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Bioinformatics and biogeography to mine natural products in metagenomes

Bioinformática e biogeografia para buscar produtos naturais em metagenomas

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Faculdade de Ciências Farmacêuticas de Ribeirão Preto/USP para obtenção do Título de Doutor em Ciências

Área de Concentração: Produtos Naturais e Sintéticos.

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Field of Study: Natural and synthetic products

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RESUMO

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Os produtos naturais microbianos (NP) tem demonstrado ser inestimáveis pontos de partida na descoberta e desenvolvimento de medicamentos aprovados pelo FDA. A abordagem tradicional para a identificação de produtos naturais microbianos exige a cultura em laboratório. Infelizmente, os métodos convencionais baseados nesta metodologia foram desestimulados devido a altas taxas de redescoberta de moléculas. Os métodos independentes de cultura que se baseiam no sequenciamento do metagenoma microbiano sugerem a ocorrência de um enorme reservatório inexplorado de *clusters* biossintéticos de produtos naturais (BGCs) no meio ambiente. Neste trabalho utilizamos uma metodologia baseada em PCR e *barcoding amplicon*-sequencing para buscar importantes famílias de produtos naturais como peptídeos não ribossomais (NRP), ácido 3-amino-5-hidroxibenzóico (AHBA), dímeros de triptofano (TD), policetídeos, aminoglicosídeos e outros. Para isto desenvolvemos um *script* chamado SecMetPrimer que nos permitiu bioinformaticamente desenhar conjuntos de *primers* contendo um gradiente de degenerâncias. No total, desenhamos 165 conjuntos de *primers.* Os *amplicons* foram obtidos por PCR padrão, tendo sido concatenados *barcodes* específicos por amostra e sequenciados através de Illumina MiSeq. Para validar, utilizamos eDNA (*environmental* DNA) de bibliotecas metagenômicas, totalizando 223 milhões de clones. Através das análises bioinformáticas, as curvas de rarefação foram calculadas e a diversidade para cada família foi determinada. Foi realizada uma re-amplificação dos domínios de adenilação de peptídeo não ribossomal e domínios de cetosintase de policetídeos utilizando eDNA isolado de 25 amostras diferentes coletadas em Mata Atlântica, Cerrado e ambiente marinho. Nossos dados indicaram a correlação entre distância geográfica e o tipo ecológico dos biomas. Deste modo, foi possível assim atribuir genes relacionados à *clusters* biossintéticos que codificam importantes produtos naturais à informações taxonômicas e metabólicas. Deste modo identificamos os melhores *hotspots* para busca de diversidade biossintética dentre as amostras analisadas.

Palavras-chave: Produtos naturais, Metagenômica, Bioinformática

ABSTRACT

FRIAS, U. A. **Bioinformatics and biogeography to mine natural products in metagenomes.** 2017. 123 p. Doctoral Dissertation. School of Pharmaceutical Sciences of Ribeirão Preto – University of Sao Paulo, Ribeirão Preto, 2017.

Microbial natural products (NP) have proven to be invaluable starting points in the discovery and development of many drugs approved by FDA. The traditional approach to identify microbial natural products requires the culturing in the laboratory. Unfortunately, conventional culture-based methods have been deemphasized due to high rediscovery rates. Culture-independent methods applying microbial (meta)genome sequencing suggest the occurrence of an enormous untapped reservoir of natural-product-encoding biosynthetic gene clusters (BGCs) in the environment. Here we have used a PCR-based approach and barcoding amplicon-sequencing derived from important families of microbial natural products such as nonribosomal peptides (NRP), polyketides (PK), 3-amino-5-hydroxybenzoic acid-containing NPs (AHBA), tryptophan dimmers (TD), aminoglycosides, phosphono-containing NPs and others. We have written an internal script called SecMetPrimer that allowed us to bioinformatically design sets of primers containing a range of degeneracy to amplify these genes. At the total, we designed 165 different sets of primers. The amplicons were obtained by standard PCR containing double-barcoded-target primers and sequenced by Illumina MiSeq platform. The validation process was conducted using eDNA from metagenomic libraries containing a 223 millions of clones. The rarefaction and diversity analyses were assigned, and the best-hit primer for each family was chosen. We have re-amplified the nonribosomal peptide adenylation domains and polyketide ketosynthase domains, using as substrate environmental DNA isolated from 25 different samples collected in Atlantic Forest, Cerrado and marine environment. Our data indicate a correlation between geographic distance and biome-type, and the biosynthetic diversity found in these environments. Thus, by assigning reads to known BGCs against taxonomic and metabolic profiles, we have identified the hotspots of relevant biosynthetic diversity among the analyzed samples.

Keywords: Natural Products, Metagenomics, Bioinformatics

INTRODUCTION

1 Introduction

1.1 The Importance of Natural Products

During most of the historic records, small molecule natural products have been a source of innovative therapeutics agents against a broad spectrum of diseases. Humans have used plant extracts for therapeutic purpose for millennia (Handelsman *et al.*, 1998; Ji *et al.*, 2009). Since the early 1800s, the identification of natural products has risen a brand-new era of medicine, and drugs started to be isolated from plants, and microorganisms (Newman e Cragg, 2016). The term natural product literally proposes any naturally occurring molecule but is commonly considered to mean a secondary metabolite, a molecule that has apparently no fundamental function in the metabolism (Williams *et al.*, 1989).

It is believed that molecules exist to enhance the reasonableness of an organism's survival by attracting or repulsing other organisms. The initial reports on natural products include strychnine, morphine, atropine, quinine, and colchicine (**Figure 1**) (Hosztafi, 1997; Cordell *et al.*, 2001; Corson e Crews, 2007; Zenk e Juenger, 2007; Kaiser, 2008; Cragg e Newman, 2014). In 1826, E. Merck revealed the first business natural product, morphine (Sertuerner, 1817) and this was succeeded by the first semisynthetic, pure drug based on a natural product, aspirin, by Bayer in 1899 (**Figure 1**) (Sneader, 2000). The next breakthrough was the identification of penicillin from the filamentous fungus *Penicillium notatum* by Alexander Fleming in 1929. This unquestionably led in the "Golden Age of Antibiotics", the period from the 1940s to the 1970s (Li e Vederas, 2009; Newman e Cragg, 2016).

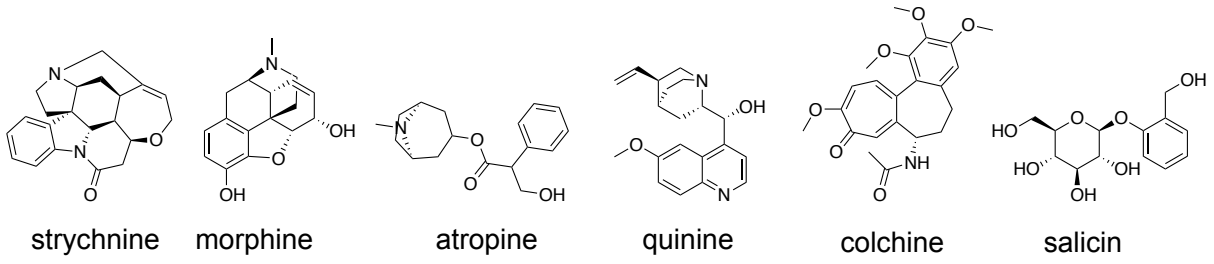


Figure 1: Natural Products breakthrough isolated from plants

Pharmaceutical companies started extensive studies of microbes as sources of novel antibiotics and led to the identification of a host of different antibacterial compounds, including the tetracyclines (e.g., doxycycline), cephalosporins, aminoglycosides (e.g., streptomycin), lipopeptides (e.g., daptomycin), glycopeptides (e.g., vancomycin), and macrocyclic compounds such as erythromycin (Newman *et al.*, 2000; Cragg e Newman, 2001). Microbial natural products have also been the source of different anti-infective drugs having antifungal (e.g., amphotericin, nystatin) and antiparasitic, (e.g., ivermectin, fumagillin) activities (**Figure 2**) (Newman *et al.*, 2000; Cragg e Newman, 2001; Newman, David J. e Cragg, Gordon M., 2012).

