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MicroOpinion

The evolution of secondary metabolism – a unifying model

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Summary

Why do microbes make secondary products? That question has been the subject of intense debate for many decades. There are two extreme opinions. Some argue that most secondary metabolites play no role in increasing the fitness of an organism. The opposite view, now widely held, is that every secondary metabolite is made because it possesses (or did possess at some stage in evolution) a biological activity that endows the producer with increased fitness. These opposing views can be reconciled by recognizing that, because of the principles governing molecular interactions, potent biological activity is a rare property for any molecule to possess. Consequently, in order for an organism to evolve the rare potent, biologically active molecule, a great many chemical structures have to be generated, most of which will possess no useful biological activity. Thus, the two sides of the debate about the role and evolution of secondary metabolism can be accommodated within the view that the possession of secondary metabolism can enhance fitness, but that many products of secondary metabolism will not enhance the fitness of the producer. It is proposed that secondary metabolism will have evolved such that traits that optimize the production and retention of chemical diversity at minimum cost will have been selected. Evidence exists for some of these predicted traits. Opportunities now exist to exploit these unique properties of secondary metabolism to enhance secondary product diversity and to devise new strategies for biotransformation and bioremediation.

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Introduction

> 'In certain scientific circles it is something of a sport to theorize about function, often with the intent of finding one overriding axiom true for all secondary metabolism. Speculations range from the notion that they are waste products or laboratory artefacts, to the concept that they are neutral participants in an evolutionary game, to ideas of chemical weaponry and signalling, through a number of other creative notions.' (Bennett, 1995)

There have indeed been many discussions of the role of secondary metabolites in microbes (e.g. Stone and Williams, 1992; Vining, 1992; Demain, 1995), yet the study and exploitation of secondary metabolites has progressed despite this lack of agreement as to why some microbes possess such chemical diversity. The fact that some secondary metabolites possess such potent biological activity is now widely regarded as being indicative of their purpose. However, sceptics of this viewpoint point to the fact that the very great majority of secondary metabolites have not been shown to benefit the producer. We contend that the finding that a few secondary products possess very potent biological activity, but that the majority do not, is not contradictory but predictable on the basis that potent, specific biological activity is a rare property for a molecule to possess (Jones and Firn, 1991). The strict structural requirements that must be fulfilled in order for a low-molecular-weight chemical to bind tightly to a target protein must have been a very important evolutionary constraint in organisms that developed a secondary metabolism. For an organism to gain fitness by producing a potent biologically active chemical, it can be postulated that the possession of metabolic traits that enhance the likelihood of producing and retaining chemical diversity would have been highly advantageous (Firn and Jones, 1996). This model for the evolution of secondary metabolism not only explains why very potent, biologically active molecules are made by some organisms, but also explains why many secondary metabolites possess unimpressive biological activity. As in the case of the immune system in animals (Lodish *et al.*, 1999), the possession of the overall machinery is crucial,

but most substances made by that machinery confer no advantage to the producer.

