria add an important new dimension to microbial natural product research. Continued efforts to characterize marine actinomycete diversity and how adaptations to the marine environment affect secondary metabolite production will create a better understanding of the potential utility of these bacteria as a source of useful products for biotechnology.

Introduction

Natural products, or derivatives there-of, remain the single most important source of new medicines (Newman et al. 2003 and references cited therein). Despite a recent de-emphasis on natural product research by the pharmaceutical industry, no other drug discovery platform has proven to be as effective at yielding unique chemical structures with either direct application in the treatment of disease or the capacity to serve as chemical scaffolds from which molecules with enhanced efficacy can be derived. There can be little doubt that myriad structural motifs remain undiscovered from natural sources and that these molecules will continue to be an important source of new medicines.

Among the potential sources of natural products, bacteria have proven to be a particularly prolific resource with a surprisingly small group of taxa accounting for the vast majority of compounds discovered. For example, of the 53 known bacterial phyla, only five are reported to produce anti-infective agents (Keller and Zengler 2004). And among these five, the Class *Actinobacteria*, and more specifically, bacteria belonging to the Order *Actinomycetales* (commonly called actinomycetes) account for approximately 7000 of the compounds reported in the Dictionary of Natural Products. Looking individually at the more than 140 currently described actinomycete genera, it becomes clear that even within this Order it is a few well-known soil genera that account for the vast majority of microbial natural products discovered. In fact, the genus *Streptomyces* alone accounts for a remarkable 80% of the actinomycete natural products reported to date, a biosynthetic capacity that remains without rival in the microbial world. Given that the *S. coelicolor* genome sequence revealed 18 biosynthetic clusters in addition to those specifying the biosynthesis of previously analyzed metabolites (Bently et al.

2002), the capacity of even this well-studied genus appears to be far from exhausted. The recent prediction that only about 10% of the natural products capable of being produced by *Streptomyces* spp. have been discovered (Watve et al. 2001) supports further studies of both new and traditional actinomycete taxa alike.

A logical extension of the search for new actinomycete natural products is the study of marine-derived strains. Although these strains appear to be a useful source of new molecules, with more than 100 compounds described to date (Blunt et al. 2004 and references cited therein), it was only recently demonstrated that some were in fact indigenous to the marine environment and not merely transient contaminants from shore. Given that large numbers of actinomycetes are undoubtedly washed from shore into the sea, distinguishing between those that have evolved in response to specific marine environmental challenges vs. those that are present as dormant spores must clearly be a priority if we are to understand how life in the marine environment affects secondary metabolism. Although few natural product studies have assessed the taxonomic novelty of marine-derived strains, those that have yielded exciting new chemistry (e.g. He et al. 2001; Feling et al. 2003) suggesting that targeting marine taxa represents a productive and rational approach to natural product discovery. This paper highlights some of our recent work with marine actinomycetes and the secondary metabolites they produce.