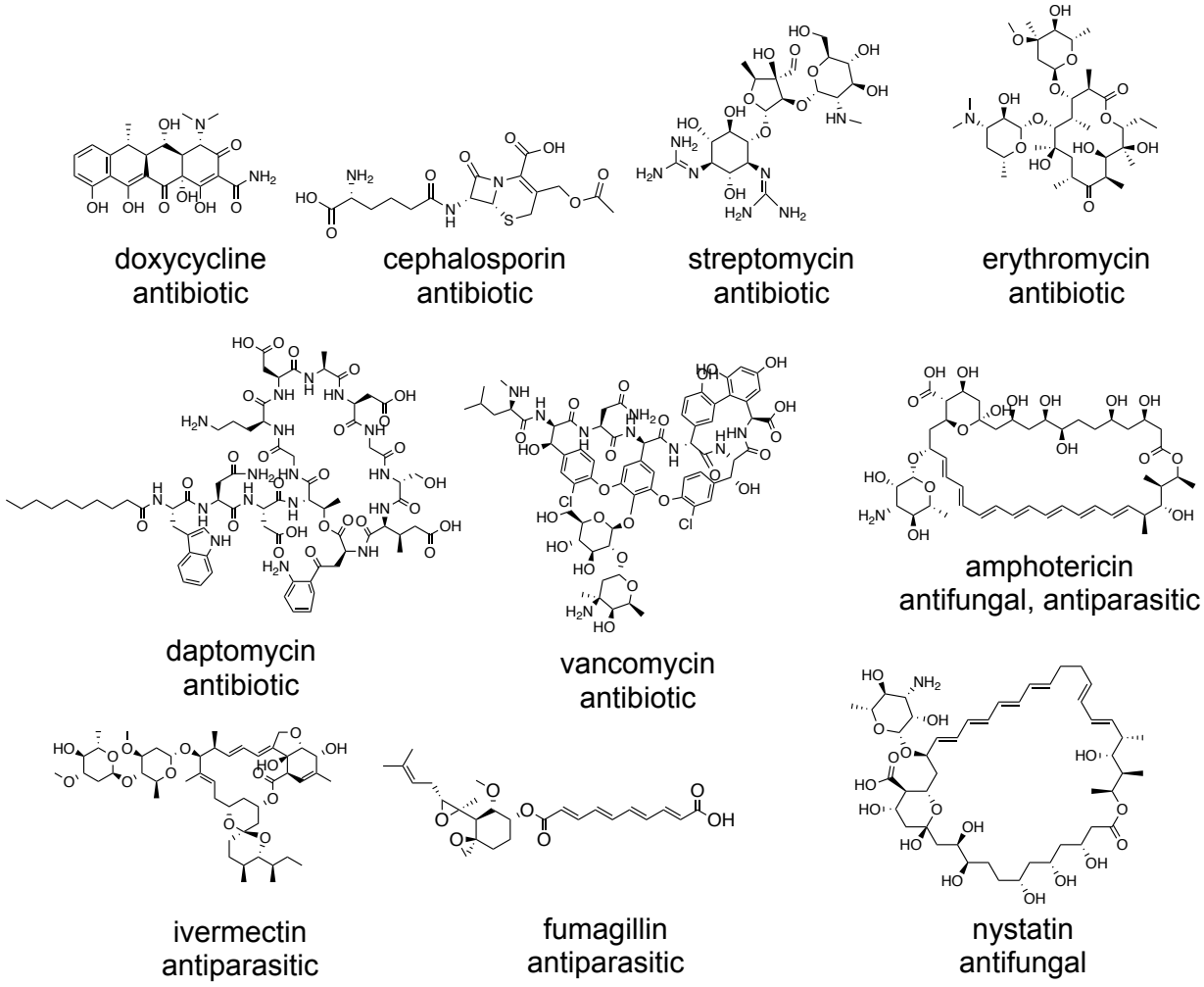
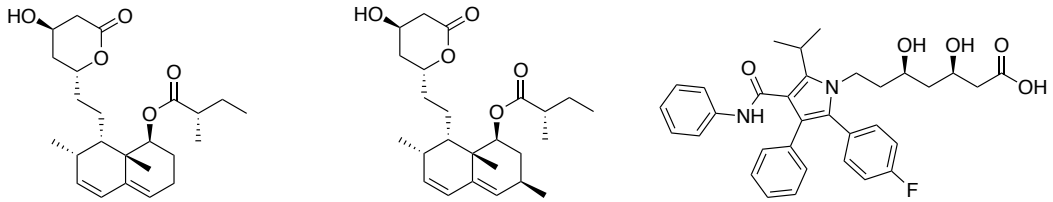


Figure 2: Natural Products as therapeutic agents

In this way, microbes have given the template for the development of anticholesterolemic drugs, such as statins (Cragg e Newman, 2014). The determination of the mevastatin (compactin) and lovastatin as inhibitors of HMG-CoA reductase led to the progress of synthetic statin analogs, such as atorvastatin (**Figure 3**).



mevastatin (compactin) lovastatin atorvastatin

Figure 3: Natural and synthetic anticholesteromic drugs

The antitumor antibiotics families of anthracyclines (e.g., doxorubicin), enediynes (e.g., calicheamicin), ansamycins (e.g., geldanamycin), peptolides (e.g., dactinomycin), epothilones (e.g., ixabepilone), mitosanes (e.g., mitomycin C), are microbial natural products that represent meaningful role in the drug discovery (**Figure 4**) (Cragg *et al.*, 2009; Cragg *et al.*, 2012; Newman, D. J. e Cragg, G. M., 2012; Newman e Cragg, 2016). The Type I polyketide rapamycin are another relevant class of immunosuppressive drugs that complement the performance of another immunosuppressant microbial natural product, cyclosporine (Cragg e Newman, 2001).

