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ORIGINAL PAPER

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Streptomyces genes: from Waksman to Sanger

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My interest in the streptomycetes began nearly 50 years ago in the summer of 1954 when, having just graduated from the University of Cambridge with a degree in botany, I was persuaded to try to develop a genetic system in a streptomycete. The motivation was the view, common at the time, that the actinomycetes represented an intermediate group with characteristics of both the bacteria and fungi and therefore likely to reveal some genetic novelties. My background reading over the summer was Waksman's 1950 treatise on the actinomycetes [24], which I read from cover to cover. Sure enough, there was great ambiguity about the relationships of this group of microorganisms. More to the point, a modern study of their genetics à la Lederberg and Tatum had not been attempted; and cytological studies purporting to demonstrate some kind of a sexual process were highly questionable. I also looked up the few available papers on variation in the actinomycetes and these reinforced the burning need for an objective test for genetic exchange. In particular, there was a group of papers by Albert Kelner, one of them describing the discovery of photoreactivation of UV damage in *Streptomyces griseus*—before its demonstration in *Escherichia coli* and other "mainstream" microbes. In another paper he wrote: "The actinomycetes have been the subject in recent years of fundamental cytological and physiological studies, but there has been little genetic study of this difficult group of microorganisms" [10].

Prompted by this sparse background of genetic investigations on the actinomycetes, several people began more or less simultaneously to isolate auxotrophic mutants—the first precisely defined genetic variants to be generated in these organisms and therefore the logical starting point on this survey of genes from Waksman to Sanger—and to use them to demonstrate the unambiguous occurrence of some kind of gene exchange process [7]. Some of us also isolated streptomycin-resistant mutants—again the result of precise and predictable genetic changes. The first linkage map of an actinomycete chromosome, that of *S. coelicolor* in 1958, had six genes, five identified by auxotrophic mutations and one by streptomycin resistance [5]. By 1965, the number of mapped genes had increased to 39 [6] and included one designated *redA* because the mutant made a diffusible red pigment instead of the blue colour typical of the actinorhodin that gives *S. coelicolor* its name. In retrospect, based on this phenotype and the gene's map location, this almost certainly represents the first example of an identified gene for a step in antibiotic biosynthesis.

Much earlier studies—for example those of Dulaney et al. in 1949 [4] on *S. griseus*—described morphological variants of streptomycetes. These strains either lacked aerial mycelium or had aberrant colony morphology. However, in the absence of the means to demonstrate a clean segregation of a discrete phenotype from a cross, their genetic status could not be interpreted. This situation started to change around 1966, when the first *bld* mutants and later the first *whi* mutants of *S. coelicolor* were isolated and mapped to defined genetic loci. The former class of mutations define steps in the origin of the aerial from the substrate mycelium; and the latter identify steps in the metamorphosis of the undifferentiated aerial hyphae into spore chains. For each class, systematic phenotypic and genetic mapping experiments began to reveal the complexity of these developmental events, with the unambiguous identification of seven *whi* loci in 1972 [3] and four *bld* loci in 1976 [17]. By the late 1970s, too, the first indication of a complex cluster of genes for an antibiotic biosynthetic pathway was obtained, with the mapping of seven phenotypic classes of mutants interrupted in actinorhodin biosynthesis (the

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act mutants [22]) to a short segment of the chromosome corresponding to the original *redA* locus mentioned above. Pertinent to Waksman's discovery of streptomycin, the first mutant blocked in a specific step in streptomycin biosynthesis was studied around this time [18].

The isolation of mutants and their genetic mapping can, by definition, identify genes; and these techniques are the basis of classic genetic experimentation. However, with the advent of gene cloning in the mid-1970s, they were no longer enough. It took until 1980 for gene cloning to be established in *Streptomyces*, but soon it was well underway with the isolation, first of easily selected antibiotic-resistance genes [2, 23] and then of even more interesting genes, for antibiotic pathway enzymes and for steps in differentiation, as defined by the *bld* [14] and *whi* mutants [16]. For the last two, the concept of a "complete" set is obviously problematical and the hunt for new genes continues unabated at the time of writing. Instead, for the clusters that include genes encoding the dedicated enzymes for antibiotic biosynthesis, in addition to resistance and pathway-specific regulatory genes, innumerable "complete" sets have been cloned following the first reported example [15]. They include a particularly detailed analysis of the streptomycin biosynthetic cluster in Waksman's *S. griseus* [20].

