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REVIEW

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New drugs from marine microbes: the tide is turning

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Abstract This is a mini-review demonstrating that investigation of the genomics of marine microbes from all three domains has the potential to revolutionize the search for secondary metabolites originally thought to be the product of marine invertebrates. The basis for the review was a symposium at the 2005 Annual Meeting of the SIM covering some aspects of the potential for marine microbes to be the primary producers of such metabolites. The work reported at that symposium has been integrated into a fuller discussion of current published literature on the subject with examples drawn from bacteria, cyanophytes and fungi.

Keywords Marine actinomycetes · Microbial metagenomics · *Prochloron* · Secondary metabolites

Microbial involvement in production of marine invertebrate secondary metabolites: introduction

Although the presence of microbes in marine invertebrates has been known for many years, over the last 25 or so years, there have been a significant number of comments in journals dealing with marine natural products, where investigators, initially marine natural products chemists, had remarked on the close similarity of compounds isolated predominately from sponges with those found in terrestrial organisms of entirely different taxa. Perhaps the widest initial divergence in taxa would have been the recognition by Perry et al. [26] of the similarities between the structures of mycalamides A & B from a *Mycale* sp. (subsequently identified as *M. hentscheli*) collected in Dunedin Harbor, South Island, New Zealand, and pederine, a toxin originally isolated from the *Paederus* beetle in South America (cf the recent work on microbial sources by Piel et al. [27]). Further work with this genus by other New Zealand-based groups [25] demonstrated that other cytotoxins were found in this population and that the composition was variable and site-specific. In addition to these there were many compounds from other marine invertebrates that had close similarities to antibiotics isolated from terrestrial actinomycetes, with a current example being ecteinascidin 743 from the tunicate *Ecteinascidia turbinata* and cyanocycline/saframycin derivatives from a variety of terrestrial actinomycetes [24].

Over the last 10 or so years, a very significant body of work has amply demonstrated that the marine environment is an almost untapped source of microbes that are in "commensal" relationships with invertebrate hosts covering probably all of the phyla in the sea and that the waters and sea bed are not the microbial deserts that was once assumed, but are populated with amazing numbers of microbes of all three domains, the bacteria, archaea and eukarya. Many representatives of these microbial groups have not yet yielded to cultivation (though an increasing number of specific groups are now being successfully cultured), but whose phylogeny can be interrogated by molecular approaches, including widely-used small subunit rRNA gene sequencing, and in certain cases, whose secondary metabolite-producing clusters can be expressed in surrogate hosts.

One of the first examples of the use of molecular techniques to study sponge microbial communities was published by Webster et al. [35] in 2001 demonstrating that a significant number of actinomycetes were present

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within the Great Barrier Reef sponge *Rhoploeides odorabile* even though culturable organisms were very low in number. Over the succeeding years, many groups, including Hill's, have continued with this type of work with demonstrations of close similarities of microbiota in sponges from distant geographic sites [11], identification of a specific sponge related candidate phylum, the "*Poribacteria*" [7], reports on variation in the microbial diversity of sponges both by direct molecular techniques using three candidate sponges from different taxonomic classes [10], and a molecular systematization of cultured microbial associates from deep water marine invertebrates by the Harbor Branch group [32].

Contemporaneously with these studies, the potential for isolation and culturing the microbes that were either free-living or associated with invertebrates was also reported by a significant number of investigators. From the aspect of culturing free-living microbes from the marine environment and expressing/identifying secondary metabolites, the work of Fenical et al. over the last 20 years has effectively led the field, leading to identification of both novel metabolites and new genera of actinomycetes (see later). In an analogous manner, the metagenomic analyses of both invertebrate-associated microbes as exemplified by the studies of Schirmer et al. [29] with the sponge *Discodermia dissoluta* from which the tubulin interactive agent discodermolide was first obtained (see later for other examples) and complete metagenomic analyses of seawater communities (see below) has shown the potential of the marine microbial world in all of its many facets.