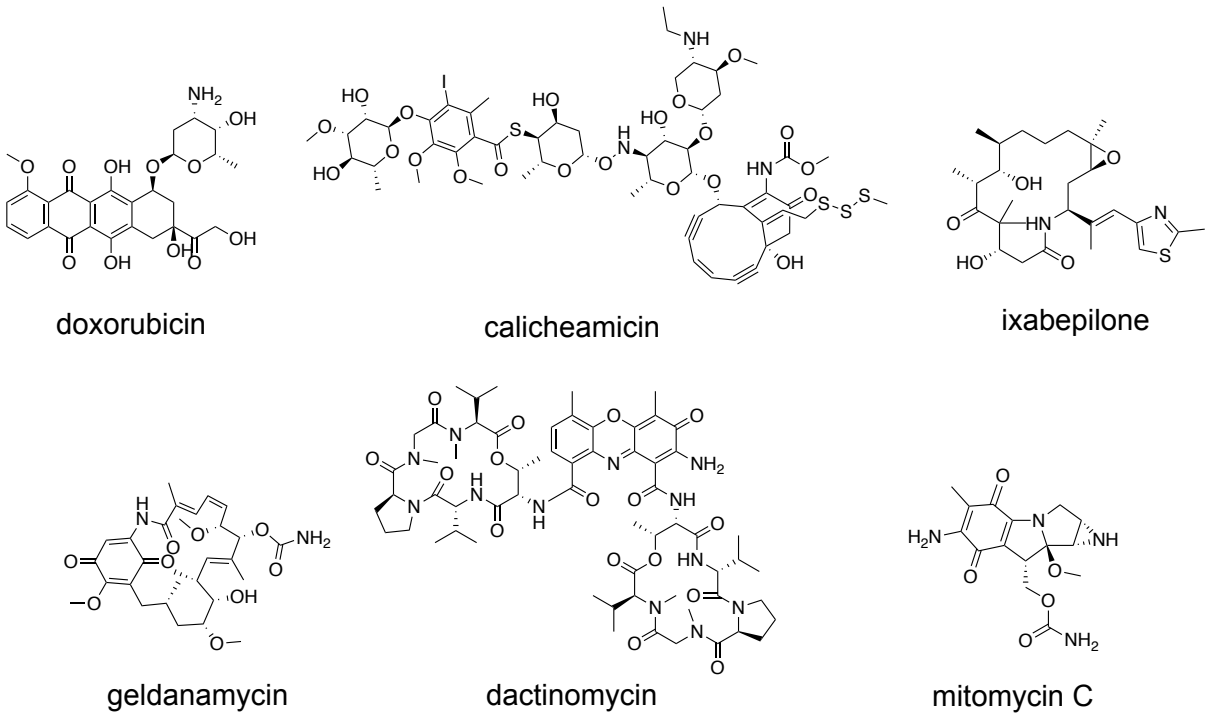


Figure 4: Therapeutic natural products isolated from bacteria

Thus, it is calculated that approximately 70% of all small molecule drugs have the origin in natural products (**Figure 5**) (Newman e Cragg, 2016). Therefore, natural products have been, and remain to be, an essential source of innovative drugs and pharmaceutical leads.

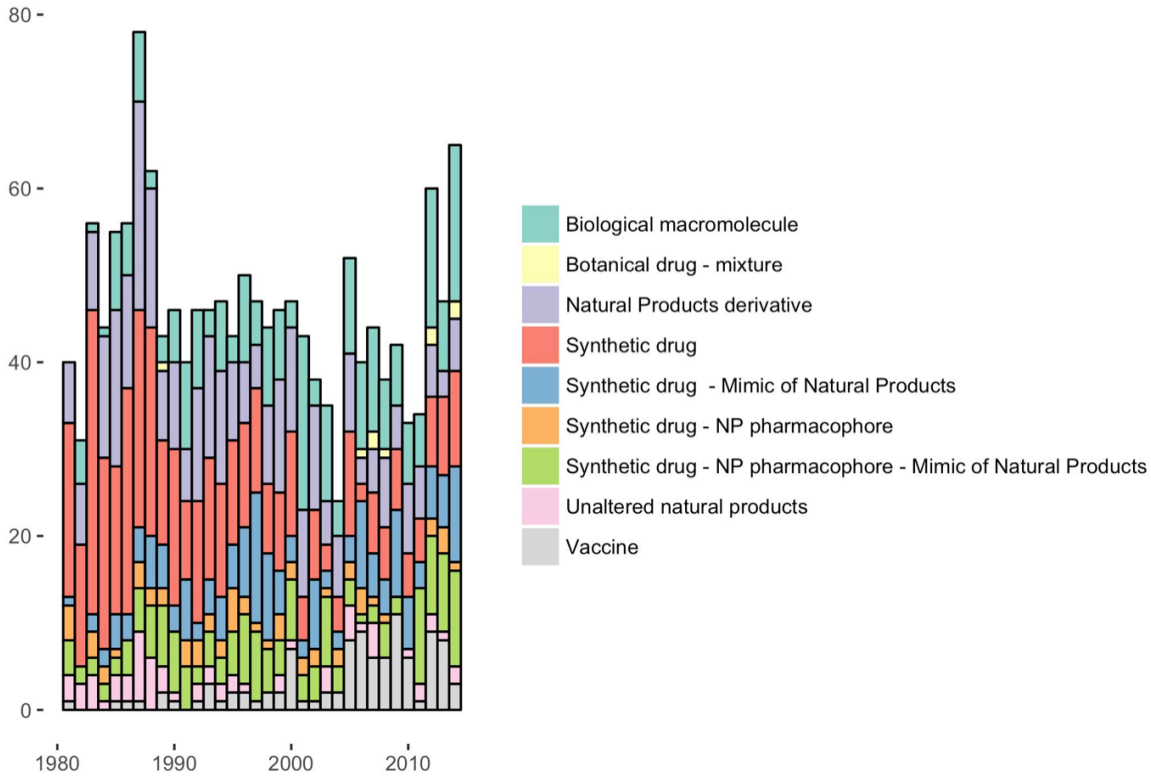


Figure 5: Distribution of 1562 drugs approved by FDA the during 1980 - 2014
This graphic represents the correlation of the amount of drugs approved by FDA (x-axis) by year (y-axis). The colors in legend represent drugs class. This data is available in reference - Newman and Cragg (2016).

Natural products isolated from cultivated microorganisms have given the large dimension of the most significant pharmacophores discovered to date (Newman e Cragg, 2016). Their bioactivity is associated, to a considerable amount, to selective pressures that crowned in the evolution of structural and chemical features of natural products to perform their function efficiently in biological contexts (Maplestone *et al.*, 1992). Presumably, in the natural microbial ecosystem the metabolites have been revealed to perform crucial functions in processes as self-defense, nutrient scavenging, and virulence (Omura *et al.*, 2001; Wolfgang *et al.*, 2003; Nougayrede *et al.*, 2006; Wyatt *et al.*, 2010). Soil-dwelling bacteria, particularly actinomycetes, have been an overflowing reservoir of bioactive natural products. Admittedly, a predicted two-thirds of all clinically valuable antibiotics were isolated from one bacterial genus singly, *Streptomyces* (Kieser et al. 2000). A single gram of soil is predicted to harbor

up 10,000 unique bacterial species. Consequently, soil-bacteria appear to represent an encouraging reservoir of new natural products with broad therapeutic potential (Torsvik et al. 1990; Rappe and Giovannoni 2003; Charlop-Powers, Owen*, et al.*, 2014; Charlop-Powers *et al.*, 2015). There are hundreds of thousands of different natural products reported in literature. The CRC Dictionary of Natural Products (DNP) lists 190,939 records (DNP, Chapman and Hall, 2005). Regrettably, there is no rigidly established design for classifying natural products. Their endless heterogeneity in structure, function, and biosynthesis is exceptionally vast to enable them to fit into a few simple categories.