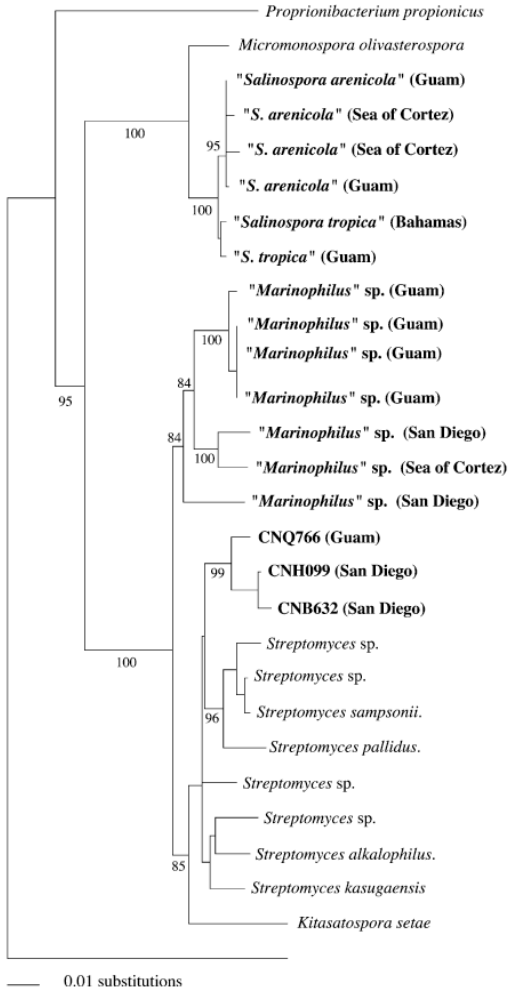

Figure 1. Neighbor-joining phylogenetic representation of cultured marine actinomycetes and their closest NCBI (BLASTn) relatives based on almost complete SSU rRNA gene sequences. Phylogenetically unique marine actinomycetes (in bold) include the new marine genera '*Salinospora*' and '*Marinophilus*' and a yet to be described taxon represented by strain numbers CNQ766. Bootstrap values (1000 re-samplings) are presented at the respective nodes and strain origin in parentheses.

Marine actinomycete diversity

An intriguing picture of the diversity of marine actinomycetes is beginning to emerge. Once largely considered to originate from dormant spores that washed in from land (Goodfellow and Haynes 1984), it is now clear that specific populations of marine adapted actinomycetes not only exist but add significant new diversity within a broad range of actinomycete taxa (e.g. Mincer et al. 2002; Stach et al. 2003). Figure 1 depicts the phylogenetic relationships of some of the marine actinomycetes we have cultured from marine sediments. These actinomycetes fall into two Families and represent multiple new genera. Despite the fact that the selective methods used to cultivate these actinomycetes targeted only mycelium-producing strains, and thereby omit important marine groups such as the mycolate actinomycetes (e.g. Colquhoun et al. 1998), it can be seen that marine actinomycetes include new

phylotypes that have clearly diverged from those known to occur on land.

Although the ecological roles of marine actinomycetes remain undefined, it is possible that, like their terrestrial counterparts, they are involved in the decomposition of recalcitrant organic materials such as chitin, a biopolymer that is particularly abundant in the sea. Given that actinomycetes living in the ocean experience a dramatically different set of environmental challenges compared to their terrestrial relatives, it is not surprising that speciation has occurred and unique marine taxa are now being recognized. It now remains to be determined not only the extent of marine actinomycete diversity but how adaptations to life in the sea have influenced the production of secondary metabolites.

'Salinospora'

In 1991, we reported the cultivation of an unusual group of seawater requiring actinomycetes isolated from marine sediments (Jensen et al. 1991). At the time, this was the first evidence that marine-derived actinomycetes could display typical marine bacterial adaptations such as a requirement of seawater for growth. Subsequent studies revealed that these strains represented a new actinomycete genus for which the name '*Salinospora*' was proposed (Mincer et al. 2002). '*Salinospora*' strains have been cultivated from marine sediments collected around the world including the Caribbean Sea, the Sea of Cortez, the Red Sea, and the tropical Pacific Ocean off Guam supporting a pantropical distribution. To date, no strains have been recovered from samples collected off San Diego or in the Bering Sea off the coast of Alaska suggesting latitudinal distribution barriers. Despite extensive cultivation efforts and the isolation of more than 2000 strains, only two species, '*S. tropica*' and '*S. arenicola*', have thus far been obtained in culture. A formal description of these new taxa is being prepared.