Biomolecular activity and the evolution of secondary metabolism

Screening programmes provide ample evidence that, for any biological target, most chemicals, whether synthetic or naturally occurring, are inactive unless tested at high concentrations (Firn and Jones, 1996). For example, when 400 000 microbial cultures were screened over a 10 year period, only three useable antibiotics were discovered (Fleming *et al*., 1982). However, the relevance of this evidence to discussions about the evolution of secondary metabolism has been challenged by Berenbaum and Zangerl (1996), who contended that the low frequency of activity found in screening trials was simply the result of using inappropriate screening methodologies. They argue that, if the 'correct' targets were used, a very high frequency of biological activity would be found. Why is there such disagreement on such a fundamental issue? The crux of the disagreement seems to lie with the definition of the term 'biological activity'. Only by defining what 'biological activity' means in terms of the evolution of secondary metabolism will it be possible to advance the debate. 'Biological activity' studied at a molecular level *in vitro* can have a different meaning to 'biological activity' studied at a whole organism level. At the molecular level, there is ample evidence that specific biological activity against a defined molecular target is a rare property for a molecule to possess – that is why high-throughput screening protocols capable of assessing the biological activity of 100 000 chemicals per day have been developed, and it is why chemical libraries with in excess of 1 million compounds are commercially available for drug screening. The experience of several decades of large screening programmes is now underpinned by a secure conceptual understanding. Ligand-binding studies reveal that high-affinity, reversible, non-covalent interactions between a ligand and a protein only occur when the ligand has exactly the right molecular configuration to interact with the complex three-dimensional structure of the protein (Lodish *et al*., 1999). We propose that this type of biological activity should be given the term 'biomolecular activity', and it should be defined as the ability of a molecule to interact with a biologically functional molecule such that that its biological function is changed significantly. There is overwhelming experimental evidence that, at low concentrations ($< 10^{-5}$ M), any one chemical has a very low probability of showing 'biomolecular activity' against any one target protein (Firn and Jones, 1996). However, it is predictable that the frequency of molecules possessing 'biological activity' will be higher if activity is assessed by targeting an organism instead of a protein. An organism contains thousands of potential protein targets; hence, if one were screening for a somewhat non-specific effect (performance or survival) on an unadapted organism, it is predictable that a higher frequency of activity will be found than in a screen based on 'biomolecular activity'. Further aggregation will occur if the chemical is tested against many diverse species. Furthermore, if the concentration of every chemical being tested against an organism is increased, the laws of mass action predict that the frequency of finding any effect will increase further. Thus, the low probability of finding potent 'biomolecular activity' against a specific molecular target at a low concentration (Firn and Jones, 1996) is entirely consistent with the view that a higher frequency of less specific activity might be found if a very wide range of unadapted organisms is screened using a high concentration of each chemical (Berenbaum and Zangerl, 1996). However, where in this continuum between the extreme definitions of biological activity (potent biomolecular activity against a specific target versus low-potency 'toxicity' against any organism) is selection operating in terms of the chemical interactions between organisms? In evolutionary terms, the only target organisms that matter are those that have had an opportunity to interact with the producer organism. An effect produced in any other organism cannot act as a focus for selection. That restriction substantially reduces the number of possible chemical–target organism combinations (Firn and Jones, 1996). Similarly, in evolutionary terms, the only concentration that matters is that which a target organism would receive under normal circumstances – physiological effects shown only at concentrations that are above those achievable in the natural environment cannot be of selective significance. In our opinion, the most common evolutionary scenario for selection operating on specific parts of the secondary metabolism will have involved few rather than many target organisms. Furthermore, we consider that selection will have favoured organisms that can produce effective chemicals at low cost, and that will favour the selection of organisms capable of producing highly potent chemicals. High potency results from a strong ligand–protein interaction, and that is necessarily dependent on a very specific ligand structure fitting a precise target site on the protein, hence giving rise to a very specific biomolecular effect (Lodish *et al*., 1999). These considerations suggest to us that the constraints that apply to the evolution of 'biomolecular activity' will have been important in the evolution of secondary metabolism.

Evolutionary strategies to optimize the exploitation of rare biomolecular activity

The genetic fixation of beneficial variation from

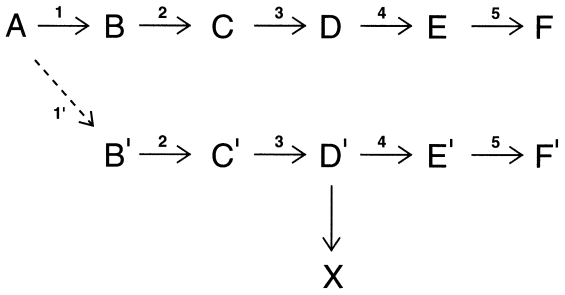

Fig. 1. The increased generation of chemical diversity after a mutational event if a broad substrate tolerance is available. The addition of one new enzyme 1′ results in six new products.

low-frequency mutational events is the basis of evolution (Lodish *et al*., 1999). Hence, the low frequency at which molecules with high biomolecular activity arises is simply another constraint within which evolution will operate. Where would the consequences of this constraint be most severe? The most obvious factor to be considered is the difficulty in evolving long biosynthetic sequences when each new enzyme that extends a pathway has a very high probability of producing a chemical that adds costs to the product but produces no benefits (Firn and Jones, 1996). The low odds of producing a beneficial new chemical would be compounded at every step in a biosynthetic sequence. Although improbable events are not impossible ones, it seems reasonable to propose that selection would favour organisms that evolved metabolic traits that reduced the costs of generating and retaining chemical diversity.