1.2 Metagenomics and uncultured microorganisms

Mapping biologically significant chemical space is of highest interest for drug discovery and development. Nevertheless, the discovery of new natural product antimicrobials has decreased over the past decades (Fox, 2006; Banin *et al.*, 2017). Multiple factors are providing this decay. The isolation of small molecules frequently relies on the decade-old methods and techniques (**Figure 6**). The conventional protocols employed to isolate and identify natural products from microorganisms usually apply extraction of bacterial cultures using organic solvents, and isolation of the molecules by activity-guided fractionation (**Figure 6**). However, the conventional culture-based model appears to be one of the main obstacles to new natural product discovery. Notwithstanding decades of historical productivity, pharmaceutical companies have deemphasized natural product discovery applications due to the rising of rediscovery rates of known metabolites produced by cultured bacteria - a rate that now outstrips 99% (Newman e Cragg, 2016). The challenges associated

with culturing environmental bacteria limit traditional culture-dependent methods to be applied to a small fraction of the bacterial species in nature. The microbial 16S rRNA sequencing efforts showed that just a tiny fraction of the microbes had been cultured through standard microbiological techniques (Torsvik *et al.*, 1990; Kaeberlein *et al.*, 2002; Zengler *et al.*, 2002; Gans *et al.*, 2005; Tringe *et al.*, 2005). In the bacterial domain, this fraction is represented by less than 1% of all species in the environment.

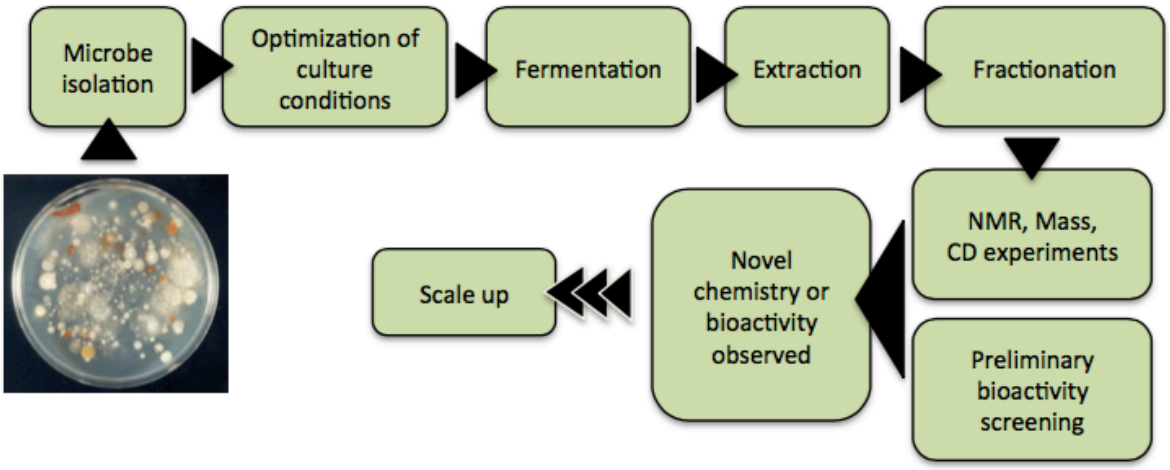


Figure 6: Traditional culture-based methods in microbial natural products isolation
Arrows guide the pipeline for isolation and characterization of natural products.
Methods could change across laboratories, however the general idea is maintained.

These likewise investigations comprehend that more than 80 dominant bacterial divisions exist, but cultured isolates less than half (Keller e Zengler, 2004; Schloss e Handelsman, 2004; Desantis *et al.*, 2006). New methods suggest that uncultured bacteria are apparent the biggest outstanding pool of genetic and chemical diversity on the planet. Hence, it is evident that the large preponderance of natural products molecules biosynthesized by microorganisms in the biosphere are exceeding the reach of the common culture-dependent model (Charlop-Powers *et al.*, 2015).

There are multiple strategies employing both culture-dependent and culture-independent methods, which are presently being extended to access this untapped reservoir of chemical diversity. Typically, culture-independent approaches require the cloning of the genetic material from an environmental sample (eDNA - environmental DNA), which carries the genes encoding small molecules (**Figure 7**). This genetic heterogeneity of group of the bacteria across given environmental sample has been termed the "metagenome" (Handelsman *et al.*, 1998). While genomics is the investigation of an individual organism's DNA, metagenomics transcends the single genome. Catching advantage of the progress in DNA-sequencing technology, this approach provides the investigation of whole populations of microorganisms simultaneously, circumventing the requirement for isolation and culture (Handelsman *et al.*, 1998).

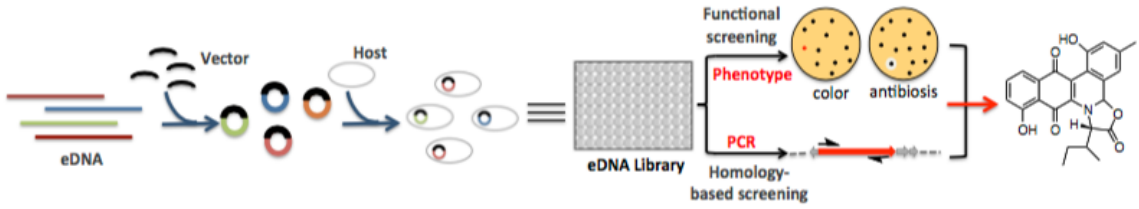


Figure 7: Metagenomic library construction and screening
The general idea is connect the eDNA with vectors using ligase and put inside a host (in general *E. coli*). The library is construct and screening by phenotypic screenings.

It consequently appears possible that we can investigate the biosynthetic potential of uncultured bacteria using metagenomics and obtains new ways to identify unknown bioactive metabolites for drug discovery applications. Metagenomics emerged fast as an option to standard microbial screening for natural product discovery. Investigating natural products biosynthetic gene clusters (BGCs) applying metagenomic approach requires eDNA (environmental DNA) cloning and

sequencing improvement techniques. The DNA obtained from an environmental sample, before-mentioned as soil and water, contains vast amounts of genetic material, including the secondary metabolite biosynthetic gene clusters.

DNA extracted from an environmental sample, such as soil and water, contains large quantities of genetic material, including secondary metabolite biosynthetic gene clusters. This eDNA is purified and cloned into a vector that provides the high-efficiency transformation of a tractable library host, typically in *Escherichia coli*. To perform this, eDNA can be ligated into a cosmid vector, packaged into lambda phage and transfected into *E. coli* (Brady, 2007). This cloning approach can capture up to 50 kb pieces of eDNA in a single clone. Considering that genes responsible for the biosynthesis of natural products are usually clustered collectively, it is possible to capture a complete biosynthetic gene cluster or a large part of it in a single cosmid (Handelsman *et al.*, 1998).

Some remarkable works have showed how the metagenomic approaches have been applied to microbial communities. As examples from soils/sediments (Rondon *et al.*, 2000; Brady *et al.*, 2002; Voget *et al.*, 2003), rumen gut (Brulc *et al.*, 2009), planktonic marine microbial associations (Beja *et al.*, 2000; Breitbart *et al.*, 2002), deep sea microbiome (Sogin *et al.*, 2006), acid mine site (Tyson *et al.*, 2004), arctic sediments and the Sargasso sea (Venter *et al.*, 2004).

