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Bioactive marine and terrestrial polyketide and peptide secondary metabolites and perspectives of their biotechnological production

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Abstract

Marine invertebrates and microorganisms are the sources of novel, bioactive secondary metabolites. Structurally, many of these compounds appear to be biosynthesized by polyketide synthase and nonribosomal peptide synthase, that have been found in terrestrial microbes and marine organisms. This review describes the scientific advances during the last 10 years in the emerging field of the molecular genetics of polyketide and nonribosomal peptide natural products isolated from marine organisms.

Keywords: marine microorganisms, marine gene cluster, polyketide synthase (PKS), nonribosomal peptide synthase (NRPS)

Introduction

Advances in biotechnology have resulted in high throughput sequencing of DNA, which in turn has revolutionized the potential of examining the genetic complexity of organisms and has led to the creation of an enormous database of both prokaryotic and eukaryotic DNA sequences. The convergence of advances in technology and biology has triggered an explosion in the field of bioinformatics, commonly referred to as the „genomic revolution“. Post-genomic research areas such as transcriptomics and proteomics have further allowed researchers to decipher the complex nature of gene expression and regulation. The “genomics revolution“ has revitalized the search for novel drug molecules from microbial sources. Sequence analyses of several bacterial genomes have revealed, that there is often discrepancy

between the number of peptide/polyketide secondary metabolite gene clusters harboured in the genome of an organism and the number of natural products that can be isolated, suggesting that genes encoding natural product biosynthesis are tightly regulated, rather than constituitively expressed. Analysis of the genomes of industrially important organisms like *Streptomyces coelicolor* A3(2) and *Streptomyces avermitilis* have revealed the presence of several such gene clusters (Bentley et al. 2002; Omura et al. 2001).

For many years it was believed, that only a very small percentage of microbes unique to the marine environment were culturable and it was not until a variety of marine-specific techniques were employed, that a much greater number of diverse microbes could be isolated from seawater, marine sediments and invertebrates (Morris et al. 2002, Mincer et al. 2002). These efforts have provided an increasing number of unique bioactive natural products from marine microorganisms which are particularly attractive due to the availability of cultured source organisms (Blunt et al. 2003). Many marine microbial metabolites possess both potent bioactivity and interesting polyketide and/or peptide structures and are therefore attractive targets for molecular genetic studies. The complete gene clusters (PKS and NRPS) that have been reported from marine organisms were isolated from cultured actinomycetes and cyanobacteria, two microbial groups that are especially well known for their ability to produce diverse natural products. All of the clusters were identified from cosmid libraries by developing probes designed from conserved regions of biosynthetic domains from terrestrial microbial pathways. Although the quantity of published marine gene clusters is relatively small, we expect to appear more examples in the near future. The above mentioned marine bacterial gene clusters demonstrate, that while PKS and NRPS biosynthetic routes are conserved between terrestrial and marine systems, in some cases there are additional, novel catalytic enzymes responsible for the unique functional groups found solely in marine natural products (Moffitt and Neilan 2003, Davidson et al. 2001).

Polyketide biosynthesis

One unifying theme in the diverse polyketide family of metabolites is their biosynthesis through the sequential condensation of small carboxylic acids, reminiscent of the synthesis of fatty acids in bacteria, lower and higher eukaryotes (Hopwood and Sherman 1990). There are three major classes of PKS systems, arranged by their mode of synthesis and structural type of product. Type I PKSs in bacteria are multienzyme complexes that are organized into

individual, linear modules, each of which is responsible for a single, specific chain elongation process and post condensation modification of the resulting β-carbonyl. The fungal type I PKSs consist of a single, giant protein that iteratively uses the same set of domains in a module for building the polyketide. Type II, or aromatic PKSs are complexes of monofunctional proteins characterized by their iterative use of a single set of distinct enzymes to construct polyketide chains which are then cyclised to produce small molecules containing aromatic ring systems. Recently, a third type of polyketide synthase was identified from bacteria belonging to the chalcone and stilbene synthase family of enzymes from plants. These enzyme systems, unlike the type I and II systems contain one protein, one domain and one active site to carry out the three central reactions of chain initiation, elongation and cyclization (Austin and Noel 2003).