Predicted metabolic traits that would enhance the generation and retention of chemical diversity

In order to increase the chances of producing the rare molecule with potent and appropriate biomolecular activity, it can be proposed that evolution should have:

1 selected for traits that enhance the generation of chemical diversity;

2 selected for traits that enhance the retention of chemical diversity;

3 selected for traits that reduce the fitness costs of 1 and 2.

In this short article, it will only be possible to consider three of a number of traits that have been proposed to account for the generation and retention of chemical diversity in organisms making secondary products (Jones and Firn, 1991).

Substrate specificity – different rules in primary and secondary metabolism. When a new enzyme variants arises by mutation to extend metabolism, it usually differs from the wild type in terms of its substrate specificity and not the type of chemical catalysis it can conduct (Petsko *et al*., 1993). New enzyme variants that arise with a broad substrate specificity will be more likely to carry out a new transformation than new variants with a very narrow substrate specificity, simply because the range of substrates available to the broad-specificity variant will be larger. Thus, it seems probable, but not inevitable, that most new enzymes will possess a broad substrate specificity, and high specificity will more usually be gained by subsequent selection. Selection to reduce the range of substrates acted upon will only occur if increased benefits or reduced costs result from improving selectivity. Judging by the fact that most (but not all) enzymes involved in primary metabolism are highly substrate specific, the benefits that accrue from increasing specificity may be very significant in primary metabolism. However, in secondary metabolism, in which some of the benefits may only accrue spasmodically and where new threats are ever present, the selection pressures would be expected to be different from those operating on primary metabolism. Selection pressures to increase substrate specificity may not exist, quite the contrary. By retaining a broad substrate specificity, the generation and maintenance of chemical diversity may be enhanced, as illustrated in Fig. 1.

In this model, a substrate A is converted by a series of enzymes into five other compounds, with each conversion being carried out by a unique enzyme. Suppose a mutation gives rise to a new variant of the organism, which produces a compound B′, which is structurally similar to B. If the enzymes in the pathway B → F now act on B′, new compounds C′, D′, E′ and F′ will arise. The addition of one new enzyme (1′) has resulted in the production of five new compounds. If any of these compounds possesses beneficial biomolecular activity and if the costs incurred are sustainable, the new variant may be advantaged during selection. The best available evidence for this model to explain secondary product diversity comes from a study of terpene biosynthesis in plants (one can justify using evidence from plant secondary metabolism because the basic principles governing the evolution of biomolecular activity are molecular, and the type of organism or the type of product should not negate these principles). A mutant of spearmint produced a mix of monoterpenes that were characteristic of peppermint (Croteau *et al*., 1991). A single gene mutation caused the spearmint to lose several compounds and to gain several more. The changes were caused by the mutant hydroxylation enzyme adding a hydroxyl to a 3-position in a cyclohexene ring (≡ B′ in Fig. 1) instead of the wild-type 6-hydroxylation (≡ B in Fig. 1). The subsequent substrate-tolerant enzymes in the pathway accepted the new substrates to give the new products. Furthermore, the generation of chemical diversity will beget further diversity. Thus, in Fig. 1, X is shown as being formed from D by some enzyme not in the A → F

pathway. This is similar to the reported appearance of a new, unexpected product in the spearmint. A microbial example of this concept is illustrated by the finding that the addition of a gene coding for phytoene desaturase from *Erwinia* into *Rhodobacter* resulted in the production of a number of new carotenoids (Garcia-Asua *et al*., 1998). This could be an example of a 'gene saving device', which Cerda-Olmedo (1994) suggested was needed to explain how so few genes could produce such large chemical diversity in some microbes. More recent evidence for such inherent biosynthetic flexibility in microorganisms comes from a study of polyketide synthases (PKS) (Hutchinson, 1999; Shen *et al*., 1999). The flexibility of the PKS pathway derives from an impressive substrate tolerance (Byford *et al*., 1997). This tolerance not only allows each unit of the modular pathway to accept a wide range of substrates, but it also allows the substitution or elimination of individual modules to give another layer of chemical diversity generation. The biochemical flexibility of the PKS pathway not only helps to explain the existence of the >3000 polyketides known in nature, but also provides a rational basis for further attempts to manipulate the PKS pathway genetically to generate new chemical diversity. The possibility of creating thousands, if not millions, of 'new' (at least to humans) polyketides (McDaniel *et al*., 1999) can be seen not to be fortuitous but an inherent trait predicted by the principles discussed in this paper. Studies of a microbial peptide synthase also showed a relaxed substrate specificity, which was considered to contribute to the generation of chemical diversity (Baldwin *et al*., 1994).