The basis of all metagenomic strategies is the isolation and subsequent examination of DNA extracted directly from naturally occurring microbial communities, which bypasses the challenges associated with culturing environmental bacteria (Handelsman *et al.*, 1998). Metagenomics is expressly fascinating to natural product investigation because a high fraction of the gene clusters are under 100 kb.

Thus, become possible to capture the BGCs on a small number of eDNA clones and by heterologous expression obtain molecules (Handelsman *et al.*, 1998).

1.3 Functional Metagenomics

In functional metagenomic approach, eDNA libraries are analyzed in simple high throughput assays intended to recognize clones that have phenotypes correlated with the biosynthesis of some small molecules, such as pigment production, antibiosis, or altered colony morphology. One of the manageable approaches employed to detect eDNA clones that might provide small molecule antibiotics has been the screening of libraries hosted in *E. coli* for clones that generate inhibition zones against pathogenic microbes in top agar overlay assays.

Initial attempts to mine metagenomes looking for natural product began with the construction of eDNA cosmid libraries in *E. coli*. The expression of a new molecule was accompanied by a visual or chromatographic screening of these libraries looking for phenotypes commonly associated with natural product expression (e.g., color, antibiosis, HPLC peak). These simple screening produced some interesting metabolites as violacein, turbomycin A, palmitoylputrescine, a long-chain fatty acid enol ester, long-chain *N*-acyl tryptophan, long-chain *N*-acyl arginine (**Figure 8**) (Rondon *et al.*, 1999; Rondon *et al.*, 2000; Brady *et al.*, 2001; Courtois *et al.*, 2001; Macneil *et al.*, 2001; Brady *et al.*, 2002; 2004; Wang *et al.*, 2010; Feng *et al.*, 2011). However, they were less productive than initially expected.

Screening a complex metagenome library for natural product biosynthesis poses a severe challenge yielded the small fraction of clones that are expected to receive biosynthetic genes of interest. An approach to avoid this is the selectively enriching of metagenomic libraries for secondary metabolite biosynthetic machinery.

This has been illustrated by the functional screening of eDNA clones for phosphopantetheine transferase (PPTase) genes using a PPTase deficient *E. coli* strain (Charlop-Powers *et al.*, 2013). PPTases are responsible for activating nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) modules by post-translational attachment of phosphopantetheine (PPT). They are required for the function of NRPS and PKS gene clusters, including those which produce the secondary metabolite iron-chelators, siderophores, required for bacterial growth under iron-limiting conditions (Matzanke *et al.*, 1984). This method conduct to the isolation of vibrioferrin and enterobactin from metagenomics libraries (**Figure 8**).

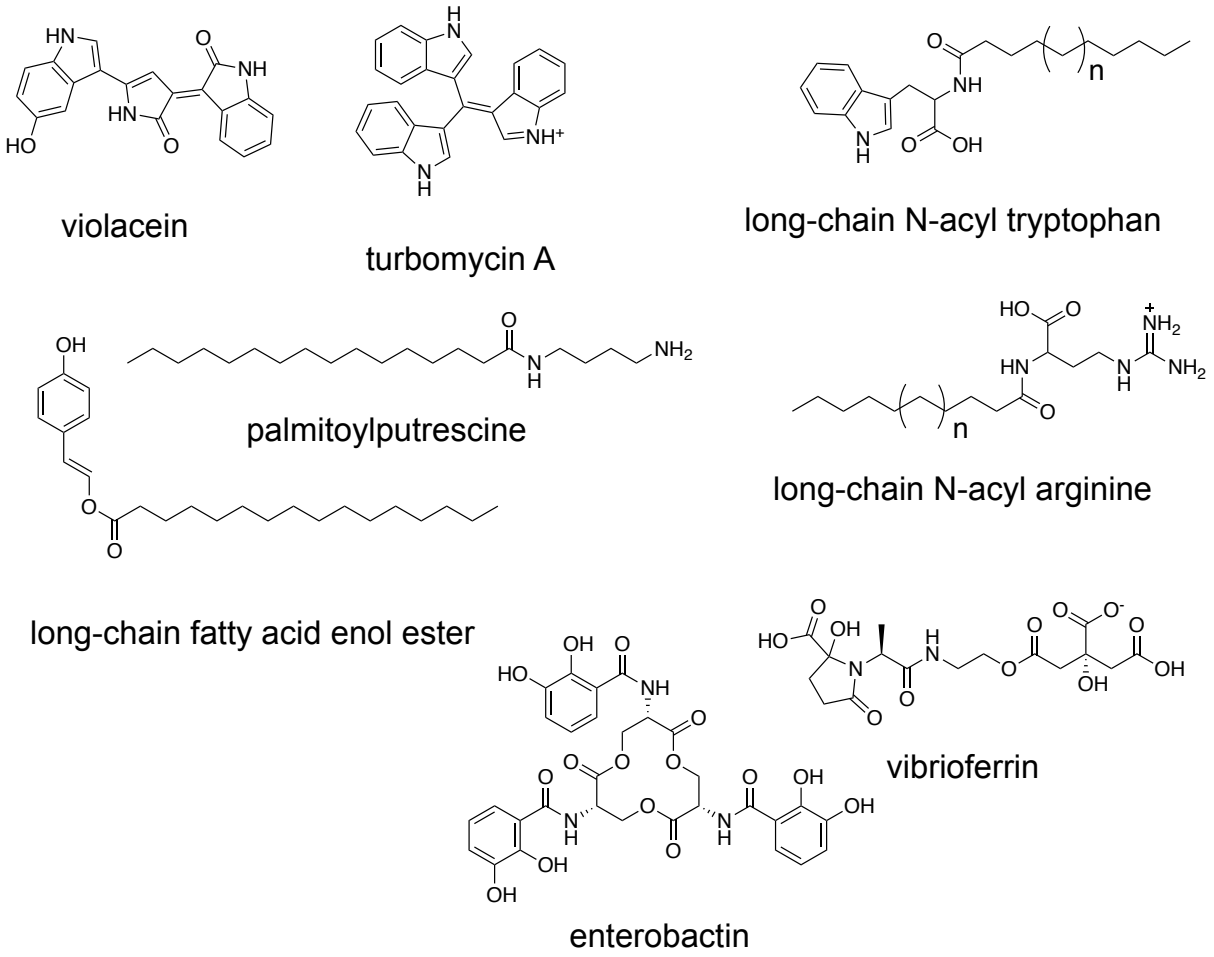


Figure 8: Natural products identified by functional metagenomic screening
This molecules were obtained from different libraries and screening using different host

This approach has led to the identification of novel longchain *N*-acylated amines, new isonitrile indole antibiotic (Brady e Clardy, 2000; 2005). Antibiotics have also been detected by monitoring pigmented eDNA clones, and also through the direct screening of fermentation extracts from randomly picked clones (Wang *et al.*, 2000; Brady *et al.*, 2001; Macneil *et al.*, 2001; Gillespie *et al.*, 2002; Lim *et al.*, 2005).

