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Integrated Approaches for Discovering Novel Drugs From Microbial Natural Products

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Summary

Historically, nature has provided the source for the majority of the drugs in use today. This owes in large part to their structural complexity and clinical specificity. However, only a small percentage of known microbial secondary metabolites have been tested as natural-product drugs. Natural-product programs need to become more efficient, starting with the collection of environmental samples, selection of strains, metabolic expression, genetic exploitation, sample preparation and chemical dereplication. A renaissance of natural products-based drug discovery is coming because of the trend of combining the power of diversified but low-redundancy natural products with systems biology and novel assays. This review will focus on integrated approaches for diversifying microbial natural-product strains and extract libraries, while decreasing genetic and chemical redundancy. Increasing the quality and quantity of different chemical compounds tested in diverse biological systems should increase the chances of finding new leads for therapeutic agents.

Key Words: Diversity; microbial natural products; drug discovery; redundancy; dereplication; synergy.

1. Introduction

The most well-known examples of natural product are antibiotics *(1)*. The "Golden Age of Antibiotics," from the 1940s to the 1970s, was sparked by the serendipitous discovery of penicillin by Alexander Fleming in 1928 and its development by Chain and Florey in the 1940s. Another remarkable milestone in the medicinal use of microbial metabolites and their derivatives was the introduction of the immunosuppressants cyclosporin A, FK-506 *(2)*, and rapamycin *(2,3)*. Other examples are the commercialization of the antihyperlipidemic lovastatin and the recent discovery of guggulsterone *(4)*. Microbial natural products have also been developed as antidiabetic drugs, hormone (ion-channel or receptor) antagonists, anticancer drugs, and agricultural and pharmaceutical agents *(5)*. Microorganisms not only produce secondary metabolites that affect cell growth, but also accumulate bioactive compounds that interact with valuable targets of cell metabolism and signaling that are not directly correlated with cell death *(6)*.

Drug discovery strategies for pharmaceutical and agrochemical applications are in a revolutionary period *(7)*. The completion of the Human Genome Project and the elucidation of dozens of microbial pathogens genomes have provided thousands of disease-

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related targets for screening. Automated instrument systems, robots, high-throughput screening (HTS) platforms, and high-throughput chemistry have provided powerful tools for screening large compound libraries in a cost-effective manner. Combinatorial chemical, synthetic chemical, and natural-product libraries provide abundant resources for target-based screening. The success and failure of drug discovery is coupled to the novelty and meaningfulness of the applied biological test systems as well as the amount and structural diversity of the test compounds available *(8)*.

In the early to mid-1990s, combi-chem companies attempted to fill the void with large numbers of new molecules. Unfortunately, it appears that the chemistry employed did not create sufficiently diverse or pharmacologically active molecules. It is clear that the future success of the pharmaceutical industry depends on the combining of complementary technologies such as natural-product discovery, HTS, genomics and proteomics, combinatorial biosynthesis, and combinatorial chemistry. The process of drug discovery for therapeutic and preventive medicines is facilitated by increasing knowledge of biological mechanisms, such as treatment efficiency, potential side effects, and the growing threat of drug resistance. A large amount of disease-relevant protein targets have been identified and validated from genomics, proteomics, and systems/computational biology approaches. Novel targets and novel HTS assays and measurement systems are emerging to allow more sensitive, reliable, and low-background searches for new potential drugs among natural products.

The advantages and challenges of natural product-based drug discovery as compared to its synthetic chemistry counterpart are summarized as follows:

Advantages:

- Natural products offer unmatched chemical diversity with structural complexity and biological potency *(9)*.
- Natural products have been selected by nature for specific biological interactions. They have evolved to bind to proteins and have drug-like properties.
- Natural products are a main source of pharmacophores. Drugs such as cyclosporin A and FK-506 are not only active as immunosuppressants but also as antiviral, antifungal, and antiparasitic agents.
- Natural-product resources are largely unexplored. Novel discovery strategies will lead to novel bioactive compounds. Natural-product extracts are complementary to synthetic and combinatorial libraries. About 40% of natural-product diversity is not represented in synthetic compounds libraries.
- Natural products can guide the design of synthetic compounds *(10)*.
- Research on natural products has led to the discovery of novel mechanisms of action—for example, those of immunosuppressants and guggulsterone *(4)*.
- Natural products are powerful biochemical tools; they serve as "pathfinders" for molecular biology and chemistry, and in the investigations of cellular functions.

Main challenges:

- The lack of systematic exploitation of ecosystems for the discovery of novel microbial compounds had resulted in random sampling and has missed the true potential of many regions *(11)*.
- Little effort has focused on the isolation and cultivation of less culturable microorganisms. The discrepancy between the number of microbes detected by molecular methods

and the number of strains in culture, demonstrates that there remains a relatively untapped source of novel strains in all ecosystems *(12)*.
- The selection of strains has traditionally been based on morphology, rather than on the more powerful approaches of chemical diversity and genotype *(13)*.
- Lack of dereplication has resulted in a redundancy of strains and compounds within many natural-product extract libraries *(14)*.
- The characterization and isolation of active compounds from natural-product extracts are extremely labor intensive and time consuming *(15)*.
- The production of adequate quantities of the active compound needed for drug profiling may require extensive media optimization and scale-up *(16)*.

This review concentrates on the challenges of efficiently diversifying a library of microbial compounds, and does not deal with other problems such as preparation of samples, scale-up, chemical identification, and so on. Systematic approaches to maximize the biodiversity of microorganisms within a natural-product library are discussed from the following three perspectives: (1) isolation and selection of samples from diverse ecosystems; (2) manipulating microbial physiology to activate microbial natural-product biosynthetic machinery; and (3) genetically modifying strains for production of unnatural microbial natural products. By manipulating all three of these approaches, the diversity of an extract collection can be maximized, and in doing so, the chance of finding a "hit" can be increased. The quality of a microbial natural-product library is built on the dynamic equilibrium between diversification and reducing redundancy of microbial natural products. Therefore, strategies for obtaining high quality of a microbial natural-product library are discussed here.

