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Microbial molecular diversity: past and present
Thom Award Lecture

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Many of us owe our livelihoods and some of us our lives to antibiotics; we know what they are and what they can do. They are the secondary metabolic products of microbes (and sometimes plants) employed to inhibit or kill those living organisms harmful to man, animals, or plants; the use of antibiotics has transformed the treatment of infectious diseases. Of course, they do have drawbacks, and no antibiotic is without some deleterious effect on the host. The overuse and indiscriminate administration of antibiotics has led to the selection of drug-resistant strains of bacteria that have contributed to a number of modern world epidemics of difficult-to-treat bacterial diseases throughout the world. But that is another story. The role of secondary metabolites in nature, of which probably only about 20% have detectable antibiotic properties, is poorly understood; it has always been assumed that they must have (or have had) roles in the function or survival of the producing cell. This is not easy to study since we cannot, in the laboratory, recreate the myriad and intricate circumstances that represent the life style of microbial populations in nature. The complexity of microbial diversity is one of the major issues facing microbiologists at this time; this diversity is surpassed only by the amazing structural and biological variety of secondary metabolites. Microbial diversity breeds molecular diversity. Our knowledge of these matters is at about the level of subatomic physics in the 1930s; the atom existed but its component parts and the forces that held them together were not known. However, as a result of the efforts of many brilliant minds and with the appropriate research support, ever more powerful techniques were conceived and employed to provide the detailed understanding of atomic structure that exists today. The rewards of this immense effort have been spectacular in terms of practical applications.

In the case of microbial diversity, a similar scientific challenge is presented and similar rewards await; the earth is populated with an enormous number of different microbes, less than a few percent of which can be cultivated and identified. (Of the microbial content of oceans, the number is less than 1%.) Microbes live an intensely cooperative and communicative lifestyle, in concert with plants and with animals. It is known that microbes play essential roles in determining physical, chemical and biological aspects of our environment, even influencing climatic conditions, and that the survival of all higher organisms is dependent on microbes, but we are not able to analyze all their biological contributions because a complete catalogue of microbes does not exist. As has been stated on many occasions, 'if you can't grow them, you can't know them'. In all likelihood many of the unidentified species produce unusual and interesting metabolites that could (perhaps) be used to ameliorate some of the infectious diseases problems that plague (no pun intended) the medical profession today, and are likely to continue to do so in the future. The practical benefits of a concerted effort in studies of microbial diversity would likely be as great or greater than the spinoffs of atomic energy research. In addition, until we have a better idea of species diversity and how it has evolved, we will not be able to understand relationships between species change and environmental stability.

It is believed that tens of millions of microbial metabolites are produced that probably play roles in regulating microbial metabolism, signaling, communication, and sexual exchange; some may actually function as antibiotics. Simple inhibition tests identify antibiotic activity in the laboratory, but is this really the role of secondary metabolites in the microbial world? I have proposed that some secondary metabolites may be very old molecules that appeared on earth at the same time as, or soon after, amino acids and nucleic acid bases and may have been important in the evolution of the biochemical processes of cells [2]. Many more secondary metabolites are likely to have mediated or effected reactions during different phases of biochemical evolution, acting as the forerunners of proteins in the roles of primordial

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catalysts or effectors in biosynthetic reactions. I realize that evolutionary theories cannot be proven; one can only propose experimental tests as simulations or seek relics of early reactions that still exist. If you have followed the development of modern physics, you will know that there are essentially two kinds of theories: those that make testable predictions and those, like superstring theory, that are speculative efforts made in an attempt to expand current understanding by providing more general descriptive concepts. The evolutionary role of secondary metabolites falls into this latter category.

Secondary metabolites are key components of the microbial global control mechanisms that are essential to diversity; a better understanding of their roles may well provide the basis for unifying models of microbial community growth. One of the reasons why we can't grow all microbes in nature is likely due to the fact that they require 'factors' from other microbes in the environment. Since it is probable that low-molecular-weight molecules have performed these functions for a very long time, an understanding of their properties may illuminate many of the 'black' boxes that exist in the schemes of cellular evolution that have been proposed.

Efforts to reproduce the conditions and components of primordial reactions ('soups') in the laboratory have led to the detection of precursor molecules (amino acids, nucleic acid bases, and carbohydrates) thought to be the building blocks for biological macromolecules. The formation of these molecules under such conditions is consistent with current theories of chemical evolution. However, these random chemical processes produce a variety of other simple organic molecules. Non-protein amino acids are also made and are rarely mentioned, but these are components of a large variety of biologically-active oligopeptides produced by microbes (Table 1, [4]). Other molecules structurally related to known secondary metabolites might also be formed by the condensation or modification of 'building block' molecules found in reactions taking place under primordial earth conditions. Even if one does not accept any of the variants of the 'warm little pond' hypothesis, there are alternative theories to provide for chemical precursors—for example, that the organic molecules which formed the basis of early chemical evolution were delivered into the earth's atmosphere from space [1]. Nonetheless, the argument that primordial soups contained an enormous array of chemical diversity still stands—primordial atmospheres must have contained simple polypeptides and other molecules that could be the forerunners of what we now call secondary metabolites; indeed, some secondary metabolites might rightly be considered chemical fossils.