Examples of compounds with bioactivity recognized from these models of investigations include the antibiotic pigments violacein and indigo recovered from soil libraries (Chang *et al.*, 2015). The functional metagenomics has also been used to distinguish clones that produce proteins with potential anti-infective activities (Schipper *et al.*, 2009). Although almost all small molecules by functional metagenomic studies have been identified in *E. coli*, it is expected that the majority of the biosynthetic diversity existing in an environmental sample is not functionally accessible employing the same heterologous host. To investigate this difficulty, a computational approach of promoters and ribosomal binding sites used by a taxonomically diverse group of sequenced bacteria detected that at most, 40% of the enzymatic features present within a conventional metagenomic sample could be reached employing *E. coli* as a heterologous host (Gabor *et al.*, 2004). Consequently, libraries originally created in *E. coli* were later alternated into different hosts including *Streptomyces lividans*, *Ralstonia metallodurans*, *Rhizobium leguminosraum*, *Agrobacterium tumefaciens*, *Burkholderia graminis*, *Caulobacter vibrioides*, and *Pseudomonas putida* (Wang *et al.*, 2000; Martinez *et al.*, 2004; Li *et al.*, 2005; Craig *et al.*, 2009; Craig *et al.*, 2010).

One primary advantage of a functional screening is that no a priori understanding of the biosynthetic enzymes is required to discover novel metabolites. In functional screenings, a secondary metabolite is immediately linked to its

biosynthetic genes providing the impartial discovery of novel biosynthetic sequences that have not been previously described. Although many novel metabolites have been revealed applying functional screenings, the expression of complete BGCs demands the coordinated composition of multiple proteins in a clone supporting laboratory culture conditions (Bentley *et al.*, 2002; Craig *et al.*, 2010; Garcia *et al.*, 2011; Milshteyn *et al.*, 2014). The biosynthetic gene clusters isolated from a metagenomic sample are of distinct phylogenetic origin. Thus, the plausibility that a biosynthetic gene cluster attends all of the requirements is very low. Consequently, the hit rates for functional screenings of metagenomic libraries are commonly around 0.01% (Courtois *et al.*, 2003; Williamson *et al.*, 2005; Guan *et al.*, 2007; Brady *et al.*, 2009). For these restrictions, functional screenings of metagenomic libraries have been planned so that they can be efficiently developed on a large number of clones (Katz *et al.*, 2016).

Although functional metagenomic screening is a powerful method to access to the chemical diversity encoded in uncultured ecosystems, the approach still needs optimization. As explained, metagenomic cloning relies fundamentally on cosmid-based vectors. However, multiple canonical biosynthetic gene clusters are too large (>50 kb) to be carried on single cosmids and are trimmed. This special barrier performs a significant restriction of using functional assays to identify new natural products from cosmid-based eDNA libraries. Apart from this, the multifariousness present in environmental samples performs the adoption of an optimal heterologous screening host considerably challenging (Torsvik e Ovreas, 2002). Heterologous expression hosts are essentially limited in their capacity to functionally process foreign DNA. The extended use of phylogenetically diverse heterologous expression hosts will continue to benefit functional screening purposes. Searching vector-host

sets that support the screening of metagenomic libraries in phylogenetically diverse microorganisms has the potential to increase the chemical diversity within functional metagenomic approaches (Katz *et al.*, 2016).

1.4 Homology-based screening for Natural Products Discovery

The expression-independent or homology-based screening of metagenomes is based on PCR amplification of conserved natural product biosynthetic gene sequences to identify and recover gene clusters from eDNA libraries. This approach enables targeted recovery of precise biosynthetic pathways from the genomes of all bacterial species present within a metagenome.

Homology-based screens rely on the similarity of the unknown, eDNA-derived secondary metabolite biosynthetic pathways to sequenced clusters of known metabolites (Milshteyn *et al.*, 2014). In these studies, the eDNA libraries are investigated to distinguish clones that contain conserved sequences correlated with a specific group of secondary metabolite biosynthetic gene. Bioinformatics analyses of the sequences obtained by homology screening provide ways to eliminate sequences associated with known gene clusters. Consequently, homology-based screening associated with phylogenetic analyses of sequences obtained in the eDNA libraries permit the discovery of biosynthetic gene clusters encoding for metabolites related to rare families of bioactive natural products (Kang e Brady, 2014; Milshteyn *et al.*, 2014).

Homology-based screening utilizes degenerate oligonucleotides to amplify homologs sequence via the polymerase chain reaction (PCR) (Seow *et al.*, 1997). Degenerate PCR primers are designed from beforehand sequenced conserved

regions of a biosynthetic gene of interest. The screening of metagenomic libraries with these degenerate primers permits the identification and recovery of uncommon clones with homologous genes from the eDNA library (King *et al.*, 2009). Those models of screening can be applied to detect new derivatives of known metabolites by targeting pathway-specific BGCs. These primers can additionally be designed to identify novel structures by targeting conserved universal biosynthetic genes observed in a class of compounds. As illustration, degenerate primers for amplifying conserved regions of minimal polyketide (PK) ketoacylsynthase alfa (KSα) gene are used to amplify KSα sequences obtained in the Texas eDNA library (King *et al.*, 2009). KSα genes eDNA-derived were applied as probes to identify and recover the cosmid clones containing type II PKS biosynthetic gene clusters from the metagenomic library (**Figure 9**).

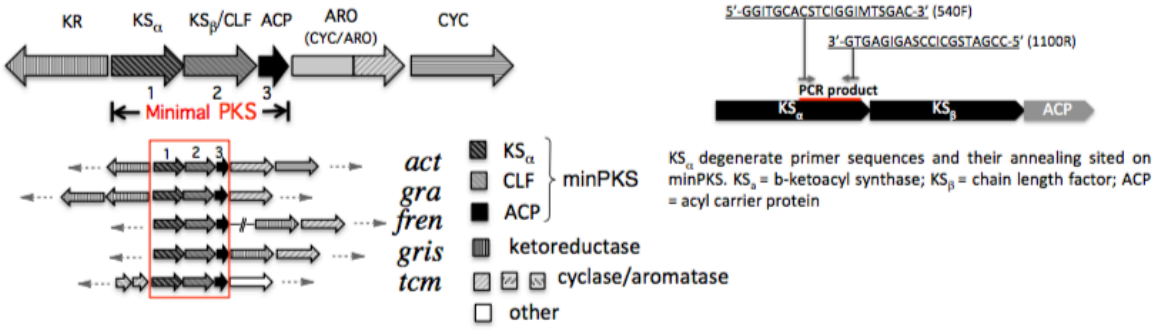


Figure 9: PCR-based screening for novel natural products
The type II PKS here described show an example of natural products gene cluster and how the PCR approach could be used to design primers targeting conserved genes.

The homology-based metagenomics screening has been proving to be an attractive approach to access the structural diversity encoded by uncultured bacteria. It has been successfully used to detect novel structures by targeting conserved broad biosynthetic genes observed in a class of molecules. One of the first groups of molecules targeted applying this approach was the iterative (type II, aromatic)

polyketides. Type II polyketides use a small number of biosynthetic enzymes to produce a distinct array of scaffolds, which are transformed by various downstream reactions (Zhang *et al.*, 2017). Although the biosynthetic gene clusters that encode the biosynthesis of this class of molecules can fundamentally differ in gene content, they all are encoded by a profoundly conserved minimal PKS formed of three proteins: ketosynthase alpha (KSα); ketosynthase beta/chain length factor (KSβ); and acyl carrier protein (ACP)(Robbins *et al.*, 2016). This “minimal PKS”, which is responsible for the iterative condensation of malonyl-CoA into a polyketide chain, maintains a conserved system and is consequently an excellent target to identify molecules using homology-based metagenomics (Hertweck, 2009).