2. Sample Collection and Selection From Diversified Ecosystems

Existing microbial natural-product drugs were originally isolated from all over the Earth. Microbes can sense, adapt, and respond to their environment quickly and help compete for defense and survival by generating unique secondary metabolites. These compounds are produced in response to stress. In diversifying microbial natural-product extract libraries, the greatest influence will undoubtedly be the genetic diversity of strains. By maximizing the types of samples collected and diversifying the isolation strategies, a highly diverse microbial collection can be generated.

2.1. Ecosystem Rationale

Collecting environmental samples for isolation of interesting microorganisms has often been conducted without defined strategies *(17)*. Such programs need to take into consideration the biogeography of ecosystems, number of samples collected, and isolation procedures. It is important to increase the number and diversity of sampling sites *(18)*, and it is especially important to look at underrepresented sites. Diverse regions such as the deep subsurface, the deep sea, and sites that have extreme temperature, salinity, or pH often generate novel microorganisms and therefore provide the potential for novel compounds *(19)*. Temperate ecosystems should not be excluded, because they also have the potential to provide many novel species, especially when novel isolation strategies are used. Cyclosporins, rapamycin, penicillin, and rifamycin, among others, were isolated from microorganisms collected in temperate regions.

It is still debated whether most microorganisms are cosmopolitan or endemic to specific geographic areas. There is a lack of detailed information in the field of geographical distribution of microorganisms *(19)*. In some cases, the presence of an endemic species can be detected; for example, several groups of bacteria appeared to be endemic to an ice microbial community *(20)*. However, the definition of a microbial species is difficult, especially for prokaryotes, which exchange parts of their genomes with sufficient ease to make difficult the biologically meaningful definition of a species *(21)*. In order to increase the chance of constructing a library of microorganisms with high diversity, the first step is to consider different geographic areas, including biodiversity hot spots *(22)*. There are more compounds in nature than possible molecular targets. One should concentrate on sampling in regions with different climates, fauna, and flora.

It is important to analyze properly the various ecosystems of a region. For example in Massachusetts, there are 13 eco-regions, from the Berkshire Highlands to the coastal regions of Cape Cod (http://www.state.ma.us/mgis/eco-reg.html), each of which has various subecosystems. Microbiologists should work closely with botanists and ecologists to obtain as many different samples and microorganisms as possible from one ecosystem to maximize the likelihood of finding novel strains and in turn novel chemicals. In almost all ecosystems, no matter how harsh, a group of organisms will grow and thrive. In these unique sites, we can expect to find unique metabolic pathways that have evolved to allow microorganisms to adapt and survive.

With the discovery that microbial symbionts were driving the metabolism of tubeworms in deep-sea hydrothermal vents, it was realized that an oasis of rich diversity could be found even in areas that were thought to be devoid of life. The deep sea, one such ecosystem, is actually a rainforest with a diversity of more than 10 million species, more than 60% of which are unknown *(23)*. In addition to the open ocean, there are diverse and dynamic areas such as mangroves, coral reefs, hydrothermal vents, and deep-sea sediments in which to search for microbes. Natural products have been isolated from marine invertebrates such as sponges, tunicates, mollusks, and bryozoans *(24)*. This not only demonstrates the numerous opportunities the oceans provide for discovering new compounds, but also validates the pharmacological value of exploring the oceans for novel compounds. There are some concerns about the isolation of marine microbes. Some researchers claim that this resource is not thoroughly explored because these organisms are hard to maintain in the laboratory environment. However, one successful case was the recent discovery of a new genus of actinomycetes found only in the marine environment, i.e., *Salinospora (25)*. One isolate produces salinosporamide A, a potent anticancer agent. Thus, the oceans can no longer be ignored *(26)*. Other regions that warrant further study are locations with extremes of pH, temperature, and salinity.

A defined sampling strategy must be adopted. To comprehensively explore a particular site, multiple discrete locations within the site must be sampled. Many types of samples should be selected in one ecosystem—e.g., soils, sediments, organic material, dung, dead animals, plants, and lichens. Soil still remains an important source because it carries higher populations of microbes than any other habitat *(27)*. DNA community analysis has proven that the number of types of organisms found within a microbial community is much higher than previously thought. One analysis of the reassociation kinetics of total bacterial DNA in a 30-g soil sample suggested that it contained more than 500,000 species *(21)*.

Plants and lichens offer niches for interaction between microorganisms and eukaryotic cells. Many natural products initially isolated from plants and animals were actually produced by microbial symbionts found within the tissue of the host *(23)*. In some incidences where the microorganism or symbiont could not be cultured axenically, the genes responsible for the production of the active compounds were attributed to the microorganism *(28)*.

Endophytes are microorganisms including unicellular bacteria *(29)*, actinomycetes *(30)*, and fungi *(31)* that spend part or all of their life cycle colonizing, either inter- or intracellularly, the healthy tissue of a plant *(32)*. Almost all vascular plants and mosses examined so far have endophytic bacteria or fungi within their tissues *(29)*. The number of strains found within the plant tissue can vary from a few to several hundred per plant. This relationship between the microorganism and the plant can range from mildly phytopathogenic to symbiotic. Endophytes produce a range of compounds *(16)*, some of which help the plant to survive and thrive in its ecosystem and some that help it fight off infection *(33)*. Plants therefore provide an obvious source for isolation of microorganisms that could potentially produce novel natural products. Of special interest are the large number of alkaloids and taxol produced by endophytic fungi *(16)*.

Lichens, symbiosis between fungi and cyanobacteria, are another source of microorganisms living in a unique and competitive environment. In each lichen sample, the fungus forms a thallus or lichenized stoma *(34,35)*. Furthermore, in addition to the symbiotic fungal strains, other fungi and bacteria live as endophytes inside the lichens, or as epiphytes on the lichens. The fungi within the lichen often produce unique secondary metabolites. Over 800 lichen secondary metabolites have been collected so far.