The symposium at the 2005 SIM meeting

In August 2005, as part of the annual meeting, a symposium on these topics was organized by the authors and a select group of investigators were asked to present their current work, emphasizing the involvement and potential of the microbiota of the marine environment as sources of bioactive compounds. The papers presented covered free-living marine actinomycetes as sources of novel chemistry and microbiology, the identification of the actual microbial producers of a variety of coral-associated terpenoid compounds, a demonstration of the potential of marine metagenomic studies as routes to novel genes and the production of secondary metabolites from the smallest photosynthetic symbiont, a *Prochloron* sp. by transfer of the biosynthetic gene cluster into a surrogate host and subsequent expression leading to production of the encoded peptidic secondary metabolites.

Marine actinomycetes

The two presentations that involved actinomycetes were presented by Jensen et al. [12] and Fortman et al. [8]. Jensen discussed the work from the Scripps Institute of Oceanography group led by Fenical, emphasizing that if the time is spent in developing specific media and growth conditions, using information as to levels of carbon and other nutrients in the specific environment from which the samples are collected, then it is possible to culture significant numbers of previously unrecognized organisms. By utilizing these techniques, coupled to excellent chemical isolation and identification technologies, this group has revolutionized the field of Gram-positive marine-derived microbes over the last 20 years, with their reports on the methods that they have used to isolate, purify and then ferment organisms from the Actinomycetales. These fermentation methods, when coupled to the novel secondary metabolites that they have isolated and identified, predominately directed against cancer due to their source of funding, has amply demonstrated that these novel organisms are highly productive and produce chemical structures not previously reported from invertebrates.

It should be emphasized that prior to the work of this group, the prevailing attitude on actinomycetes isolated from marine samples, irrespective of the genera/species, was that "these are spore-formers and what you are isolating are wash-offs from terrestrial sources."

However, from this presentation and from work reported by Fenical, Jensen and co-workers [20] over the last 10 or so years, this is no longer a valid criticism as they have identified, published and had recognized, the novel, previously undescribed genus, *Salinispora*, a member of the Micromonosporaceae that requires seawater for growth (and production of secondary metabolites).

By use of 16S phylogenetic studies on the 2000 plus organisms that they isolated from tropical and subtropical regions, they have demonstrated three very closely related species, named *Salinispora tropica, Salinispora arenicola* and *"Salinispora pacifica"*, with each genus and species appearing to be a monophyletic clade. This assignment was confirmed by sequence studies of the *gyrB* protein and no additional clades were resolved. In addition to their presence in marine sediments, *Salinospora* species have been found in sponges (note that Kim et al. [13] used the older name in their paper).

From a secondary metabolite aspect, the *Salinispora* species have proven to be very interesting with the report of the novel proteasome inhibitor, Salinosporamide A by Feling et al. [6] obtained by fermentation of *S. tropica*. This compound is approaching Phase I clinical trials under the aegis of Nereus Pharmaceuticals, and the fermentation has been successfully scaled up to production levels in a seawater-based medium. It is also remarkable that instead of the old paradigm "secondary metabolites are strain-specific," what appears to occur, at least in the *Salinispora* species, is that in these closely related species, the phylotype can predict the chemotype. Thus from these three species, the following chemotypes appear to be species-specific.

Salinispora tropica produces salinosporamides and sporolides; *S. arenicola* produces staurosporines, rifamicins and salinoketals and "*S. pacifica*" produces cyanosporaside.

Of the newer metabolites, the most work has been reported on the salinosporamides, both from fermentations under slightly different conditions and from chemical syntheses, thus obtaining significant structure activity relationships and the original papers should be consulted for the structures and activity information [6, 18, 36].

From these phylogenetic analyses, they have been able to classify the marine sourced organisms into 15 "MAR" groups covering ten actinomycete families, with "MAR1" being the *Salinispora*. Of the remaining 14 groups, two others "MAR2" and "MAR4" may well be recognized as new genera within the *Streptomycetaceae* and similarly to the *Salinispora* they also produce very interesting and apparently phylotypic metabolites.