As mentioned, BGCs of interest can be obtained from the particular pool of metagenomic libraries, sequenced, and analyzed in heterologous expression studies (Milshteyn *et al.*, 2014; Katz *et al.*, 2016). This concept was illustrated using a multi-million membered metagenomic library created using eDNA isolated from Arizona desert soil (Owen *et al.*, 2013). The screening utilized degenerate NRPS- and KS-targeting primers to identified tags concerning BGCs to encode congeners of a class of bioactive molecules. One BGC predicted a glycopeptide-like antibiotic and was obtained by heterologous expression using *Streptomyces toyocaensis*:ΔStaL as host. This provided the isolation of three novel glycopeptide congeners.

Strong promoters, such as the *erm*E* erythromycin resistance encoding promoter, can also be located in close of positive regulatory elements, or individual biosynthetic genes to activate the pathways (Laureti *et al.*, 2011; Kallifidas *et al.*, 2012; Luo *et al.*, 2013). Thus, resulting in the production of the secondary metabolites. This method has been used to produce 6-epialteramides, candicidins, antimycins (Luo *et al.*, 2013) and the 51-membered macrolide, stambomycin (Laureti

et al., 2011) and tetarimycin A (Kallifidas *et al.*, 2012) in a heterologous host. Some investigations have shown complete refactoring of biosynthetic pathways as a way of obtaining metabolites from cryptic cluster families, resulting in the heterologous expression of spectinabilin (Shao *et al.*, 2013) and several new polycyclic tetramate macrolactams (Luo *et al.*, 2013). Recently, yeast homologous recombination was used to production of the indolotryptoline and the antiproliferative agents, lazarimides A and B (Montiel *et al.*, 2015).

Using Trans-activation response element (TAR) and shuttled into *Streptomyces albus* for heterologous expression led the identification of seven novel epoxyketone protease inhibitors natural products: clarepoxins A-E and landepoxins A and B (Owen *et al.*, 2015). Some metagenomic discovery efforts were focused on the screening of eDNA libraries searching for Tryptophan dimmers (TD) BGCs using degenerate primers based on an alignment of chromopyrrolic acid synthase (CPAS) genes, which encode the enzymes involved in the dimerization of oxytryptophan (Chang e Brady, 2013; Chang *et al.*, 2013). These investigations produced the identification of some TD BGCs and the characterization of both known the antitumor molecule BE-54017 (Chang e Brady, 2011), and novel TDs (e.g., erdasporine (Chang, 2011), borregomycins (Chang e Brady, 2013). Some clusters were heterologously expressed to produce hydroxysporine: a pyrrolinone indolocarbazole core containing TD that had been described as a synthetic compound but never seen in nature. The second cluster, which was correlated with a phylogenetically novel CPAS sequence tag, encoded the new TD, reductasporine, which includes a new pyrrolinium indolocarbazole core (Nakano e Omura, 2009; Chang *et al.*, 2013). Thus, these investigation supports the premise that “outlier” sequence tags have a high potential of being associated with functionally novel gene clusters (Katz *et al.*, 2016).

This approach has led to the identification of a number of natural products with new or rarely ring systems such as arimetamycin A, tetarimycin A, arixanthomycin A, calixanthomycin A; UT-X26, AZ154 (King *et al.*, 2009; Feng *et al.*, 2011; Kallifidas *et al.*, 2012; Kang e Brady, 2013; 2014), further illustrating the utility of targeted screens in conjunction with a metagenomic natural product-mining pipeline to discover diverse new secondary metabolites (**Figure 10**).

The application of sequence‐based metagenomic natural product discovery allowed to use these metagenomic analyses to other environments. As example, the symbiotic bacteria found associated with organisms like marine sponges and tunicates have pointed to the characterization of diverse metabolites, such as the antitumor polyketides, bryostatins (Davidson e Haygood, 1999; Davidson *et al.*, 2001; Hildebrand *et al.*, 2004; Trindade *et al.*, 2015), onnamide (Hori *et al.*, 1993; Wilson *et al.*, 2014), and polytheonamides (Hamada *et al.*, 2010; Wilson *et al.*, 2014). In the case of the cytotoxic natural product calyculin A, it took a targeted sequence-based metagenomic method to identify the symbiotic organism and associated BGCs responsible for its production almost three decades after its initial discovery from extracts of the marine sponge *Discodermia calyx* (Kato *et al.*, 1986).

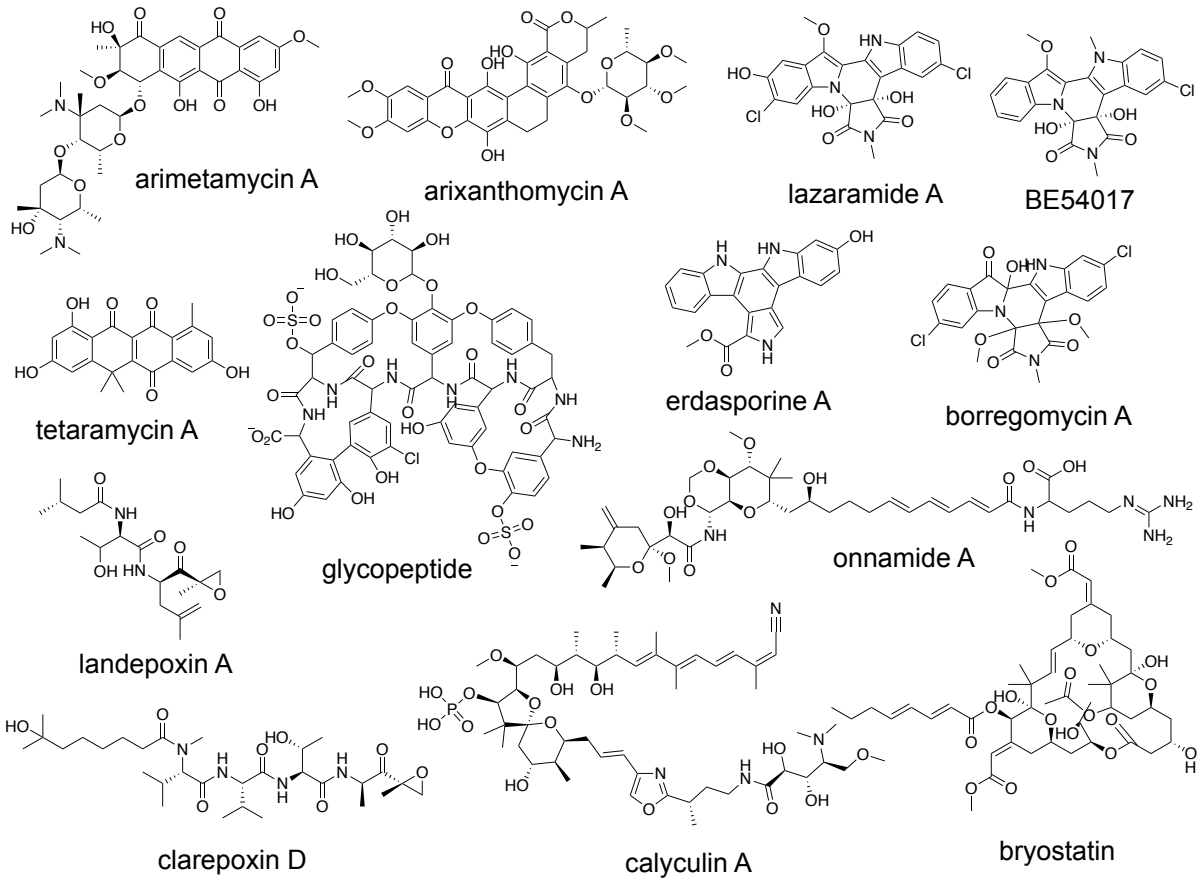


Figure 10: Some natural products molecules from homology screenings
The examples described were obtained from different metagenomic libraries. The homology were also detected using PCR-based approach