2.2. Isolation Strategies

Microbial diversity in the environment is far greater than reflected in most strain collections, due to the number of organisms that cannot be cultured using standard culture conditions *(36)*. Therefore, a vast majority of microorganisms in many samples remain unexplored. Molecular techniques allow detection of organisms that were missed using culture-dependent methods. Culture-independent methods, such as DNA clone libraries, have allowed identification of vast numbers of new organisms that are different from anything previously cultured. It is estimated that as few as 0.1–1% of the organisms living in the biosphere have been cultured and characterized in the laboratory setting. In one study, approx 10^7 bacteria were counted in 1 g of soil *(37)*, but as few as 0.1% were culturable using standard culture techniques. The other 99.9 % of the population may represent novel genetic diversity *(14)*, and may produce novel natural compounds. In 1987, when Dr. Carl Woese *(116)* proposed the five-kingdom phylogenetic tree, the bacteria were divided into twelve groups. The initial evaluation was done primarily with bacteria in culture. By 2000, the number of groups had expanded to 36, of which 13 do not have a representative in culture *(38)*. Approximately 6000 bacterial species have been described, but the number of bacteria that exist in nature is predicted to be as high as 600,000 *(39)*. The situation may even be more extreme for fungi. The currently accepted number of described species of fungi is 72,000, but the estimated figure of fungi that exist in nature is 1.5 million. This suggests that there are diverse novel microorganisms in the natural environment that could be used as sources for drug discovery. The argument against putting effort into culturing less-culturable organisms is that it is very time consuming and the techniques used for one organism

may not be applicable to others. With this in mind, several biotech organizations are now seeking means to harness the potential of these less-culturable strains.

Microbial community analysis has revealed that the microorganisms in culture not only represent a small part of the population, but may not be the most prevalent in the natural environment *(12)*. It is expected that one of the largest efforts in the next decade will be exploring means to culture less-culturable organisms. It is thought that the reason for the enormous discrepancy between the total viable cell counts and those of culturable cells may be due to the following: (a) cell damage by oxidative stress, (b) formation of viable but nonculturable cells, (c) inhibition by high substrate concentrations, (d) induction of lysogenic phages upon starvation, and (e) lack of cell-to-cell communication in laboratory media *(41)*. Two main approaches have been used to enhance the resuscitation of less-culturable strains. The first is the addition of cell-signaling molecules and the second is the use of oligotrophic isolation media.

Microorganisms use pheromones to communicate with each other, both within and across species *(42)*. Microorganisms may require signaling from other organisms in order to grow, even if provided with the appropriate nutrients. The addition of growth factors to culture medium has been used successfully to increase the resuscitation of greater numbers of microorganisms and thus higher microbial diversity. The addition of pyruvate or catalase to reduce oxidative stress during isolation can increase the numbers and diversity of strains isolated *(43)*. The addition of cyclic AMP *(44)* and *N*-acyl-homoserine lactones have both been shown to increase the resuscitation of starved cultures under laboratory conditions *(41)*. In enterobacteria, cAMP is involved in the regulation of the majority of the genes expressed under starvation, including those coding for high-affinity sugar-transport systems *(45)*.

A second approach for increasing the resuscitation of less-culturable strains is the use of oligotrophic isolation media. It has been well documented that conventional media have extremely high concentrations of complex organic compounds compared with those present in the natural environment. Most isolation media allow for growth of only a selected group of strains and inhibit the majority of the natural population. Oligotrophic media not only allow the growth of less-culturable microorganisms but also prevent the overgrowth of fast-growing "microbial weeds" *(46)*. Using unamended site water as a growth medium, unique populations of microorganisms have been cultured *(47)*. A variation of this method is encapsulation of single cells within gel microdrops that contain low-nutrient media *(46)* or within specialized growth chambers incubated in site water *(42)*.

One argument against applying less-culturable strains to a drug-discovery program has been that they could not be cultured at a high enough cell density. The argument for including them is that although they may initially require the addition of growth factors or oligotrophic growth conditions, there is evidence that once cultured, the organisms can be grown out in nutrient-rich media. Using 960 cells cultured in microdrops, 67% of the cultures were able to grow to densities of $>10^7$ cells/mL *(46)*. This allows the cells to be cultured in a manner that could be easily applied to drug-discovery platforms.

An alternate approach to access unculturable microorganisms is to clone the DNA directly from uncultured microorganisms (*see* **Subheading 3.1.).**

3. Manipulating Microbial Physiology to Activate Microbial Natural-Product Machinery

In order to exploit the true potential of microorganisms, the physiological growth conditions used for generating extracts need to be diversified. And microbial metabolism can be influenced to produce qualitatively and quantitatively different chemical compounds. The physiology of secondary metabolism has often been neglected. Very few of the regulatory features of secondary metabolism have been elucidated *(48)*. The global situation in physiological regulation is very complex, as a result of the variety of microbes, the variety of biosynthetic pathways, and the variety of controls. Environmental conditions remain, however, a key element in the discovery and production of secondary metabolites. Strategies have to be developed in order to exploit the full metabolic potential of each microorganism in order to maximize chemical diversity. Biochemical pathways, induction, and regulation of secondary metabolism by internal molecules (such as the autoregulators) have been reviewed previously *(49,50)*.

3.1. Various Optima

The optimal conditions for biosynthesis of secondary metabolites are usually not identical to the ones for growth. In general, the optimal zones are narrower for secondary metabolite production. Physiological regulations vary with different microorganisms and different metabolic pathways. The qualitative and quantitative aspects for secondary metabolite production must be taken into consideration.

There are usually differences between the optimal carbon sources for growth and those that are good for secondary metabolism *(51)*. For example, glucose is an excellent carbon source for growth in most cases, but depresses the production of a series of secondary metabolites such as actinomycin, cephalosporin, ergot alkaloids, and tylosin. However, glucose does not interfere with the production of aflatoxin, aminoglycosides, or chloramphenicol *(52)*, and the production of anticapsin by *Streptomyces griseoplanus* is maximal at a concentration of glucose as high as 100 g/L *(53)*.