The actions of the essential "core" domains present in types I and II PKS systems are presented schematically in Fig. 1. The initiation module begins with the actions of the acyl transferase domain (AT) selection of an activated acyl-CoA monomer (usually malonyl or methyl malonyl CoA). Following this selection step, the AT domain transfers the acyl-CoA to the adjacent "swinging arm" of the post-translationally (phosphopantethiene) modified acyl carrier protein (ACP) (Schwarzer et al. 2003). The chain is transferred from the ACP to the upstream ketosynthase domain (KS) required for catalyzing the decarboxylation of the carboxylic acid and subsequent Claisen condensation between the growing chain (or starter unit) and the downstream ACP tethered extender unit. In type I PKSs, structural diversity is introduced partly by optional reductive domains acting upon the newly formed β-carbonyl after each extension reaction. The "reductive loop" can consist of one or more of the following activities: ketoreductase (KR) reduces the carbonyl to a hydroxyl group, a dehydratase (DH) dehydrates the alcohol to form a double bond and enoylreductase (ER) is responsible for the reduction of the double bond to a fully saturated methylene. The incorporation of unusual starter molecules (amino acids, fatty acid derivatives, *etc.*) and extension units also plays an important part in generating immense diversity of polyketide structures (Moore and Hertweck 2002). Additional "auxiliary" domains consist of methyl transferases (MT) or other embedded activities. The complete elongated and functionalized chain is often transferred to a final thioesterase domain (TE) capable of catalyzing the hydrolytic release of a linear compound or a hydrolytic release coupled with cyclisation to generate a macrolactone structure (Rix et al. 2002).

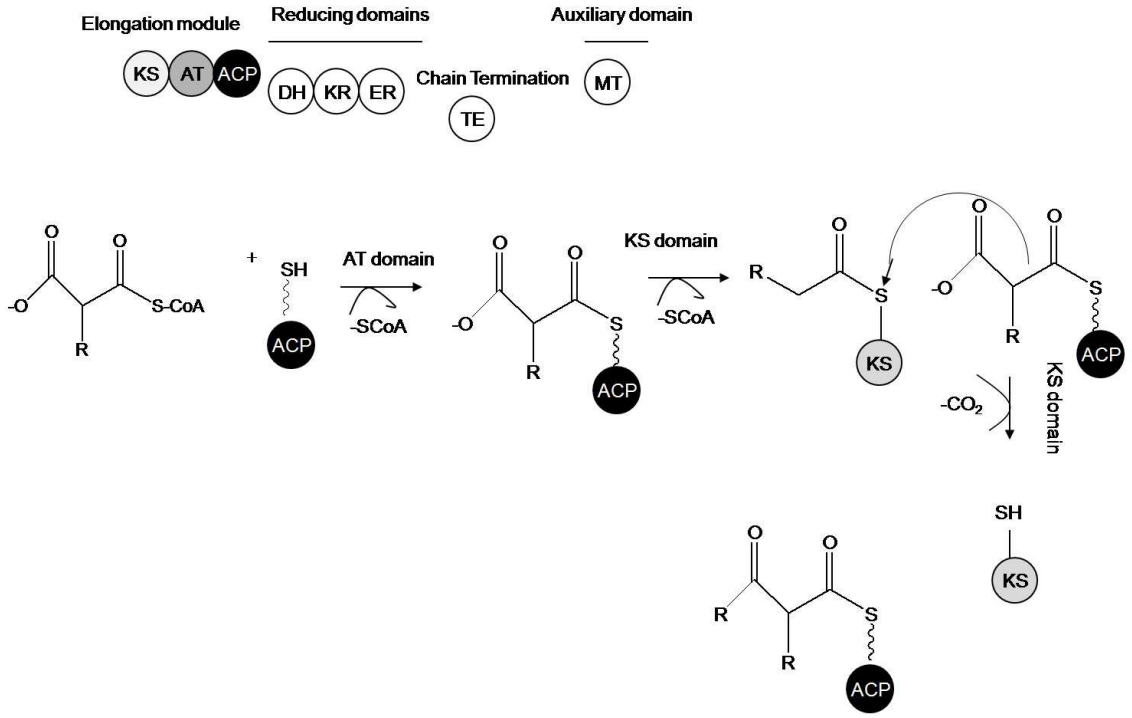


Fig. 1 Organization and mechanisms of modular biosynthetic pathway systems in polyketide biosynthesis (PKS). KS: ketosynthase, AT: acyltransferase, ACP: acyl carrier protein, DH: dehydratase, KR: ketoreductase, ER: enoylreductase, TE: thioesterase, MT: methyltransferase. Schwarzer *et al.* 2003.

Nonribosomal peptide biosynthesis

Nonribosomally produced peptide metabolites display a remarkable spectrum of activities and are extremely important as pharmaceuticals. Examples of some of the most clinically useful peptides are the antibiotics vancomycin and penicillin, the immunosuppressive agent cyclosporine and the antitumour compound bleomycin. Compounds synthesized by NRPSs can be distinguished by the presence of non-proteinogenic branched D-amino acids, which are often cyclic in structure (Mootz et al. 2002, Walsh et al. 2001).