Enzymes producing more than one product? It was postulated (Jones and Firn, 1991) that the use of enzymes that produce more than one product, or the incorporation of non-enzymic reactions into secondary metabolic pathways, would be advantageous in terms of generating and retaining chemical diversity at low cost. Striking new evidence to support this prediction has been found in studies of sesquiterpene synthases in plants (Steele *et al*., 1998). One enzyme produced 34 different compounds from a single substrate, and another produced 52 products from a single precursor. A microbial example of this concept is illustrated by isopenicillin N synthase from *Cephalosporium*, which has the ability to convert one substrate into six different β-lactam products (Baldwin *et al*., 1984).

Matrix pathways. It was proposed that matrix pathways would be an excellent way of reducing the cost of producing chemical diversity (Jones and Firn, 1991). The most elegant verification of this prediction comes from a study of carotenoid biosynthesis in the marine bacterium *Agrobacterium aurantiacum*. β-Carotene was converted by two enzymes into nine different carotenoid products in a matrix of sequential conversions (Misawa *et al*., 1995).

Some implications of this unifying model

The genetic manipulation of secondary product pathways

If some enzymes involved in secondary metabolism do have broad substrate specificities, the transfer of a gene coding for an enzyme involved in one secondary product pathway into another organism, which already maintains its own chemical diversity, may have unexpected consequences. It is quite possible that several new products could arise, some quite unexpectedly (in the manner of the example in Fig. 1). The gene transfer studies outlined above that have manipulated the pathways involved in carotenoid biosynthesis and the PKS pathway have begun to explore this concept. The opportunity to generate new chemical diversity by this means may help to produce new compounds with a biomolecular activity that can be exploited. However, the generation of chemical diversity, with unknown biological activity, could give rise to unknown and unpredictable hazards in genetically engineered organisms (Firn and Jones, 1999).

Seeking novel biotransformation capacity

Humans have long valued the ability of microbes to produce a wide range of desirable scents and flavours in foods and drinks, and attempts are being made to harness the versatile enzymatic capacity of microbes further (Hagedorn and Kaphammer, 1994). If many enzymes involved in secondary metabolism have a low substrate specificity, it should be possible to find enzymes capable of useful biotransformations in somewhat unexpected places. The fact that a synthetic substrate is not known to be metabolized or transformed by an organism should not preclude the possibility that the organism might make enzymes capable of acting upon a chemical.

The microbial breakdown of synthetic and natural products

Although it is widely assumed that many exogenous chemicals are degraded by microorganisms as a source of nutrients, the very low concentration of these compounds and the relative abundance of other substrates (especially in soil) requires that we consider the possibility that some microorganisms might degrade some exogenous compounds fortuitously. Is it possible that some enzymes exploited by microorganisms to produce secondary products also participate in the degradation of chemicals in their environment? The genes coding for

these enzymes would act a reservoir of broad-specificity enzymatic activity to be drawn upon if new chemical resources become available, and they might also be drawn upon as a way to degrade potentially harmful compounds made by competing organisms. If this scenario is valid, it is not surprising that microbes have been so successful at degrading most synthetic compounds – microbes have evolved to be versatile and diverse chemists. Hence, the 'design' of microbes to degrade toxic wastes (Timmis *et al*., 1994) should be feasible because the largely hidden versatility of secondary metabolism can be drawn upon.

Conclusion

Reynolds (1998) suggested that combinatorial biosynthesis was a lesson man learned from nature. Combinatorial chemistry or combinatorial biochemistry are simply attempts to reduce the constraints that result from the fact that specific, potent biomolecular activity is a rare property for a molecule to possess. By basing a model for the evolution of secondary metabolism on this molecular principle, it is possible to explain why organisms evolved combinatorial biosynthesis. Recognizing that some of the evolutionary constraints that operate on secondary and primary metabolism differ allows one to identify traits that are unique to each of the two types of metabolism. It is essential to develop an understanding of traits characteristic of secondary metabolism if the exploitation and manipulation of secondary metabolism is to be achieved successfully. The ability to change or enhance the enzymatic complement of microbes by genetic manipulation presents a unique opportunity both to test the proposed model of metabolic evolution and to explore further the potential of these organisms to generate new, potentially useful chemicals.

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