Secondary metabolic pathways are often negatively affected by nitrogen sources favorable to rapid growth. For example, ammonium salts inhibit the production of cephamycin, fusidin, and rifamycin *(54)*; however, some biosynthetic pathways are not affected, such as that for pyrroindomycins in *Streptomyces rugosporus (55)*. Optimal production of gibberellic acid by *Gibberella fujikuroi* in a defined medium requires a concentration of 22.5 m*M* ammonium sulfate. Complex natural sources of nitrogen such as soybeans and casamino acids are also good. The influence of amino acids on secondary metabolite production is very variable and can depend on the precursor or the natural inducer. Inorganic phosphate suppresses the synthesis of many secondary metabolites. Thus, the optimal phosphate concentration needed for production of secondary metabolites is generally lower than that required for growth. However, the optimal concentration can vary drastically between strains. The concentration can be as low as 0.08 m*M* for the synthesis of bacitracin by *Bacillus licheniformis*, or as high as 8 m*M* for the production of novobiocin by *Streptomyces griseus (56)*. In some incidences, high phosphate concentrations can even induce the biosynthesis of some metabolites *(57)*. Secondary metabolism often requires trace elements such as iron, zinc, and manganese. Once again, the optimal concentrations vary from process to process, but often range from less than 0.1 to 1×10^{-3} *M (58)*.

Optimal temperatures for the production of secondary metabolites are, in general, lower than for growth, but can vary considerably. For example, a temperature of 21°C is optimal for biosynthesis of cyclosporin by *Tolypocladium inflatum*, 25°C is optimal for the synthesis of streptomycin by *Streptomyces griseus*, and 28°C is best for nebramycin formation by *Streptomyces tenebrarius*. Most of the known secondary metabolites are produced under standard aeration conditions, but some require lower and some higher dissolved oxygen concentrations *(59)*. Extremely high aeration is required for optimal production of secondary metabolites by *Streptosporangium (60)*.

Incubation time is another key point and is dependent on the growth characteristics of the microorganism and the culture conditions. For example, actinomycetes can vary from 3 d for the maximum production of arylomycins by a strain of *Streptomyces (61)* to 12 d for maximum production of pramicidin S by a strain of *Actinomadura (62)*. The addition of adsorbents such as XAD-16 resin to liquid cultures can also enhance the concentration of secondary metabolites produced *(63)*. Most programs for the discovery of novel metabolites from microorganisms use liquid shaken cultures for cultivation of microorganisms. This provides an easy and well-controlled system. Solid-phase fermentation allows the biosynthesis of other metabolites, mainly related to the sporulation process *(64)*. Both types can be scaled up effectively *(65)*.

3.2. Selection of Culture Conditions

The optimal conditions for secondary metabolite production vary from microbe to microbe. The composition of media and the culture conditions have a great impact on the production of secondary metabolites. In a discovery program, one is working with a large series of unique and ubiquitous microorganisms. Multiple conditions are necessary in order to allow the expression of secondary metabolites. Both static and shaken liquid cultures should be incorporated. Different incubation temperatures must be chosen. Addition of elicitor compounds such as heavy metals, oils *(66)*, microbial or fungal cell-wall components, and dimethyl sulfoxide (DMSO) *(67)* can increase the biosynthesis of certain secondary metabolites. The media can include carbon and nitrogen sources at different concentrations, as well as other nutrients such as phosphate and trace elements or elicitors at various levels, and using a Greco-Latin square format. The goal is to have a good ratio between the number of strains (genetic diversity) and the number of culture conditions (metabolic expression) for each microbe in one collection.

Another powerful tool is the preselection of strains based upon growth in a series of media in microcultures or in small vials *(68)*. A standard format that is amenable to automation allows more than 20 media to be easily tested with each microorganism, at different temperatures, and in liquid as well as on solid media. This allows the selection of the best conditions for each strain, which can then be used to scale up to get larger volumes required for initial structure determination. This system is also adequate for a quick optimization program.

For each group of strains, a series of conditions can be chosen incorporating both shaken liquid cultures and stationary solid cultures. Typically, five media types are used for each batch of cultures, and three to five incubation conditions are chosen to include various temperatures. In order to enhance the chance of success in such a random process, the conditions used for one group must be rotated. The results of chemi-

cal profiling and scores in screening should be constantly analyzed in order to improve the system.

3.3. Physiological Exploitation of Talented Strains

Some microorganisms are able to produce a variety of compounds from different chemical families and are termed *metabolically talented (69)*. Most of the recently described microbial compounds are produced by actinomycetes, mostly *Streptomyces* strains, and by saprophytic filamentous fungi. One metabolically talented microorganism, *Streptomyces* sp. strain Go.40/10, synthesizes at least 30 different secondary metabolites, many of which are new compounds *(70)*. Some strains can synthesize more than 50 compounds, which can be detected only by classical chemical methods. Myxobacteria and fungi are also considered to be talented microbes *(71)*.

The genomes of actinomycetes (8 Mb) *(72)*, fungi (13–42 Mb) *(73)*, and myxobacteria (12 Mb) *(74)* are much larger than needed for all basic functions. Therefore, it is widely thought that part of the genome may contain genes for alternative metabolic pathways. For example, *Streptomyces coelicolor* A(3)2 is designated as a potent producer of secondary metabolites. It produces methylenomycin, prodigiosin, actinorhodin, and a calcium-dependent antibiotic. In addition, several formerly unknown gene clusters (polyketide syntheses type I and II, nonribosomal peptide synthases) have been found in its genome *(75)*. In *Streptomyces avermitilis* ATCC 31267, the producer of avermectin, 24 additional gene clusters have been sequenced *(76)*. Genomic data have suggested that the myxobacterium *Stigmatella aurantiaca* DW4/3-1 has a much broader capacity to produce a much broader group of natural products than those isolated to date from this organism *(77)*. Genes responsible for production of many compounds can be found in the genomes of nonproducing strains. The questions arise: Are these genes nonfunctional? Are the detection methods not powerful enough? Are these genes not expressed under standard growth conditions? Do the genes require external signaling to turn them on? More efficient detection methods, biochemical assays such as capillary electrophoresis *(78)*, or chemical methods will allow the discovery of large numbers of novel compounds *(79)*. As briefly described in **Subheading 3.1.** small changes in the culture conditions can have a major influence in the spectrum of secondary metabolites synthesized. For example, the fungal strain Sphaeropsidales strain F-24'707 is a producer of the antifungal compound cladospirone bisepoxide. When this strain was grown in a combination of different media and cultivation types, eight new spiro naphthalenes were isolated. There were previously only six known members of this class of compounds. The addition of inhibitors, such as tricyclazole, inhibited some pathways and therefore allowed the production of other compounds, such as two new spirobisnaphthalenes and a rare macrolide, mutolide *(79)*.