Just like the type I PKS systems, NRPSs are large, multifunctional enzyme complexes, that build growing chains from individually selected building blocks. The NRPS is also organized into modules, each of which is responsible for one cycle of elongation by the incorporation of a single amino acid into the growing peptide chain. Each elongation module contains three essential domains catalyzing adenylation, thiolation and condensation. The adenylation domain (A) selects a specific amino acid and activates it as an amino acyl

adenylate. The activated amino acid is then transferred to the phosphopantethiene group ("swing arm") of the post-translationally modified peptidyl carrier protein (PCP) or thiolation domain (T). The condensation domain (C) catalyzes the peptide bond formation between amino acids in adjacent modules. The chain is elongated successively and released at the end of the "assembly-line" by the action of an integrated thioesterase (TE) domain or a separate TE generating either a linear or cyclic peptide. Additional structural diversity is introduced by modification of the growing chain catalyzed by a variety of embedded auxiliary domains such as E (epimerization), MT (*N*-methylation), CY (cyclization), OX (oxidoreduction), F (*N*-formylation) and R (reduction) domains (Walsh et al. 2001). These domains provide further methods to deviate from the nonribosomal code, providing the incorporation of diverse amino acid functionality such as thiazoles, oxazoles, thiazolidines, oxazolidones, as well as a full array of *N*-methylated and D-amino acids not found in any other system throughout nature (Walsh et al. 2001).

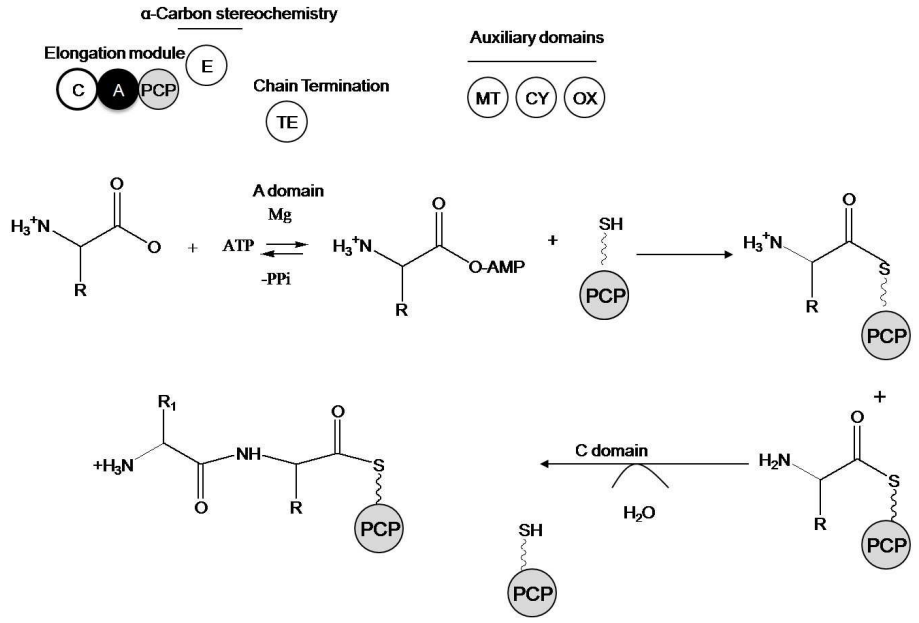


Fig. 2 Organization and mechanisms of modular biosynthetic pathway systems in nonribosomal peptide synthase (NRPS). C: condensation, A: adenylation, PCP: peptidyl carrier protein, E: epimerase, CY: cyclase, OX: oxidoreductase, TE: thioesterase, MT: methyltransferase. Adapted from Schwarzer *et al.* 2003.

Symbiosis between invertebrates and marine microorganisms

The experts focused on marine natural products ask themselves, whether the secondary metabolites isolated from marine invertebrates are produced by microbes. This long standing question has often been proposed for metabolites isolated from sponges, ascidians, soft corals and bryozoans (Davidson et al. 2001, Faulkner et al. 1993, Schmidt et al. 2000**).** Many compounds from marine macro-organisms bear structural resemblance to those from terrestrial (and marine) microbes (Bewley and Faulkner 1998, Moore, 1999). The above mentioned observations invariably invoke the hypothesis, that microbial symbionts are the biosynthetic sources of the compounds identified from the invertebrate. The circumstantial evidence for microbial origins of these compounds abounds, though examples of direct confirmation are rare (Kerr et al. 2003). Fig. 3 and 4 illustrate the resemblance between marine invertebrate and microbial compounds that are presumably produced by PKS and NRPS pathways. The figures display natural products from a variety of marine organisms, highlighting compounds isolated from marine macroorganisms *versus* terrestrial analogues produced entirely by microorganisms. In some cases the compounds are identical, such as westiellamide (Prinsep et al. 1992) and cycloxazoline (Hambley et al. 1992) found in a terrestrial cyanobacterium and marine tunicates, respectively. More commonly, there are specific functional groups or skeletal systems that are similar, for example: the enediyne "warhead" moiety in calicheamicin (Lee et al. 1987) and namenamicin (McDonald et al. 1996), the isoquinoline ring system of saframycin B (Arai et al. 1979) and ecteinascidin-743 (Wright et al. 1990), and the overall structural analogies between nodularin (Rinehart et al. 1988) and motuporin (Desilva et al. 1992).