In contrast the Sherman group [8] used a modification of the old streptomycete isolation technique of cellulose discs applied to a medium and watching for the "grow out" of the streptomycetes on agar media. They used a seawater modification of the known ST21C× medium with elimination of the yeast extract [19], and were able to isolate an assemblage of actinomycetes from sediments collected in Papua New Guinea. From morphological considerations, three groups (I, II, III) were defined on the basis of microscopy and then followed up by 16S rRNA gene sequence determination, ribotyping and cell-associated fatty acid content.

Group I, (the PNG1 clade) though resembling the MAR1 or *Salinispora* clade reported by Jensen in that they cluster closely with these organisms within the *Micromonosporaceae*, appeared to be a distinct grouping, with their closest relative being *Micromonospora nigra*, originally isolated from a terrestrial salt pond. In Group II, the representative organism was coded UMM539, which clustered close to *M. carbonacea subsp. aurantiaca* and a representative from Group III, UMM518 clustered well outside of the major grouping (PNG clade 1) but still formally within the *Micromonosporaceae*. Further analyses using fatty acid profiles, respiratory quinones and cell wall sugars demonstrated that Groups I and III may well be representatives of new genera, and the authors suggested that *"Solwaraspora"* be the genus name for the PNG 1 clade (Group I) and that UMM518 (Group III) be given the genus name "*Lamerjespora*".

In contrast to the *Salinispora* neither of these groups has an obligate requirement for salt but will complete full development cycles, including sporulation, in submerged culture in media containing 3% NaCl, consistent with their ability to grow and divide in a saline environment. Further, growth in both rich and minimal media gave extracts that were biologically active against a range of resistant pathogenic microbes but no details were given as to chemotypes produced.

Production of secondary metabolites by Corals and Ascidians

Soft corals are well known "producers" of terpenes with both known and putative value as antiinflammatory agents, the best developed of which are probably the complex of sugar-substituted terpenes known as the pseudopterosins, which are elaborated by *Pseudopterogorgia* species, and whose use in Estee Lauder's Resilience™ was perhaps the first successful demonstration of the potential of direct marine-sourced compounds for "cosmeceutical" (pharmaceutical?) use, which was also coupled to the possibility of sustained harvesting directly from coral beds. Subsequently a simple derivative of pseudopterosin progressed to Phase II clinical trials as an anti-inflammatory but no further work has been reported. Terpenes from gorgonian corals show structural diversity and include additional compounds such as fuscols, eleutherobins and kallolides, with supply being a limiting factor in the commercialization of many of these compounds.

Work over the last few years by Kerr and colleagues at Florida Atlantic and the University of California, Santa Barbara, demonstrated that the pseudopterosins were produced by a *Symbiodinium* sp. by demonstrating conversion of ^{3}H-GGPP to the cyclized product, elizabethatriene and pseudopterosins, initially reported by Mydlarz et al. [22] in 2003. Continuing with similar experiments but using different soft corals, and looking at the metabolite levels in the whole organism, dinoflagellate and holobiont fractions, corals such as *Pseudopterogorgia bipinnata*, yielded cyclic terpenes such as the kallolides and bipinnatins in different levels in each type of extract. Similar results were demonstrable with *Eunicea fusca*, where terpenes such as fuscol, eunicol and the fucosides demonstrated anti-inflammatory activity in the mouse ear 4-β-phorbol-12-myristate 13 acetate (PMA) model and also were selective inhibitors of leukotriene production.

The results from the holobiont fractions led to an investigation [23] with *E. fusca* of the biosynthetic potential of the crude microbial preparation, using methods similar to those reported for the pseudopterosins, and from studies with ^{3}H-GGPP plus microbial extracts, labeled eunicol, fucoside A, eunicene A and fuscol were recovered. Using PCR, the DNA preparations from coral, *Symbiodinium* and bacteria were investigated using coral, bacteria and *Symbiodinium*-specific primers. Only in the bacterial case were amplicons observed for all three fractions. Thus attempts were made to ferment the mixed microbe population in the presence of labeled GGPP and though there was no eunicol production after 2 weeks, at 4–8 weeks, both fuscol and eunicol were present by GC-MS and HPLC, with production levels of

18 mg/L with structures being confirmed by GC-MS and NMR.