Many microorganisms do not readily express natural-product gene clusters when grown in the laboratory *(75)*. We have to find the right physiological signal to stimulate the molecular machinery. A systematic fermentation program should be conducted with "talented" strains and with representative strains of poorly known genera in order to maximize the number of compounds produced by each strain. Such an approach should include:

- Cultures grown in shaken liquid vessels and on solid media.
- Cultures grown with media of different composition

- Incubation at two or more temperatures.
- Incubation at two or more shaker speeds.
- Incubation for at least two different time periods.
- Media with at least two pH levels.
- Absorbents, enzyme inhibitors, elicitors, precursors, precursor analogs, and high concentration of salts should be added to the most productive fermentation media.

After selection of one or two potent media, the influence of the other factors can be analyzed using an experimental design, such as fractionated factorial or Plackett–Burman design *(80)*.

3.4. Co-Cultivation

Microbial communities also hold potential for the production of novel compounds. In nature, microorganisms do not exist alone; they are part of tiny ecosystems. There is expected to be diverse signaling and cross-feeding going on between organisms that will elicit production of novel compounds. Although the longstanding argument against this type of research has been that getting a stable mixed culture is almost impossible, it may provide a means for exploiting the true potential of the consortia as a whole. An example of using co-cultivation of two microorganisms producing related products has been suggested as a suitable way towards diversification of microbial structures *(81)*.

4. Genetically Modifying Strains to Produce "Unnatural Microbial Natural Products"

4.1. Expressing the Heterologous Metagenome in a Surrogate Host

Culturable organisms provide only a finite pool of secondary metabolites *(82)*. One approach to maximize the diversity of natural-product extract libraries has been to access the DNA directly from uncultured microorganisms. DNA can be isolated directly from an environmental sample, digested into large fragments with restriction enzymes, and cloned into an artificial vector *(14)*. The vector is then transformed into a surrogate host *(83)*. Environmental DNA libraries can be prepared with large fragments of DNA from a wide range of uncultivated bacteria within an environmental sample *(36)*. This is described as screening the metagenome, the genomes of the total microbiota in an environmental sample *(84)*. The recombinant approach thus obviates the need for culturing diverse microorganisms and provides a relatively unbiased sampling of the vast untapped genetic diversity present in various microenvironments. As an additional advantage, the genes encoding a product of interest are already isolated and can be analyzed using the tools of bioinformatics, thus providing a potential boost to the efforts of analytical chemists to identify the product. Furthermore, the possibility of regulating the expression of isolated environmental gene clusters or combining them with genes for other pathways to obtain new compounds could furnish a further advantage over traditional natural-product discovery methodologies *(113)*. However, it must be noted that these biosynthetic and regulatory genes could be dormant in the host, and optimal induction conditions may be required for the production of novel natural products.

Advances in DNA-sequencing and bioinformatics technologies now make it possible to rapidly identify the clusters of genes that encode bioactive compounds and to make computer predictions of chemical structure based on gene sequence information

(85). A high-throughput genome-scanning method has recently been developed that allows discovery of metabolic loci, independent of expression. Genome sequence tags (GSTs) are genes involved in natural-product biosynthesis. These GSTs are used as probes to screen for the presence of these genes within a clonal library. Any clone that contains a GST can then be screened for novel natural-product gene clusters. More than 450 natural-product clusters have been identified in this manner *(85)*.

4.2. Gene Mixing (Combinatorial Biosynthesis)

The traditional way to diversify unnatural microbial natural products is by random mutagenesis or by culturing microbes with nonnatural precursors. However, the discovery in prokaryotes that the genes for natural products are usually clustered, made it possible to clone an entire pathway into a vector *(14)*. Many natural-product genes are modular and produce multifunctional enzymes. They have a high degree of plasticity. By interchanging and moving around genes within these clusters, hybrid enzymes can be produced that are capable of synthesizing an unlimited set of new molecules *(82)*.

The modular nature of many secondary metabolite genes provides an ideal system to genetically engineer formation of unnatural microbial natural products by incorporating genes from different pathways. An example of such an approach involves the polyketide synthases (PKSs), which are large, multi-domain enzymes that produce polyketides including antibacterials, immunosuppressants, and cholesterol-lowering agents *(86)*. PKSs are encoded by a cluster of continuous genes and have a linear modular organization of similar catalytic domains that build and modify a polyketide backbone *(87)*. Microbial genes can be engineered to produce enzymes with novel catabolic activities *(82)*. The cloning of biosynthetic pathway genes from *Streptomyces* allowed the production of novel compounds by mixing the antibiotic systems of different antibiotic-producing strains *(72)*. Novel compounds were produced by gene transfer between strains producing the isochromanquinone antibiotics actinorhodin, granaticin, and medermycin *(88)*. This pioneering work has been developed by many others for the production of novel enzymes and unnatural natural products *(83,89,90)*. These modular PKS clusters have been manipulated through introduction of different loading domains that specify a branched chain or cyclic substrate and direct inactivation or insertion of individual catalytic domains to produce new enzymes *(87)*. Combinatorial biosynthesis has also been used effectively to generate novel compounds and enzymes in type I and type II PKS systems. This type of approach can be applied to many other modular enzyme systems *(91,92)*.

Genetic engineering and pathway modification will undoubtedly be important in strain optimization in the future. These methods provide a targeted approach for construction of novel pathways and in turn the potential of novel natural products *(93)*.