TABLE 1

Non-protein amino acids found in primordial soups*

Sarcosine	γ-Aminobutyric acid
N-Ethylglycine	*N*-Methyl-β-alanine
N-Propylglycine	*N*-Ethyl-β-alanine
N-Isopropylglycine	Pipecolic acid
N-Methylalanine	α-Hydroxy-γ-aminobutyric acid
N-Ethylalanine	α,β-Diaminobutyric acid
β-alanine	α,β-Diaminopropionic acid
α-amino-*n*-butyric acid	Isoserine
α-Aminoisobutyric acid	Norvaline
β-Amino-*n*-butyric acid	Isovaline
β-Aminoisobutyric acid	Norleucine
	Allothreonine

*Many are components of secondary metabolites.

In recent years, tens of thousands of molecules have been isolated in screening procedures for antibiotic or other biological activity in the laboratory. Research on the useful properties of secondary metabolites is primarily a retrospective and artificial analysis; the roles that these molecules play in nature is not a consideration. A large number of biologically-active natural products have never been subjected to proper scientific scrutiny. Unfortunately, only a small proportion of the secondary metabolites isolated from microbes in industrial laboratories have been revealed to the scientific community due to proprietary needs of the companies that discovered them. The majority lie stored on shelves because, although they may possess biological activity, it was not the type of activity sought, or did not prove useful following testing in animals. It would be a great contribution to science if all the secondary metabolites isolated in pharmaceutical screening procedures could be collected and made available for academic research; we do not even have a catalogue! A great deal of useful information might come from detailed microbiological and biochemical studies of these compounds without the requirement that they behave as antibiotics or antitumor agents. The survival of microbes depends on sensitive responses to changes in their environment; they must respond rapidly if they are to feed, undergo gene exchange, and survive. Many secondary metabolites are secreted and induce a variety of responses in microbes; some will undoubtedly prove to be important in cell signaling. As an example, the homoserine lactones such as A-factor and similar compounds have been shown to be important as bacterial signaling agents and pheromones [14]. It has been reported that certain classes of well-known antibiotics can stimulate gene transfer in bacteria [9], which is consistent with the notion that many of the activities of secondary metabolites are involved in the global control of metabolism, both at intra- and inter-cellular levels. Plant and microbial secondary metabolites may also have important functions for species unrelated to the producer; certain antibiotic secondary metabolites have remarkable effects on insects and cause parthenogenetic wasps to become bisexual [10].

The identification and taxonomic classification of microbes is still a major problem. Only limited catalogues are available and although 16S rRNA gene sequencing has been a tremendous technological advance, it is essential that novel methods of isolation and/or detection and identification be sought to be used in biochemical and evolutionary studies in the future. Not only are we faced with definitions of speciation which become more and more complex the more

we study the topic, but the division between prokaryotes and lower eukaryotes may become increasingly blurred, as witnessed by the fact that gene exchange between pro- and eukaryotes can be demonstrated in the laboratory [5]. If it happens in the lab, it must also happen in nature! Simple prokaryotes can often be excellent models of higher cell function. Is it possible to study the biology of complex eukaryotic functions in simple bacterial systems? Microbes have a very wide range of adaptive responses for which the mechanisms are not well understood; they have been shown to possess receptors for hormones and cytokines and some even produce analogues of these (eukaryotic) physiologically-active peptides [7]. The intricate mechanisms of cell regulation in microbes present excellent subjects for study and may well provide models for processes that take place in all types of cells.

The bottom line is that we are incredibly ignorant about the numbers, roles, and lifestyles of microbes in nature—or the chemistry and biology involved. Efforts to redress this situation will pay enormous dividends, not just for microbiology and environmental science but in all aspects of industry, especially the pharmaceutical industry. After all, the more microbes we can screen, the more useful secondary metabolites we will find.

Back to secondary metabolites and their biological functions. I mentioned earlier that evidence that molecules such as antibiotics may have played roles as effectors in biochemical and cellular evolution cannot be obtained by direct experimentation. We have to rely on circumstantial relationships; can low-molecular-weight compounds be demonstrated to play roles in 'primordial' reactions under laboratory conditions? What defines such a laboratory model? As with most attempts at practical evolutionary science, the experimenter determines the plausible conditions for a model primordial reaction. It seems obvious that the re-creation of primordial reactions requires experiments using soluble cell fractions and not the study of activity of whole cells. Studies have shown that many antibiotic inhibitors of cellular functions have quite different activities when they are tested in crude cell extracts. Might this 'anomalous' behavior be an indication of potential roles in biochemical evolution?