In an effort to determine if our cultivation efforts effectively recovered the extant species diversity within the genus '*Salinospora*', a culture independent study was undertaken. This study faced a number of challenges in that it proved difficult to detect '*Salinospora*' sequences in clone libraries generated from PCR amplified community DNA using general bacterial or even actinobacterial-specific primers. In response, a semi-nested PCR method was developed using an actinobacterial-specific amplification step followed by a second round of amplification using a genus-specific forward primer that incorporated signature nucleotides diagnostic for '*Salinospora*'. Clone libraries generated from the semi-nested PCR products displayed RFLP cutting patterns characteristic of the two cultured species '*S. tropica*' and '*S. arenicola*' and sequenced SSU rDNA inserts all fell within their phylogenetic clades. There was initial evidence for new '*Salinospora*' species diversity in the clone libraries however this diversity was ultimately attributed to PCR-induced nucleotide misincorporation. These results suggest that the cultivation methods employed succeeded in recovering the full extent of '*Salinospora*' species diversity in the sediments studied and that some species-level diversity detected using culture-independent techniques was due to PCR error. Furthermore, differential lysis techniques used to isolate environmental DNA indicate that '*Salinospora*' were present as actively growing mycelia in only two of 13 sediments tested. The successful isolation of cultured strains from all of these sediments suggests that in most samples '*Salinospora*' occur largely as spores. Interestingly, no intragenomic 16S rRNA gene heterogeneity, as has been demonstrated for other members of the *Micromonosporaceae* (Wang and Zhang 2000), could be detected in any of seven cultured strains examined.

Our initial chemical studies of '*Salinospora*' strains quickly led to the discovery of an unusual bicyclic β-lactone γ-lactam containing metabolite that we have called salinosporamide A (**1**, Figure 2, Feling et al. 2003). Salinosporamide A is an extremely potent inhibitor of the chymotrypsin-like proteolytic activity of the mammalian 20S proteasome (IC_{50} 1.3 nM), an important target in cancer chemotherapy. This compound also displays highly selective cytotoxicity in the National Cancer Institute 60-cell panel with a four-log range among the least and most sensitive cell lines (mean $IC_{50} < 10$ nM) and is currently advancing through pre-clinical development at Nereus Pharmaceuticals, a San Diego based biotechnology company.

Although salinosporamide A shares a core bicyclic ring system with the proteasome inhibitor