5. Monitoring Diversity by Dereplicating Microbial Strains and Chemical Extracts

The cost of screening microbial natural products is high, and false positives waste the limited resources available for isolation and structural characterization. In order to increase the output, it is important to spend more time upfront on the prescreening of strains and extracts. In order to focus on the extracts of most interest as quickly as

possible, and to avoid repeatedly isolating the same common natural products, efficient dereplication process are of the utmost importance. One may argue that high-throughput screening, in which thousands of extracts can be screened in little time, makes the use of duplicate cultures redundant. However, we must consider that screening a more diverse set of cultures will increase the diversity of the extract library and in turn increase the chance of finding a "hit." Dereplication of the extracts can be done either at the level of the microorganism or the chemical extracts. Database-linking of microbial genetic taxonomy with extract diversity is extremely important (**Fig. 1**). Cluster analysis of an unknown sample and comparison of its taxonomy and extract chromatography to internal databases as well as published literature will provide valuable information to determine whether an extract and/or activity is novel or not.

5.1. Characterization and Selection of Microbial Strains

Ecopia BioSciences Inc. developed an automated genomics platform that predicts the chemical structures of natural products by reading the sequences of the gene clusters that direct their synthesis. By surveying the genome, all of the natural products that a microorganism can make are identified before fermentation studies begin, and the downstream production and purification strategies are specifically tailored to isolate likely new chemical entities (NCEs) and avoid the re-isolation of known compounds. The integration of new genomics technologies greatly increases the efficiency of discovery and makes it possible to build a robust pipeline of NCEs from a small collection of microorganisms, providing a new paradigm for natural-product discovery *(85)*. They scan the genomes of selected microorganisms that were reported to produce known, structurally diverse natural products, to build a database of gene clusters covering the full range of natural-product chemical diversity without sequencing entire genomes. This enables a strategy to prescreen in mini-scale for one microbe of interest in many more media and growth conditions, and look only for a specific compound property, such as molecular weight, ultraviolet (UV) absorbance, and lipophilicity predicted by the specific gene clusters. Then scale-up technologies could be used to identify and purify the specific compounds. The knowledge of the biosynthetic pathway of the compounds will guide rational design or mutagenesis to improve their yields.

Any library of microorganisms is likely to have a high number of duplicate strains. Although identical strains from the same site may be excluded on isolation, many strains of the same species may be collected over time from a wide range of collection sites. Although these strains may be useful later in strain optimization, they are redundant and costly in the initial screening phase and decrease the probability of finding novel compounds. Thus, it is important to dereplicate the culture collection. However, one must consider that strains of the same subspecies may produce different compounds.

Many methods can be used to dereplicate cultures, but molecular techniques are especially well suited to this type of analysis. However, bacterial taxonomists have not yet reached a consensus for defining the fundamental criteria of biological diversity to the species *(94)*. Prokaryotes exchange chunks of their genomes too frequently to make any meaningful species definition *(21)*. An accurate definition of a fungal species is also problematic. Certainly, for all types of microbes, the basic unit is the "ecotype" *(94)*, also called the "geovar" *(20)*. This is the reason why dereplication of strains has

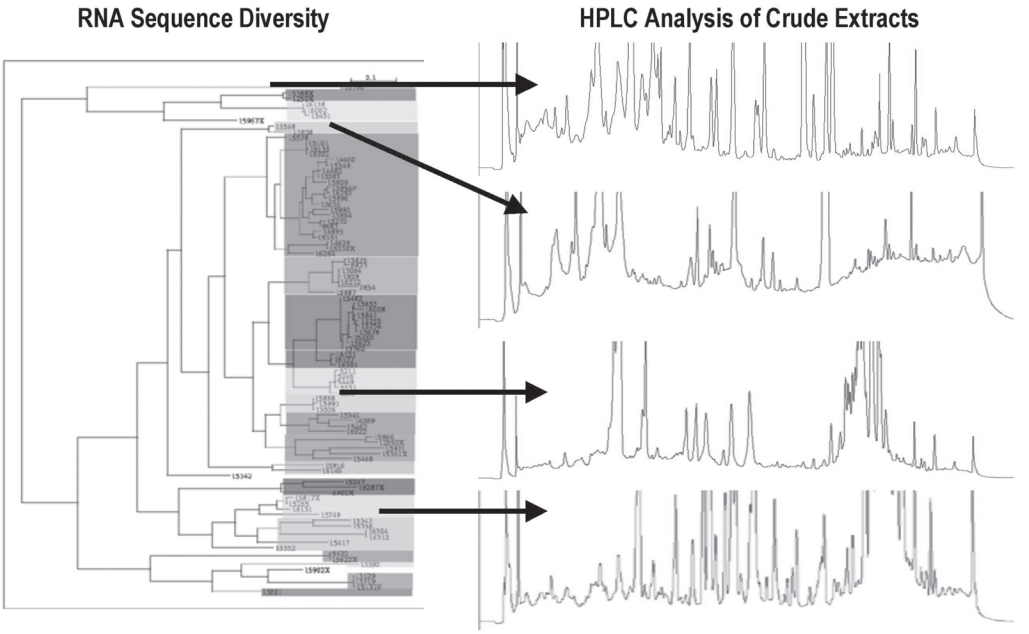

Fig. 1. Database linking microbial genetic taxonomy with extract diversity. The microbial genetic tree is generated based on ribosomal RNA sequence analysis. Chemical extract chromatography is linked to the taxonomy data by a bio-informatics approach.

to be considered only within populations isolated from a particular geographical/ecological region. A strain of *Streptomyces hygroscopicus* isolated from California is not necessarily identical to one isolated from China. Indeed, more than 200 secondary metabolites have been isolated from various *Streptomyces hygroscopicus* strains.

When building a library of microorganisms for use in HTS, thousands of strains are isolated and have to be chosen and characterized. The first step is careful morphological observation. This allows the cultures to be separated into taxonomic groups. Further speciation can be done using molecular or biochemical methods. Biochemical culture-dependent techniques such as fatty acid analysis, pyrolysis mass spectrometry *(95)*, and FT-IR analysis *(96)* were developed initially for clinical isolates and cannot be easily applied to environmental samples that require prolonged growth periods. Changes in the media, incubation temperature, and growth period can alter the profile of the organism, and hence results can be compared efficiently to one another only within one experiment. However, if the culture conditions can be standardized, the use of pyrolysis mass spectrometry analysis can reflect similar clustering of taxonomic groups as molecular methods *(97)*.