By use of conventional isolation techniques, the mixed culture was separated into approximately 600 cultures, which were then screened for the production of the target terpenoids. This led to the identification of a Gram-positive coccus that appeared to be pure by microscopic examination, and when clones from liquid cultures were sub-cultured, amplified and 16S rRNA gene sequences obtained, all were similar and gave 99.5% similarity to a Gram-positive bacterium. Since related diterpenes have been reported from both different gorgonian taxa and from similar taxa but in different geographic areas, it is possible that the actual producers in each case may be similar Gram-positive bacteria.

Finally, a simple but rather effective experiment was performed with the *Symbiodinium* cultures from *P. elizabethae* that produced the pseudopterosins. On addition of antibiotics to these cultures, although the dinoflagellates grew, no pseudopterosins were produced. Thus there is the possibility that these terpenoids as well are also produced by a microbial commensal. Work to confirm or deny this possibility is ongoing.

In contrast to the work reported above on coral-associated metabolites, in the ascidian *Lissoclinum patella* (and other didemnids), there is an easily recognizable photosynthetic symbiont present in large amounts. This is the cyanophyte, *Prochloron*, which is the smallest cyanophyte containing both chlorophyll A and chlorophyll B so far identified and is an obligate symbiont of chordates such as the ascidian mentioned above. To date, none of these symbionts have been cultivated, but particularly from *L. patella*, the microbe can be isolated by simple dissection of the host ascidian and gives a relatively pure (though not necessarily axenic) culture.

Lissoclinum patella and similar didemnids produce a series of closely related cyclic peptides, with usually 7 or 8 amino acid residues in the ring, some being heterocyclic and others being prenylated. Reports from different groups debated as to whether these peptides were produced by the ascidian or the symbiont and if they were secreted or intracellular [5, 28, 33], but no definitive data was available before late last year / early this year.

Schmidt [30] reported that as part of a program to sequence *Prochloron didemni*, the ascidian *L. patella* was collected in Palau in 2002, processed to obtain the commensal cyanophyte and the DNA extracted while still in Palau. Following successful sequencing, the group was able to demonstrate the "*pat*" cluster that produced the known ascidian metabolites, the patellamides and reported the original results in early 2005 [31].

What was unusual about this cluster was that rather than the peptides being produced by non-ribosomal protein synthetases, they were initially produced by conventional ribosomal interactions followed by formation of the heterocyclic aminoacids via oxazoline / thiazoline precursors to give thiazole or oxazole containing aminoacids, followed by cyclization involving a protease-mediated amide formation, with the major genes involved in these processes being *patA* and *patG*. Although there are other examples of ribosomal cyclic peptides in the literature, with the best examples being the "cyclotides" from plants which are not further modified, microcin J25 with a "lassoed tail" (which might be the closest homologue) and microcin B17, which though containing thiazole aminoacids, is not cyclized [3, 15, 37, 38], this was an unexpected finding, and further work demonstrated the absence of putative ascidian genes in the genome, confirming that the symbiont was the source of these cyclic peptides.

The *pat* cluster was subsequently cloned into *E. coli* and expressed detectable levels of patellamide A. A similar cluster was expressed, also in *E. coli* by the use of shotgun cloning techniques by Long et al. [17] from an Australian *Lissoclinum* sp. collected on the Great Barrier Reef, reported originally at the November 2004 SIM meeting in San Diego and then published in detail in 2005 demonstrating production of patellamides.

Using information from the *Prochloron* work, Schmidt's group has also begun to explore the genome of *Trichodesmium erythraeum* and have identified a pathway similar to *pat* but to date, no chemistry has been reported from this organism. However, by using the predictive techniques pioneered by Challis et al. [2], a putative structure of a cyclic peptide named "trichamide" was proposed and from a MALDI-TOF experiment, an ion of the correct mass {1,099 for M + H} was identified and structural elucidation is underway.