Marine compounds with terrestrial analogues

There are currently several examples of identified gene clusters from terrestrial microbes that encode compounds similar or identical to those from marine sources. In Table 1 there is a brief description of each suggested pair of compounds, their source organisms and biosynthetic pathways. The availability of analogous genes/ clusters makes these marine compounds the most tractable targets for molecular genetic studies. Given the clustering and role of horizontal transfer of genes in prokaryotes, it is unlikely that the genes encoding the

same secondary metabolite in the two organisms could arise separately by the process of convergent evolution, but it cannot be ruled out **(**Salomon et al. 2001, Walton, 2000).

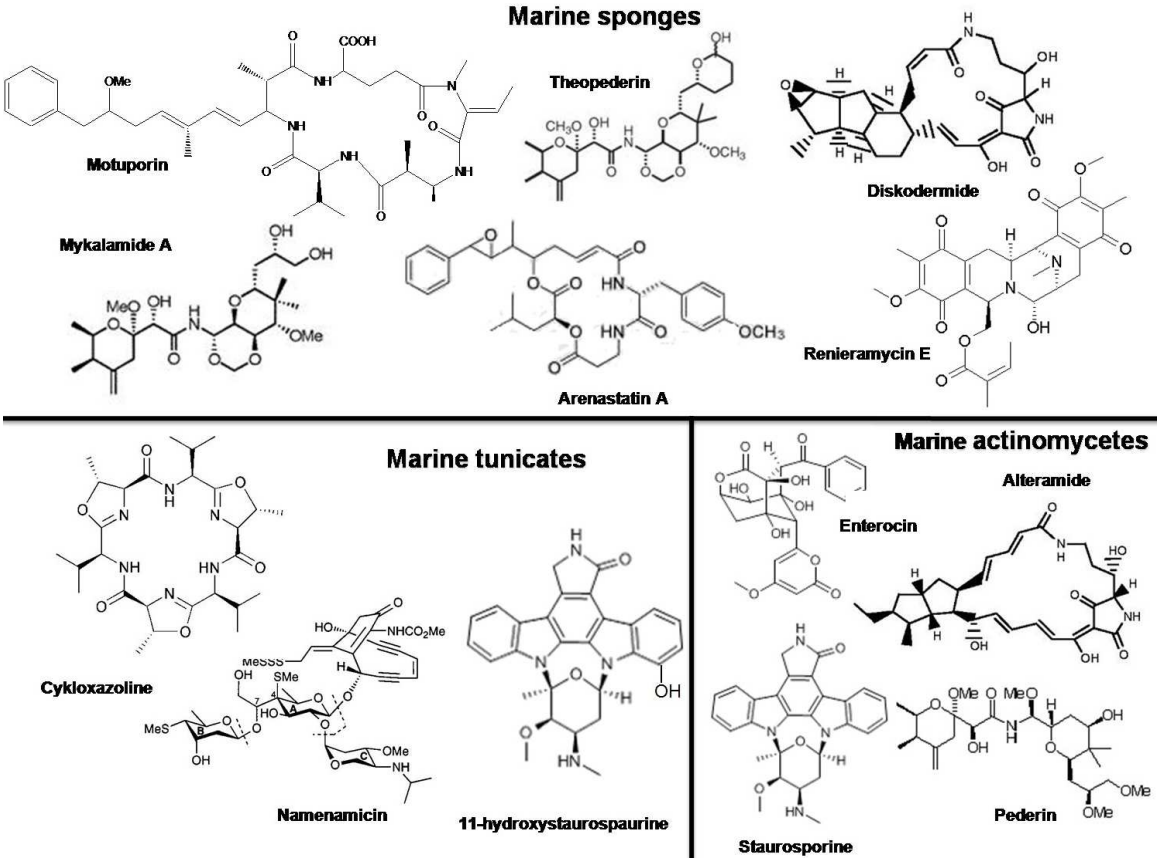
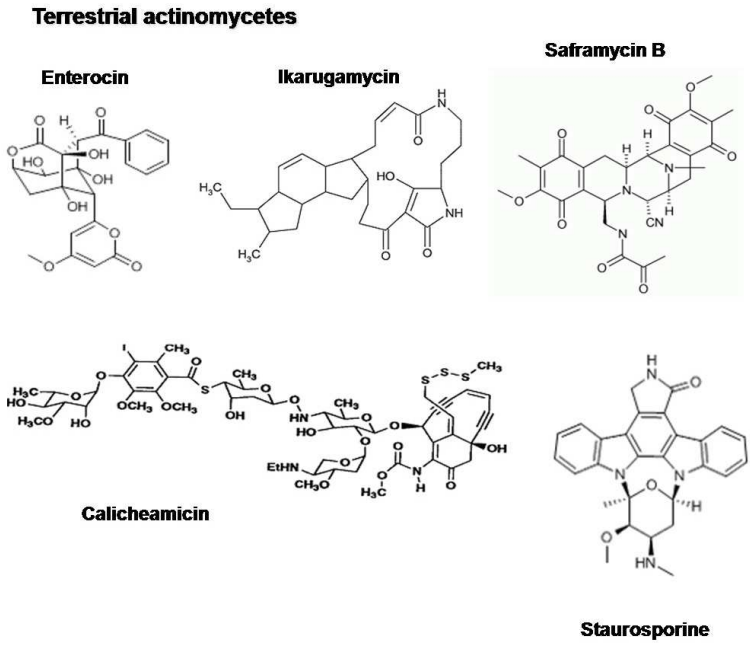