The morphogenic groups can be separated further using molecular methods such as restriction fragment length polymorphism (RFLP), which could differentiate strains to the subspecies level *(98–100)*. Ribosomal genes, including the intragenic spacer regions, have been used routinely to differentiate both fungal and actinomycete strains. Thousands of sequences are available in GenBank and the Ribosomal Database Project that can be used to phylogenetically identify interesting organisms. The pitfalls of relying

on polymerase chain reaction (PCR)-based rRNA analysis as a measure of microbial diversity in environmental samples have been emphasized *(101)*. Sequencing of other molecular markers, although costly, does however allow identification to the species level. When used in combination with RFLP, strains can be separated to the subspecies level *(100)*.

5.2. Characterization and Choice of Microbial Extracts

Strains of the same species may generate different chemistry in the same media. The first physical characteristics of unknown natural products are determined during the chemical extraction and concentration steps. Solvent-based and acid-base partitioning experiments can help define hydrophobicity and types of functional groups of the natural-product structures.

The most effective selection method from the metabolic aspect is the chemical profile analysis by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) data *(102)*. Identical HPLC retention time or TLC R_f values may not tell you whether two compounds are exactly the same, but different values definitely indicate they are different. Micro-scale extraction procedures have been developed *(103)* and can be automated. The first selection criterion is the metabolic creativity of strains, as the number of peaks revealed by HPLC with detectors of evaporative light scattering detection (ELSD), vs photodiode array (PDA), chemiluminescent nitrogen (CLND), and time-of-flight mass spectrometry (TOFMS) *(104)*. Nielsen et al. proposed a method for identification and confirmation of chemical compound classification based on single- or multiple-wavelength chromatographic profiles *(102)*. Chromatographic matrices from analysis of previously identified samples are used for generating reference chromatograms, and new samples are compared with all chromatograms by calculating resemblance indices *(111)*. In addition, the method allows identification of characteristic sample components by local similarity calculations:

http://www.esainc.com/products/HPLC/Optical_Detectors/esa_ChromaChem.html

Detection from ELSD is based on the universal ability of particles to cause photon scattering when they traverse the path of a polychromatic beam of light. The liquid effluent from HPLC is first nebulized, and the resultant aerosol mist containing the analyte particles is directed through a light beam. A signal is generated that is proportional to the mass present, and is independent of the presence or absence of chromophores, fluorophores, or electroactive groups. Since essentially every compound can be separated by HPLC or micro-HPLC and detected by ELSD, the ESA ChromaChem is equipment that every chromatography laboratory should have. It is a mass-sensitive device, which provides a response directly proportional to an analyte's mass in the sample. The presence of functional groups or chromophores is not necessary for detection. Relative amounts of compounds can be easily assessed by evaporative light-scattering technology. Any nonvolatile analyte can be detected, and gradient elution can be employed to optimize the separation. Aqueous as well as solvent-based mobile phases can be used to detect compounds that are not generally "seen" by other detection techniques. This detector can be used in conjunction with mass spectrometers to provide a complete analysis of the sample. The ChromaChem's unique nebulization system and temperature-controlled drift tube provides sensitivity, reliability, and reproducibility.

The unit's small footprint requires a minimum of bench space, allowing use under space-limited conditions.

The basic methods to compare microbial extracts are HPLC-DAD (diode-array detection) and HPLC-MS. Researchers at Eli Lilly developed a rapid (about one sample per min) surrogate measure of microbial secondary metabolite production computed from the electrospray mass spectra of samples injected directly into a spectrometer *(105,106)*.

The development of a multi-channel mass-spectrometry interface has allowed analysis at high-throughput level. In most cases, LC-MS (liquid chromatography-mass spectrometry) is the most sensitive method for obtaining dereplication information about a compound. A recent development is an eight-way fully automated parallel LC-MS-ELSD system for the analysis of natural products *(107)*. LC-NMR (liquid chromatography nuclear magnetic resonance) should become operational in the near future, allowing the on-line identification of organic molecules *(108,109)*. LC-NMR, although it has lower sensitivity than LC-MS, provides a powerful tool for rapid identification of known compounds and identification of structural classes of novel compounds. LC-NMR is especially useful in instances where the data from LC-MS are incomplete or do not allow confident identification of the active component of a sample.

For strains and chemical tracking, an in-house database has to be built to integrate with commercially available ones such as Antibase database (Wiley Publishers, 2003) or the Dictionary of Natural Compounds (Chapman & Hall, London). There is no single technique that gives 100% confidence to differentiate any two natural-product chemical profiles, but computer-enhanced structural determination methods could integrate various spectral data and raise confidence.

5.3. Chemical Dereplication to Prevent Repeated Discovery

Key elements in the success of a natural-product discovery program are quick identification of bioactive compounds, early elimination of known or unwanted metabolites, and rapid determination of the structure of novel compounds. Dereplication strategies use analytical techniques and database searching to determine the identity of an active compound at an early stage. Dramatic improvements have been achieved during the past years mainly due to the impact of combinatorial chemistry. Natural-product chemistry has to take advantages of these recent developments. The final separation-purification procedures are not discussed here since the procedures are complex and depend on the characteristics of the targeted compounds; this is beyond the scope of this chapter.

In the search for new microbial natural products, we have to consider the frequency of re-isolation of already-described metabolites from microbial cultures. Rough estimates suggest that we have isolated only a minute fraction of the compounds that exist in nature. Full identification of a natural product should be done only after partial purification to determine whether this type of compound is already known or has potential as a useful drug.

Natural-product samples need to be normalized by concentration or weight. Common "interfering" groups of compounds such as detergent-like or toxic compounds should be removed. Samples should be grouped into related chemical classes and then prioritized for further fractionation. The hit profile coupled with genetic and morpho-

logical characterization of the strains will build an increasing level of confidence in a putative structure.

The first physical characteristics of unknown natural products are determined during the chemical extraction and concentration steps. Partitioning experiments with solvents exhibiting a range of polarities and pH values will shed light on hydrophobicity and charged functional groups of the natural products.

If the natural-product extract contains a reported commercially available compound, the sample and the reference standard should be co-injected for TLC and HPLC. Identical retention times (HPLC) or Rf values (TLC) may not tell you that they are exactly the same, but different values definitely indicate they are different. If a standard is not available, the hypothesis could be tested by employing a physical test such as MS, looking for ions of the same approximate molecular weight. The chromatographic behavior could also reveal the nature of the compounds of interest, such as logP, surface area, and dipole moment.