The current dogma of molecular evolution recognizes a key role for RNA-based reactions. Together with Renée Schroeder and her group in Vienna, we have started to investigate the effects of low-molecular-weight secondary metabolites on catalytic RNA reactions and have found that several different chemical classes of secondary metabolites interact specifically with group I introns. We have demonstrated inhibition of group I intron splicing by low concentrations of certain inhibitors of translation and believe that this is consistent with the concept of an evolutionary association between secondary metabolites and RNA—could the antibiotics have played roles in modulating or effecting RNA reactions under primordial conditions [3]? In addition, since most of the antibiotics we have found to be effective as inhibitors of ribozyme functions (aminoglycosides, peptides) act on the level of the small ribosomal subunit (which is a potential ribozyme (16S ribosomal RNA)), we believe that there is likely to be an evolutionary relationship between the two types of ribozyme [8].

The peptide antibiotics are produced, not by ribosomal protein synthesis, but by the action of peptide synthetase enzymes that specifically bind activated amino acids and catalyze peptide bond formation between them. The peptide antibiotics contain a large variety of amino acid residues, a large number of which are not found in proteins [6]. I pointed out earlier that many of the same non-protein amino acids are detected as products in reactions designed to reproduce 'primordial soup' conditions. If, as proposed by the 'primordial soup' school, proteins were formed in these reactions, surely molecules similar to the peptide antibiotics would have formed from their precursors under the same primordial condensations. The peptide antibiotics have been found to possess a wide range of biological activities consistent with their roles as pre-protein catalysts (or effectors). The reactivity of viomycin and related peptides towards catalytic RNA is different from that of the aminoglycosides, since the peptide antibiotics not only inhibit group I intron splicing, but under some conditions enhance the splicing reaction and 'catalyze' the formation of head-to-tail RNA intron polymers [12]; thus some of the peptide antibiotics may have been capable of 'effecting' a variety of different catalytic RNA reactions.

The mechanism of antibiotic interaction with catalytic RNA is not known; contact with specific nucleotide bases in the RNA has been demonstrated by chemical protection studies by von Ahsen and Noller [11] and the peptide antibiotics have been modeled into putative binding sites on intron RNA, but the molecular details of the interactions are still unclear. As an interesting extension to the demonstration that secondary metabolite translation inhibitors bind to specific sites within RNA molecules, Zapp and co-workers [13] have shown that neomycin binds to the rev responsive element RNA of human immunodeficiency virus (HIV) which blocks the binding of the rev protein to its site on the viral RNA and prevents expression of late messenger RNA species encoding structural proteins of the virus. Continued studies of this type, employing other natural products that interact with RNA, might lead to new classes of secondary-metabolite inhibitors of ribozyme-catalyzed reactions. In addition, I suggest that evolutionary arguments of the type presented can be used to identify potential interactions between low-molecular-weight natural products and biological macromolecules as an alternative approach to rational drug design.

Although tens of thousands of secondary metabolites have been isolated and tested for biological activity in a variety of assays, we are seeing only the tip of the proverbial iceberg, since such a small proportion of the microbes in nature can be grown in the laboratory. In addition, it is impossible to examine all possible biological reactions as targets; as new assays are developed, new functions for old molecules are found. A very substantial investment is required for work in this field but unless this is done, the

diversity of biological effects of secondary metabolites will never be realized. However, it is unlikely that the US Congress (or any other government) will ever consider supporting studies of microbial diversity in the same way that particle physics has been funded. The roles of microbes tend to get forgotten; the Rio conference on biodiversity did not mention microbes, although their co-existence with plants and animals is vital. We should be as concerned about the destruction of the microbial species on this planet as we are about the loss of plants and animals. But who ever speaks about microbial conservation? The existence of visible biodiversity depends on invisible biodiversity but microbes are afforded scant attention in contrast to plants and animals; if there were no microbes, there would be no botanists or zoologists! The British government has steadfastly refused to increase support over its present, paltry, levels; substantially more money was invested in the making of the extinct animals for the film 'Jurassic Park' than is provided for microbial diversity research in Europe! We as microbiologists should lobby for support of research in this area, since there are enormous benefits to be gained. An increase in the number of culturable microbes up to 10% of the total number of species on this planet would be a 10-fold increase over what we know, with a correspondingly large increase in availability of novel secondary metabolites! This understanding will not come easily, but requires more subtle, imaginative, and novel approaches to the examination of microbes and their functions under natural conditions, certainly as communities and not as isolated laboratory cultures. As Einstein once stated: 'Knowledge is not enough, we need imagination.'

There is still a great deal to learn in the exciting field of microbiology and uncovering some of the biological roles of secondary metabolites would not be a bad place to start.

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