Fig. 3 Marine natural products isolated from sponges, tunicates and actinomycetes.



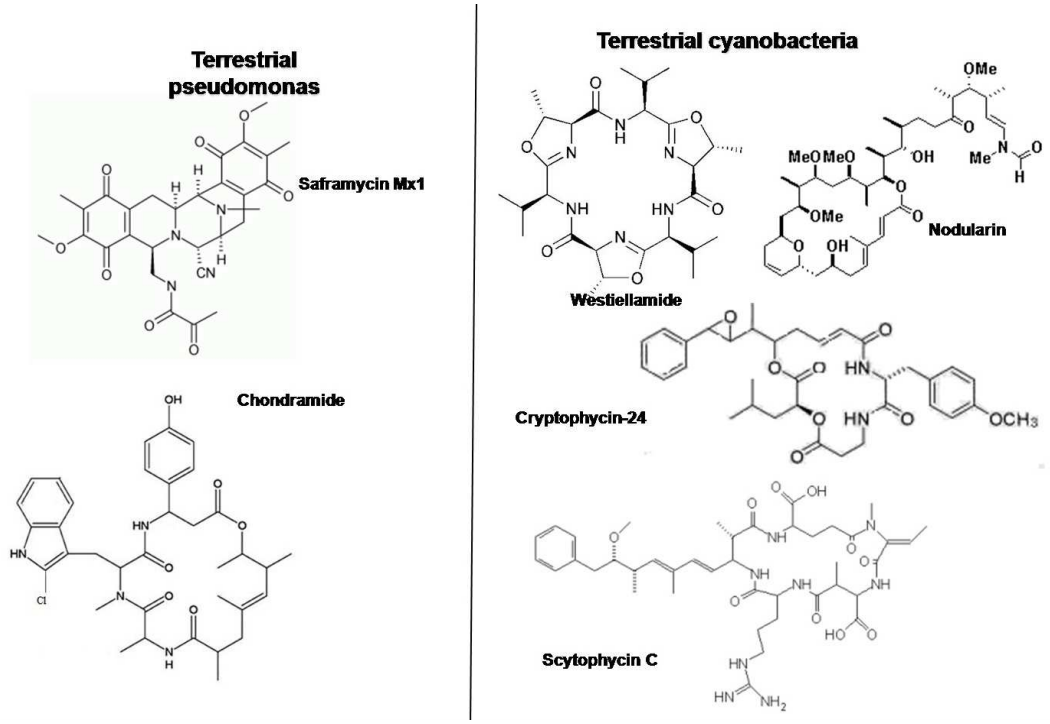


Fig. 4 Metabolites isolated from terrestrial microbes with structures ressembled to marine natural products presented in figure 3.

Table 1 Terrestrial compounds with known gene clusters that are structurally similar to marine natural products. Adapted from Salomon et al. 2004

Terrestrial compound	Microbial source (s)	Biosynthetic pathway	Related marine compound	Microbial/invertebrate sources
Staurosporine	*Streptomyces staurosporeus*	Shikimate pathway	Staurosporine 11-hydroxystaurosporine	Marine actinomycete Ascidian (*Eudistoma* sp.)
Saframycin Mx1	*Myxococcus xanthus*	NRPS	Renieramycin E	Sponge *Reniera* sp.
Saframycin B	*Streptomyces lavendulae*		ET-743	Ascidian *Ecteinascidia turbinata*
Pederin	Endosymbiont of the *Paederus* blister beetle, most similar to *Pseudomonas aeruginosa*	Mixed type I PKS/NRPS[1]	Mycalamide A Onnamide A Theopederin	Sponge (*Mycale* sp.) Sponge (*Theonella* sp.) Sponge (*Theonella* sp.)
Calicheamicin	*Micromonospora echinospora*	Iterative type I PKS[2]	Namenamicin Shishijimicin B	Ascidian (*Polysyncraton lithostrotum*) Ascidian (*Didemnum proliferum*)
Nodularin	*Microcystis aeruginosa* *Planktothrix agardhii*	Mixed type I PKS/NRPS[3]	Motuporin	Sponge *Theonella swinhoei*

[1] Piel et al. 2004
[2] Ahlert et al. 2002, Liu et al. 2002
[3] Christiansen et al. 2003

For marine compounds with close terrestrial analogues that do not have any known gene cluster available, it may be worthwhile to focus first on obtaining specific molecular probes and preliminary sequence information from the pure terrestrial microbial culture. Some examples of these marine natural products with terrestrial analogues are shown in Table 2.