Intelligent screening approaches towards microbial natural products are also required. One of the major limiting factors in the drug-discovery industry is that pharmaceuticals have been traditionally designed to target individual factors in a disease system, but diseases are complex in nature and vulnerable at multiple attack points. Therefore, a systematic novel synergistic drug-screening approach based on a multifactorial principle is urgently needed. Many drugs could be more effective at a reduced dosage if low dosages of other synergistic compounds are introduced simultaneously. Many marketed traditional medicines have demonstrated great efficacy and safety profiles in their long history. However, when efforts were made to purify a single molecule, the activity often was lost. SynerZ Pharmaceuticals Inc. has developed a drug-discovery approach consonant with the systems biology framework, and complementary to the target-based approach. Synergistic co-drugs from natural products will enable existing drugs to be more effective and contribute to our better understanding of multiple pathways to cure disease.

Ketoconazole is commonly used to treat *Candida* infections. However, at clinical doses, ketoconazole is associated with important toxic side effects, including hepatitis. In addition, resistant strains often emerge during long-term or prophylactic treatment as a result of the necessarily high concentrations of drug required. The concentration of ketoconazole alone at 0.01 µg/mL gave only about 20% inhibition of growth (**Fig. 2**). When ketoconazole was tested at 1 µg/mL, it gave 90% inhibition of growth. However, the combination of ketoconazole at 0.01X with F0101604 achieved about 95% inhibition (better than 100-fold the ketoconazole amount used here) and the mode of action was cidal, showing a synergistic effect of the two components rather than an additive effect. The natural product SNZ101 purified from F0101604 not only improved the efficacy of a much reduced dosage of ketoconazole, but also broadened its spectrum on drug-resistant strains and reduced its side effects (data not shown). It is clear that natural product F0101604 would be disregarded in conventional screening technology for antifungal lead discovery, because by itself it failed to show any growth inhibition on fungal pathogen *Candida parapsilosis* ATCC 22019.

6. Closing Remarks

One prerequisite to natural-product discovery that remains paramount is the range and novelty of molecular diversity. Currently, natural products are going through a

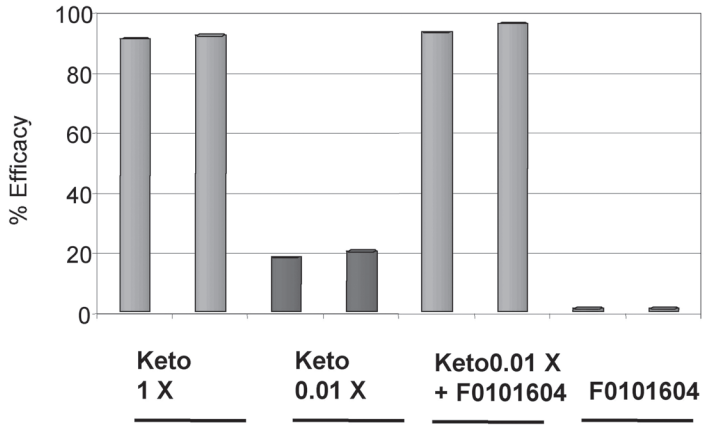

Fig. 2. Synergistic effect of F0101604 with a low dosage of ketoconazole (X = 1 µg/mL). Equal amounts (10^4/mL) of Candida parapsilosis ATCC 22019 are cultured in Mueller-Hinton (MH) broth with Alamar Blue dye in the presence and absence of a subclinical concentration of ketoconazole. Samples are treated as labeled in duplicate. Fluorescence reading after overnight incubation at 35°C in a moist chamber is measured at Ex 544 nm and Em 590 nm, and converted to percentage of growth inhibition.

phase of reduced interest in the drug-discovery field *(112)*. However, new developments may reverse this negative perception.

The systematic exploitation of selected ecosystems, combined with the development of new techniques and media for isolation of novel microorganisms, will allow the collection of representative strains from a large part of the micropopulation. This maximized biodiversity will deliver chemical diversity for an ecosystem.

The direct expression of environmental DNA in heterologous surrogate hosts is progressing. There is a need for rapid and sensitive detection and characterization of new metabolites as well as their gene clusters.

Physiological manipulation should be based on experimental design and measurement of secondary metabolism. Co-cultures will give novel insight into secondary metabolism and require the development of new vessels for stable mixed-culture fermentation.

Gene mixing coupled with the genetic engineering power of PKS, for example, will allow the generation of hybrid or "unnatural microbial natural products."

Total synthesis of natural products with interesting biological activities is paving the way for preparation of new and improved analogs. Combinatorial chemistry permits the selection of the best drug from a large number of candidates. Beyond synthesis and evaluation of organic molecules, a number of new bioorganic methods are emerging on the horizon.

In natural-product chemistry, the rapid and accurate differentiation of chemical compound profiles is based on on-line measurement by LC-ELSD, DAD, MS, and NMR. Automated comparisons of the metabolite profiles of microorganisms can be used as a valuable method for building libraries of natural products for drug discovery *(114)*.

Today, more than 30,000 diseases are clinically described, but less than one-third of these can be treated symptomatically, and only a few can be cured *(110)*. New chemi-

cal entities as therapeutic agents are urgently desired. Natural products can play a main role in drug discovery. New strategies for natural products-based drug discovery will increase chemical diversity and reduce redundancy *(115)*. Maximizing the discovery of new compounds and minimizing the re-evaluation of already known natural products will be crucial.

Acknowledgments

Thousands of people have contributed to our understanding of microbial natural products for drug discovery in the past century, and I acknowledge their efforts even though they may not all be cited in this chapter. I am indebted to members of my laboratory, past and present, who have made several of the major contributions cited in this review. I gratefully thank Springer-Verlag GmbH for granting permission that for this chapter, I could expand the contents of my previously invited review "Diversifying microbial natural products for drug discovery," published in Appl Microbiol Biotechnol (2003) 62:446–458 (DOI 10.1007/s00253-003-1381-9).

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