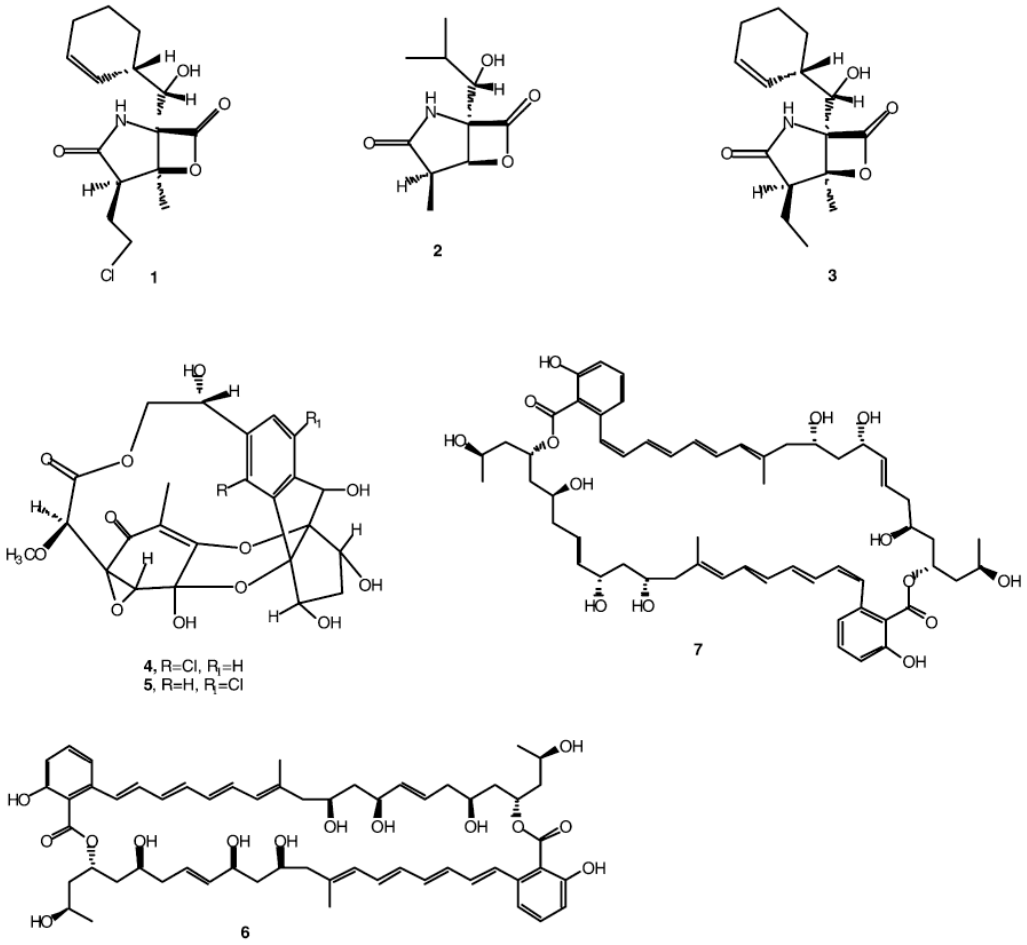

Figure 2. Structures of compounds isolated from marine actinomycetes. Salinosporamide A (**1**), omurolide (not marine, **2**), salinosporamide B (**3**), sporolides A and B (**4, 5**), marinomycins A and B (**6, 7**).

omuralide (**2**), it is 35 × more potent. Salinosporamide A is also uniquely functionalized at three positions including the carbon adjacent to the gamma-lactam carbonyl where a methyl has been replaced with an ethyl chloride. Halogenation is a common feature of many marine secondary metabolites and the extent to which it occurs may provide one indication of marine adaptation. The loss of chlorine from salinosporamide A, (salinosporamide B, **3**) results in a 500-fold reduction in biological activity indicating that the incorporation of this abundant seawater element is essential for the full biological activity of the molecule.

In additional to salinosporamides A and B, a second new series of compounds has also been discovered from '*Salinospora*' strains. These compounds, which we have called sporolides A and B (**4, 5**), appear to be formed from two independently produced polyketides and differ only in the position of the chlorine atom. Although this chemotype does not display any biological activity after limited testing, it exemplifies the level of structural novelty being isolated from the geuns '*Salinospora*'.

When considering which chemotypes are produced by which '*Salinospora*' species, an interesting pattern emerges. Although the data are still

preliminary, we have only observed salinosporamide and sporolide production from '*S. tropica*' while '*S. arenicola*' produces two known chemotypes (staurosporine and rifamycin) and what appears to be a third new chemotype that is still under investigation. So there appears to be a correlation between phylotype and chemotype in these sympatric species. There is also a biogeographical component to this story as thus far salinosporamide A has only been detected in '*S. tropica*' recovered from the Bahamas while the staurosporine and rifamycin chemotypes are present in '*S. arenicola*' regardless of location. More detailed studies of the biogeography, phylogeny, and secondary metabolite production by members of these two species are ongoing.

'Marinophilus'

Following the discovery of the genus '*Salinospora*', we began to examine other actinomycetes cultured from marine sediments to assess their level of taxonomic novelty. Using the requirement of seawater for growth as a guide and SSU rRNA gene sequences to assess phylogenetic relationships, we soon discovered another genus-level taxon that appears to reside exclusively in the sea. This taxon, for which the generic epithet '*Marinophilus*' is being proposed, exhibits significant intraclade diversity ($>5\%$) and appears to be comprised of at least three species (Figure 1). This level of species diversity is somewhat remarkable considering that, to date, we have only cultured seven strains that belong to this group. Although relatively few '*Marinophilus*' have been isolated, they add significant new diversity to the Family *Streptomycetaceae* which is currently comprised of only three formally approved genera. At this point, it is not clear if '*Marinophilus*' are rare in marine sediments or if the selective isolation methods used were not optimized for their recovery. Recent experiments suggest the latter to be correct and hopefully future cultivation efforts will lead to the recovery of large numbers of '*Marinophilus*' strains and a better understanding of their diversity and ability to produce novel secondary metabolites.