Table 2 Marine compounds with structurally similar terrestrial analogues (unknown gene cluster). Adapted from Salomon et al. 2004.

Marine compound	**Microbial / invertebrate sources**	**Proposed biosynthetic pathway**	**Terrestrial compound**	**Microbial source(s)**
Arenastatin	Sponge (*Dysidea arenaria*)	Mixed type I PKS/NRPS	Cryptophycin-24	*Nostoc* sp.
Alteramide	Bacterium (*Alteromonas* sp.)	Mixed type I	Ikarugamycin	*Streptomyces* sp.
Jaspamide	Sponge (*Jaspis splendins*)	Mixed type I PKS/NRPS	Chondramide	*Chondromyces crocatus*
Sphinxolide	Sponge (*Neosiphonia superstes*)	PKS	Scytophycin	*Scytonema pseudohofmanni*
Sphinxolide	Sponge (*Neosiphonia superstes*)	PKS	Rhizopodin	*Myxococcus stipitatus*

Identification methods of metabolic products derived from cryptic gene clusters

Many microbial natural products, in particular complex polyketides and nonribosomal peptides, are assembled by biosynthetic assembly-lines involving modular synthases and megasynthases (Fischbach and Walsh, 2006). In many cases the number of modules in the assembly-line corresponds exactly to the number of metabolic building blocks incorporated into the final product, although several exceptions to this paradigm have emerged in recent years (Haynes and Challis 2007). The presence or absence of domains with ‘tailoring’ activities in individual modules often allow prediction of the way in which an initially selected metabolic building block gets modified during the process of its incorporation into the natural product (Fischbach and Walsh, 2006). Models that predict the stereochemical outcome of some of these tailoring reactions, e.g. ketoreduction, have also emerged relatively recently (Caffrey, 2003; Reid et al. 2003). Models that predict the substrate specificity of the adenylase (A) and acyltransferase (AT) domains responsible for building block selection in each module of nonribosomal peptide synthase (NRPS) and polyketide synthase (PKS) assembly-lines have also been reported and continue to be developed (Haydock et al. 1995;

Banskota et al. 2006a, b; Stachelhaus et al. 1999; Challis et al. 2000; Rausch et al. 2005). Insight into the structural features of the metabolic products of cryptic biosynthetic assembly lines can often be derived by application of the above bioinformatics analyses (Banskota et al. 2006a, b; Bentley et al. 2002; Challis and Ravel 2000; Chen et al. 2007; de Bruijn et al. 2007; McAlpine et al. 2005; Minowa et al. 2007; Nguyen et al. 2008; Paulsen et al. 2005; Sudek et al. 2006; Tohyama et al. 2004; Udwary et al. 2007; Zirkle et al. 2004). Such structural insights can lead to the prediction of putative physico-chemical properties of a metabolic product of a cryptic biosynthetic system. The search of fermentation broths for products of cryptic pathways can be narrowed to target only metabolites with the predicted physico-chemical properties, thus simplifying the analytical challenge (Fig. 5a). Several new metabolic products of cryptic biosynthetic gene clusters have been discovered using such methodologies (Lautru et al. 2005; McAlpine et al. 2005; Banskota et al. 2006a, b).

Two other approaches that have been applied to cryptic biosynthetic systems, where the substrates of enzymes in the pathways can be predicted, are the 'genomisotopic approach' and in vitro reconstitution. In the genomisotopic approach, stable isotope-labelled putative precursors of the metabolic product are fed to the organism containing the cryptic biosynthetic gene cluster and 2D NMR experiments are used to screen extracts of the fermentation broth to identify metabolites containing the labelled precursors (Gross et al. 2007) (Fig. 5b). NMR detection of the labelled metabolites can be used to guide fractionation of the extracts to facilitate their purification. This approach has been applied to isolation of the orfamides, novel macrocyclic lipopeptides predicted to be produced by *Pseudomonas fluorescens Pf-5* from analysis of its genome sequence (Gross et al. 2007).

In the in vitro reconstitution approach, the predicted substrates of a biosynthetic enzyme, which has been produced in pure recombinant form, are incubated with it and the structures of the products are determined (Fig. 5c). Epi-isozizaene is an example of a new compound that has been identified as the product of a cryptic sesquiterpene synthase discovered by the *Streptomyces coelicolor* genome sequencing project using the in vitro reconstitution approach (Lin et al. 2006). This metabolite has recently been shown to be an intermediate in the assembly of the known *Streptomyces* sesquiterpene albaflavenone (Zhao et al. 2008).