In order to further study the pathway evolution of these synthetic clusters, Schmidt reported that work is ongoing using a number of samples of the didemnid ascidian, *Didemnum molle*, collected from a range of sites around Papua New Guinea comparing them with materials collected in Palau. Some of the ascidians are known to produce peptides, and some do not, and currently there does not appear to be a 1:1 relationship between the presence of peptides and the presence of *Prochloron* or other cyanophytes by 16S rDNA analysis, thus no generalizations can be applied, as evidence of microbial involvement has to be obtained before the actual source(s) of these peptides can be determined in each organism.

The marine metagenome

Although over 200 genome projects are either completed or are currently underway following the publication of the first whole genome in 1995, there are some significant questions that have arisen as to what is actually being sequenced, as these studies are limited to culturable microbes. Metagenomics approaches, where one can now investigate whole community genomics and/or particular environmental genomics, can yield new insights and has the following potential advantages. The

technique is culture independent, it is hypothesis independent, and is also an hypothesis generator and finally, the data supplies a resource (data mining) which may be usable by a large scientific community.

Heidelberg [9] demonstrated the power of "data mining" by reporting on a variety of studies. The first was use of the datasets generated from metagenomic studies with uncultured Monterey Bay microbes. Here he reported that carbon monoxide (CO) is known to be produced a greater rate than the flux to the atmosphere, hence some process in the ocean is utilizing CO and though CO-dehydrogenase (CODH) is a potential pathway for chemoautotrophic growth in marine microbes, it is inefficient, and the amount of CO required by cultured microbes is tenfold greater than in the surface water. From data-mining, a CODH gene was identified in the genome of a marine heterotroph, which though perhaps not suitable for carbon fixation, may well be linked to ATP production, thus giving a method of producing energy.

Secondly, by combining data on the prevalence of proteorhodopsin genes in the SAR86 group (γ-proteobacteria) and comparing these with data from many other sources, it was found that proteorhodopsin genes are widely distributed amongst divergent marine bacterial taxa [4] and when the Sargasso Sea metagenomic data [34] and the initial data from the early collections in the current global survey by J.C. Venter (a repeat of the original Challenger expedition) were used (data unpublished), then the number and variety of taxa containing these genes increased significantly. Since these data sets only covered the Sargasso Sea and the initial sites through to the Galapagos Islands, the potential for future discoveries related to these and other genes is significant.

Finally, the potential of comparative metagenomics was briefly mentioned. In this technique, comparisons of environmental Bacterial Artificial Chromosome (eBAC) data are made in a manner analogous to the techniques used to demonstrate the differences between pathogenic and non-pathogenic bacteria of the same taxa. By using this technique and comparing the percent nucleic acid identity between two eBACs from aerobic, anoxygenic phototrophic (AAP) bacteria, the similarities and differences in recognizable genes may be compared. Such comparisons may well lead to a better understanding of the roles of these organisms in their particular environment, but require significant computational power and access to the genomic information.

Commentary

From inspection of both the published literature referred to above and the presentations given at the 2005 SIM Meeting [8, 9, 12, 23, 30], it is now quite obvious that the microbiota of the marine environment have a vast and almost untapped potential for the discovery of basic microbial information of all types, and as sources of secondary metabolites of utility to man in manifold diseases.

In addition to the examples given in the text, there have been some very recent publications, which should be consulted by the interested reader, that show some examples of the compounds that have been ascribed to marine invertebrates and their associated microbial flora [14] and in particular, work analogous to that published by Schmidt [31] and Long [17] but using the thiocoraline gene cluster from two invertebrate-derived actinomycetes, *Micromonospora* sp. ML1 (originally isolated from a mollusk) and *Micromonospora* sp. ACM2-092 (from a soft coral) and expressing these genes in both *S. lividans* and *S. albus* [16].

In closing, although little has been reported on the genetic composition of marine-derived fungi associated with marine invertebrates, a very recent paper [1] describing a fascinating control system for secondary metabolite production in terrestrial *Aspergillus nidulans*, implies that an initial foray into marine-sourced *Aspergillii* may well provide some surprises in secondary metabolism, plus application of the secondary metabolite search techniques described by McAlpine et al. [21]with *Streptomyces aizunensis* to marine actinomycetes will surely demonstrate that the search for bioactive metabolites from marine microbes has only just begun.

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