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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.

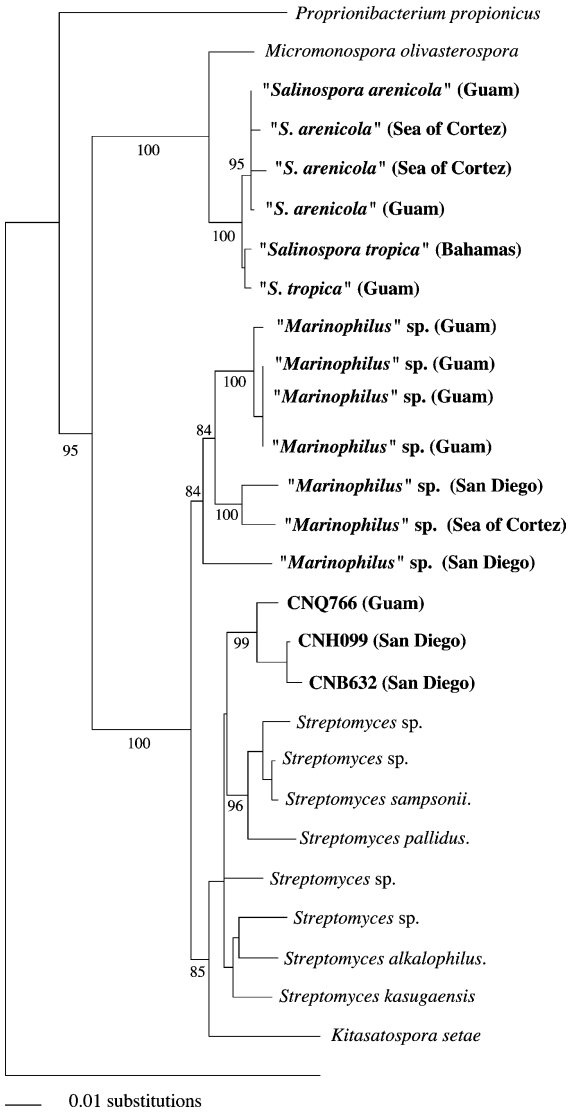

Figure 1. Neighbor-joining phylogenetic representation of cultured marine actinomycetes and their closest NCBI (BLASTn) relatives based on almost complete SSU rRNA gene sequences. Phylogenetically unique marine actinomycetes (in bold) include the new marine genera '*Salinospora*' and '*Marinophilus*' 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

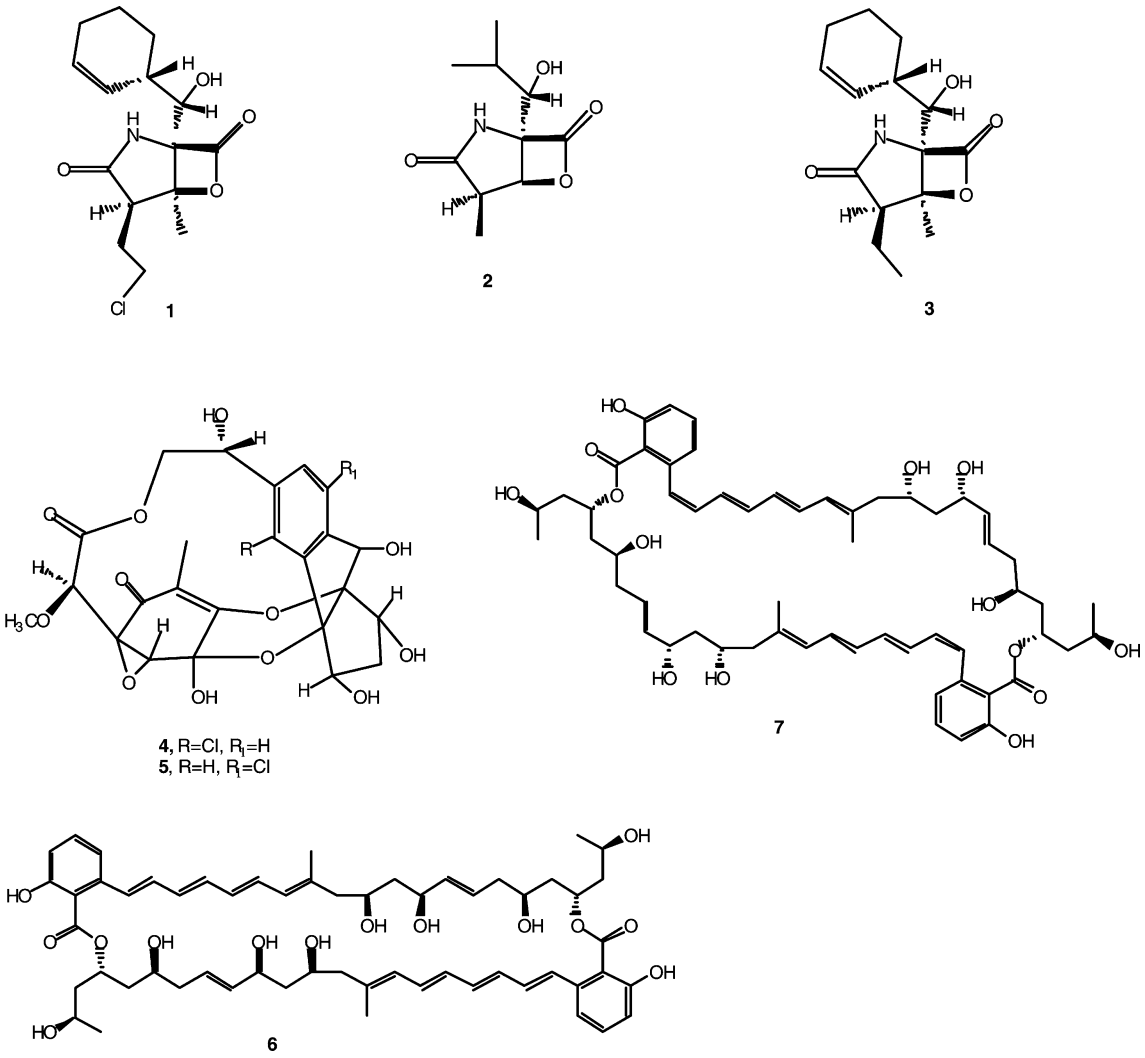

Figure 2. Structures of compounds isolated from marine actinomycetes. Salinosporamide A (**1**), omurolide (not marine, **2**), salinosporamide B (**3**), sporolides A and B (**4, 5**), marinomycins A and B (**6, 7**).

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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