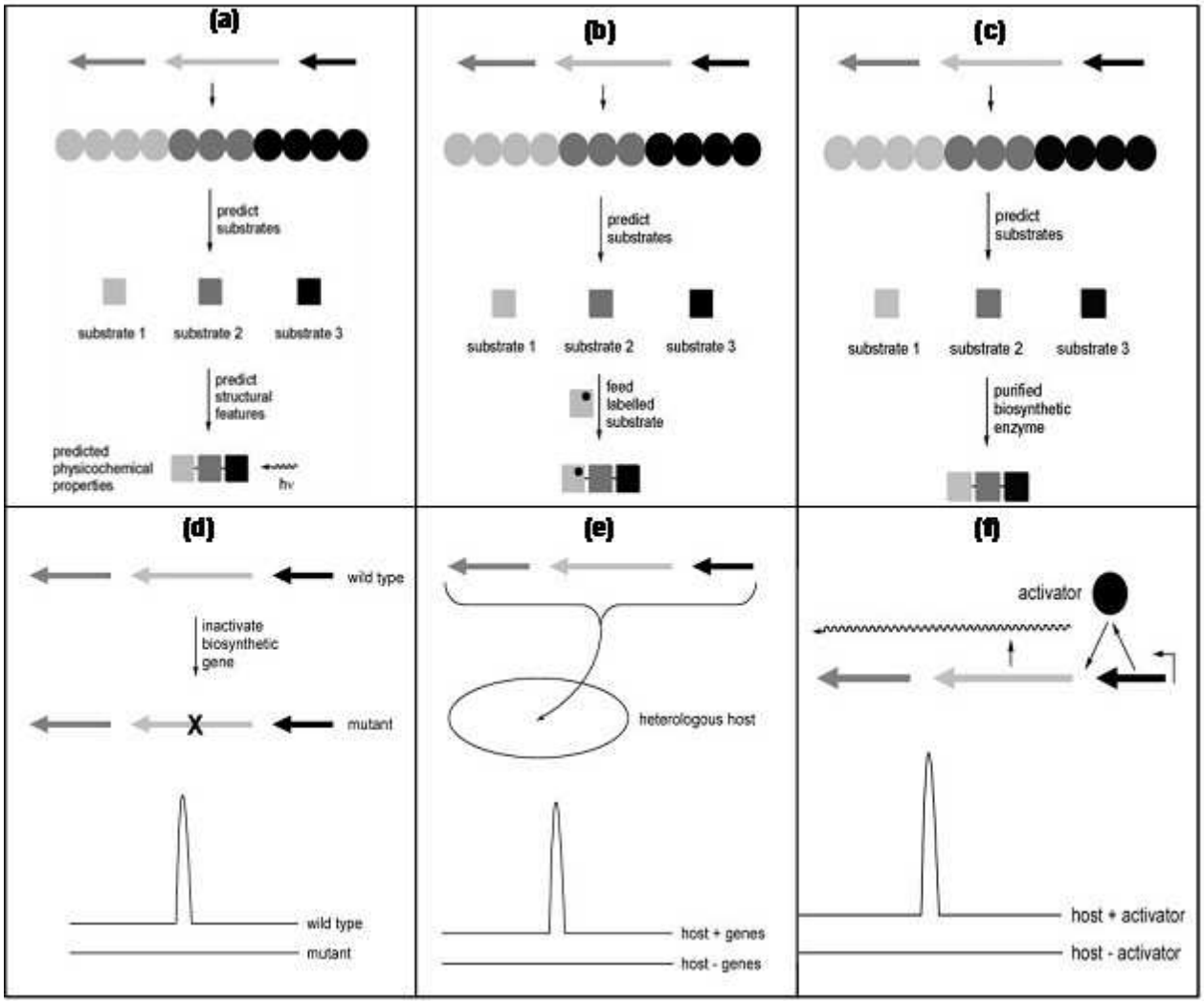


Fig. 5 **(a)**. Method of identifying the product(s) of cryptic biosynthetic gene clusters by predicting likely physico-chemical properties of the product from sequence analyses. **(b)**. Principles of the genomisotopic approach for identifying the product(s) of cryptic biosynthetic gene clusters. **(c)**. The in vitro reconstitution approach for identifying the product(s) of cryptic biosynthetic gene clusters. The structure(s) of the product(s) resulting from incubation of the predicted substrate(s) with the purified enzyme are determined. **(d)**. The gene knockout/comparative metabolic profiling approach to identifying the product(s) of cryptic biosynthetic gene clusters. **(e)**. The heterologous expression/comparative metabolic profiling approach to identifying the product(s) of cryptic biosynthetic gene clusters. **(f)**. The expression of pathway-specific activator/comparative metabolic profiling approach to identifying the product(s) of cryptic biosynthetic gene clusters. Placing a pathway-specific activator gene under the control of an inducible promoter results in activation of transcription of the normally silent biosynthetic gene cluster (Gross et al. 2007, Challis, 2008).