Our initial chemical studies of cultured '*Marinophilus*' quickly led to the discovery of a series of structurally unique antitumor-antibiotics that we have named marinomycins A and B (**6, 7**). The structure eluciation of these compounds proved difficult due to their symmetry and many stereo centers, however these issues have now been resolved. Although the marinomycins are polyene-like macrolides, they do not possess the antifungal activities typically associated with polyene antibiotics. They do, however, display potent cytotoxicity in the NCI 60-cell panel with mean GI_{50} values of 18.6 and 12.6 nM, respectively for compounds **6** and **7**. These compounds are currently being subjected to additional testing at the NCI. They also possess antibacterial activities with MIC values ranging from 0.125 to 0.625 μg/ml vs. vancomycin-resistant *Enterococcus facieum* and methicillin-resistant *Staphylococcus aureus*. Careful chemical analyses of all of the '*Marinophilus*' strains that we have thus far cultivated indicate that most produce polyene-like macrolides with no two strains producing the same molecules. Thus, as with '*Salinospora*', there appears to be a correlation between phylogeny and biosynthetic capacity, however more strains must be examined to better define this correlation and determine if '*Marinophilus*' species and chemotypes are regionally endemic or cosmopolitan. A formal taxonomic description of this group has the potential to add a new dimension to our understanding of the diversity of the biomedically important family *Streptomycetaceae*.

Discussion

It is now clear that major populations of marine actinomycetes reside in ocean sediments and that these bacteria display highly evolved marine adaptations including the requirement of seawater for growth. These findings will hopefully encourage additional studies addressing the ecological roles of actinomycetes in the marine environment, their diversity, distributions, culture requirements, and evolutionary responses to life in the sea. These aspects of marine actinomycete biology must become better understood before the potential of these bacteria to produce new secondary metabolites can be fully appreciated.

What we know from our experience with the two marine actinomycete genera that we have discussed in this paper is that they both produce

secondary metabolites that possess new carbon skeletons. In addition, in the case of the genus '*Salinospora*', there is a clear correlation between species and the class of compounds produced. Thus, these preliminary studies argue in support of the search for new marine taxa as a strategy for secondary metabolite discovery. If biosynthetic pathways are rapidly transferred horizontally among species however, the importance of taxonomy to actinomycete secondary metabolite discovery would be greatly diminished. In the case of the salinosporamides, this does not appear to be the case. However, this question needs to be addressed in more detail using additional species, more informative phylogenetic markers, careful chemotyping, and knowledge of the molecular basis for the biosynthetic pathways responsible for compound production.

Although many new secondary metabolites have been reported from marine-derived actinomycetes, we have little way of knowing if their production is a direct result of adaptation to life in the sea. Surely it is possible that new compounds could be discovered from marine-derived strains that existed in the ocean entirely as dormant spores and that these same strains and compounds would have been found if the original soils had been sampled. In the early days of our program, we paid little regard to taxonomic novelty or marine adaptations and as a result spent a great deal of time isolating molecules that had previously been reported from terrestrial strains. Now that we are focusing on marine taxa, the discovery rate has improved, as has the level of structural novelty of the compounds isolated, however the results from these efforts are still too few to draw any firm conclusions. Without knowing more about the evolution of secondary metabolic pathways, how they are transferred among diverse taxa, the ecological roles of secondary metabolites in the marine environment, and the correlations between taxonomic and biosynthetic novelty, we will not be able to draw any firm conclusions about how life in the sea affects microbial secondary metabolite production. At present, however, it appears that marine-adapted actinomycetes produce a relatively high rate of new secondary metabolites and that these bacteria do in fact represent a natural product resource worthy of thorough exploration.

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