The analysis of eDNA libraries and metagenomic sequencing data for families of known BGCs is likely to be a valuable approach for recognizing new structural analogs of multiple bacterially derived antimicrobials, conceivably granting ready access to molecules with a refined spectrum of activity (**Figure 11**). Kang and Brady identified a collection of unique bioactive pentangular polyphenols, calixanthomycin A, arenimycins C-D, arixanthomycins A-C (Kang e Brady, 2013; 2014) through homology-based screening. Applying related strategy, the antibiotic fasamycins were isolated from soil eDNA and found to inhibit FabF of type II fatty acid biosynthesis (Feng *et al.*, 2011; Feng *et al.*, 2012). Expression of a pathway encoding the biosynthesis of a new erdacin derivative was identified with the unknown pentacyclic skeleton **(Figure 11)**. This confirmed the hypothesis that uncultured bacteria contains

a chemical diversity different from that detected in cultivated bacteria (King *et al.*, 2009).

A set of fluostatins and the methicillin-resistant *Staphylococcus aureus* (MRSA)-active antibiotic tetarimycin A have also been isolated from soil libraries (Feng *et al.*, 2010; Kallifidas *et al.*, 2012) (**Figure 11**). In extension to isolating type II polyketides, homology-based metagenomic screening has been fortunately used to detect novel tryptophan dimer analogs from eDNA libraries (Chang e Brady, 2011; Chang *et al.*, 2013; Chang e Brady, 2014; Chang *et al.*, 2015).

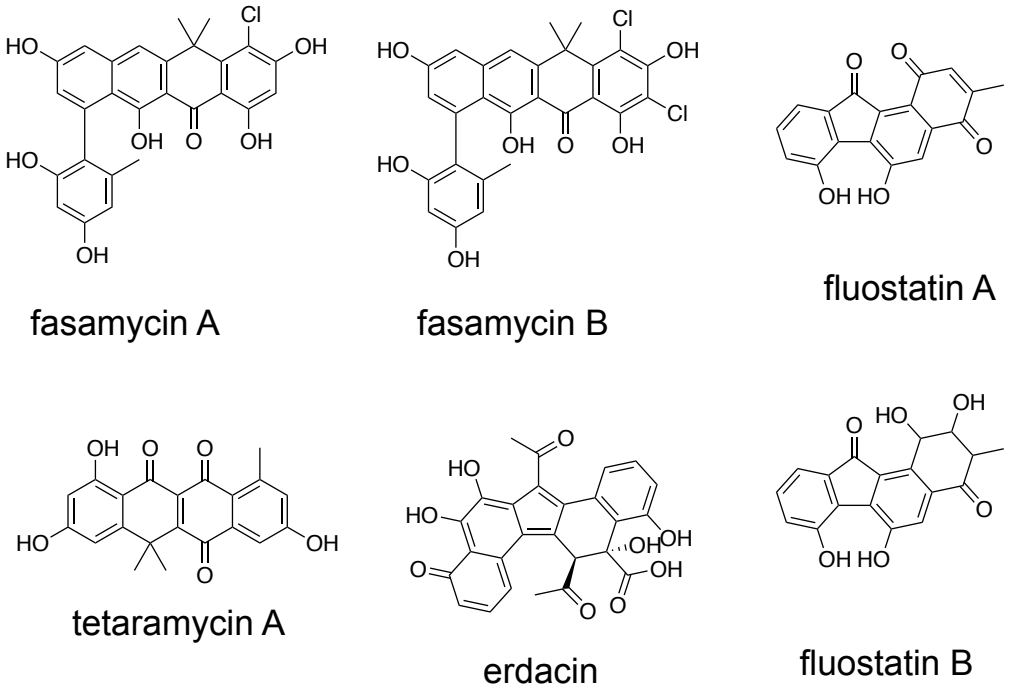


Figure 11: Metabolites isolated in homology-based metagenomic screening

Thus, homology-based metagenomic approaches have been used in a high number of studies to identify new derivatives of known compounds (Donia *et al.*, 2006; Ziemert *et al.*, 2010). Banik et al. applied a PCR screening targeting OxyC, a glycopeptide oxidative coupling enzyme, to detect new glycopeptide biosynthetic gene clusters from soil libraries (**Figure 10**) (Banik e Brady, 2008; Banik *et al.*, 2010). From this metagenomics library, novel mono, di- and trisulfated glycopeptide

congeners of teicoplanin aglycone were isolated (Figure 8) (Banik e Brady, 2008). In another homology-based metagenomic study, Kang et al. identified an eDNA-derived cluster encoding for arimetamycin A, an anthracycline obtained to be more efficient than clinically used natural anthracyclines against multidrug-resistant cancer cells (**Figure 10**) (Kang e Brady, 2013).

CONCLUSIONS

5 Conclusions

The detection of innovative natural products molecules have demanded new approaches, and the metagenomic methods provided a powerful way to access a brand-new environment in the natural products research. Here we have applied two methods to search different molecules in nature. In the first part, we created the SecMetPrimer package to design efficiently degenerated primers for natural products targets. SecMetPrimer designed 165 set of primers for polyketide, nonribosomal peptide, aminoglycosides, phosphono, and tryptophan dimmers families. Besides, the validation of the primers was developed using eDNA as template a metalibrary with 223 millions of clones. For this work, the annotations of AHBA and tryptophan dimmers were developed. The data revealed a new clade of AHBA and tryptophan dimmers in the phylogenetic analysis, showing a new group of those molecules. We also described the presence the ansamycins ansamitocin, ansatrienin, chaxamycin, divergolide, hygrocin, macbecin, napthmycin, rifamycin and rubradirin. In the second section, we have used the biogeography approach to mining the information about biosynthetic gene clusters in Atlantic Forest, Cerrado, and Marine environment samples. The data revealed that no strong correlation of nonribosomal peptides was observed across the samples. This suggested that the microbial biodiversity is much complex than expected. Additionally, the search for clinically relevant nonribosomal peptides revealed the presence of the vancomycin-like glycopeptides in samples from Cerrado and Marine sediment. This is the first study to apply a bioinformatic approach to automatically design degenerated primers capable of amplifying unknown groups of natural products. In the same way, the use of biogeography approach to prioritizing samples for further metagenomic screening is novel, and this

is the first time this type of description was provided. In conclusion, this study demonstrated the creation and use of a bioinformatic pipeline to reach unknown groups of natural products and the biogeography of soil samples collected in different Brazilian biomes.

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