For some types of biosynthetic system, substrate specificity cannot be predicted with any degree of confidence from bioinformatics analyses. In these cases the directed approaches described above are not useful for finding the metabolic products of cryptic biosynthetic pathways and more generic approaches are required. Two more related generic approaches, that have been used successfully to discover the products of cryptic biosynthetic gene clusters, are gene knockout/comparative metabolic profiling and heterologous gene expression/comparative metabolic profiling (Corre and Challis 2007). The first of these involves inactivation of a gene within the cryptic biosynthetic gene cluster hypothesized to be essential for metabolite biosynthesis, followed by comparison of the metabolites in the culture supernatants or extracts of the wild-type organism and the non-producing mutant using an appropriate analytical technique such as liquid chromatography-mass spectrometry (LC-MS). Metabolites present in the wild type but lacking in the mutant are likely products of the cryptic gene cluster (Fig. 5d), which can be isolated and structurally characterized. Germicidins are an example of metabolites discovered using this strategy (Song et al. 2006).

In the second approach the entire biosynthetic gene cluster is cloned, often in a single cosmid or BAC vector, and expressed in a heterologous host. The profile of metabolites in culture supernatants or extracts of the heterologous host containing and lacking the cloned cryptic biosynthetic gene cluster are compared using LC-MS or other appropriate analytical techniques. Metabolites present in the host containing the gene cluster, but absent in the host lacking the cluster, are likely products of the cryptic biosynthetic pathway (Fig. 5e), which can be purified and structurally characterized as in the first approach. CBS40 is an example of a novel metabolite that has been identified by this approach (Hornung et al. 2007). One potential obstacle which often has to be overcome in the heterologous expression/comparative metabolic profiling approach is that natural product biosynthetic gene clusters are often large (.40 kb) and therefore it can be difficult to clone the entire cluster in a single vector. The use of multiple, mutually compatible expression vectors is one approach overcoming this problem (Challis, 2006), although it has yet to be applied to the discovery of new metabolic products of cryptic biosynthetic gene clusters.

Gene clusters that are differentially expressed in the mutants compared with the wild-type are identified as putatively involved in secondary metabolic biosynthesis. This approach offers potential for the discovery of new metabolic products of cryptic biosynthetic pathways because overexpression of laeA causes increased expression of some *Aspergillus* cryptic gene

clusters. However, this potential has yet to be demonstrated by the discovery of a novel *Aspergillus* natural product. The second approach involves expression of putative pathway-specific activator genes from within silent cryptic biosynthetic gene clusters under the control of an inducible promoter (Bergmann et al. 2007). This approach (Fig. 5f) has been shown to cause expression of a normally silent gene cluster in *Aspergillus nidulans* upon addition of the inducer. Aspyridones, the metabolic products of this gene cluster, were identified by comparative metabolic profiling of the wild-type and mutant strains and spectroscopic analyses showed them to have novel structures, thus proving the utility of this approach for discovering new natural products of cryptic biosynthetic gene clusters, that are not expressed in laboratory cultures (Bergmann et al. 2007)

The various strategies summarized above for identifying the metabolic products of cryptic biosynthetic gene clusters have different strengths and weaknesses, depending on how much can be deduced about the structure of the products from bioinformatics analyses, the size of the gene cluster, and whether the gene cluster is well expressed in laboratory cultures. These factors need to be carefully considered when choosing the best approach to take in attempting to identify the products of a cryptic biosynthetic gene cluster. Doubtless further approaches will be added to this already impressive array as research activity in this exciting new field continues to increase.

Conclusion

Marine macroorganisms and microorganisms are clearly an extraordinary source of complex and bioactive secondary metabolites. The continuous discovery of new compounds from these metabolically talented organisms will provide a range of novel bioactive natural products which may be developed as potencial therapeutics. This molecular perspective focuses on some of the most pharmaceutically useful and structurally interesting terrestrial microbial metabolites belonging to the biosynthetic classes of polyketide synthases (PKSs) and nonribosomal peptide synthases (NRPSs). The ability of cloning and heterologous expressing of gene cluster enconding valuable metabolites may help to alleviate the critical supply problems associated with most marine natural products and provide contributions towards the molecular toolbox used in the genetic engineering of new drugs.

A lot of new information in the biosynthetic chemical processes has been discovered over the past 20 years by sequencing-based approaches. Progress in this area has accelerated

thanks to the avalanche of genomic data since the turn of the century. Doubtless, much new, exciting and intriguing biosynthetic chemistry remains to be discovered in the future through the continued exploitation of information from genome sequencing projects.

Acknowledgements

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