By the early 1990s, the *S. coelicolor* chromosome map included not only over 100 genes identified by auxotrophic and antibiotic resistance mutations but also six *bld* loci, nine *whi* loci and three clusters of genes for antibiotic biosynthesis [8]. It was a purely genetic map. However, by 1992, pulsed field gel analysis and the hybridisation of cloned genes to the gel-separated restriction fragments had introduced a physical dimension to the map [12]. Depending on the lengths of the fragments to which the genes were assigned, the resolution of the genetic map was in the range of a few tens to several hundreds of kilobases. This was dramatically improved with the construction of an ordered cosmid library for the *S. coelicolor* chromosome and the location of over 200 genes on the resulting high-resolution physical map. Taking into account the average length of the unique and overlapping chromosomal segments represented by the clones, the average resolution all around the map was about 12.5 kb [21].

The ultimate resolution of a genetic map—to the level of individual base pairs of DNA—comes, of course, with a complete genome sequence. This was achieved for *S. coelicolor* in July 2001 at the Sanger Centre and published in journal form in May 2002 [1]. There were many surprises concerning the genetic endowment of the organism, revealed by annotation of the 7,825 protein-coding sequences predicted in the 8,667,507-bp genome. Many can be plausibly related to the generalist life-style of such an organism, adapted, as it evidently is, to survive and compete in the highly variable environment of the soil. There is a huge complement of transcriptional regulators of many kinds, including extraordinary numbers of RNA polymerase sigma factors and two-component regulators, transporters of molecules into and out of the mycelium, and clusters of genes for the utilisation of all manner of nutrients, including complex polymers like cellulose, chitin and xylan.

With particular relevance to this conference, the *S. coelicolor* genome sequence also contains a much larger number of putative secondary metabolic gene clusters than most of us would have expected [1]. They include the gene sets for the three known *S. coelicolor* antibiotics, actinorhodin (made by a type II polyketide synthase or PKS), the prodiginine complex, the calcium-dependent antibiotic (a non-ribosomal peptide) and the grey spore pigment, also made by a type II PKS. In addition, three chemically distinct siderophores were predicted, which were only later identified chemically. Current studies, largely by the group of Greg Challis at Warwick University, are defining the phenotypes of knockout mutants for these compounds and assessing both their biosynthesis and their different roles in iron-scavenging. Then, there are gene clusters for two classes of complex lipids (unsaturated fatty acids and hopanoids, with likely roles in influencing the physical properties of membranes), two further pigments, a chalcone and a carotenoid, a couple of potential signalling molecules, geosmin and the already identified γ-butyrolactone complex. For future elucidation, there are no fewer than eight further gene clusters, predicted to encode two modular type I PKSs, another non-ribosomal peptide synthetase, two more chalcone synthases, at least one more terpenoid cyclase and a deoxysugar biosynthetic gene cluster.

These findings serve to underline the enormous potential of *Streptomyces* genomes for the "mining" of gene sets to use in the growing field of combinatorial biosynthesis of unnatural natural products that has already been indicated with the piecemeal cloning of gene clusters from many different streptomycetes. It is further reinforced with the discovery of even more secondary metabolic gene clusters in the *S. avermitilis* genome [19]. All except a small number of them are different from the *S. coelicolor* clusters. The future indeed looks bright for the harvesting of useful genes, as whole genome sequencing becomes faster and cheaper and with the increasing ability to predict, just from a series of As, Cs, Gs and Ts, the small molecules likely to be made by the enzymes encoded by the gene clusters.

Nor do we need to confine our attention to the secondary metabolic gene clusters themselves. Many other genes might be harnessed to the task of engineering novel chemical structures. The cytochrome P450 monooxygenases are a case in point. These enzymes are major players in hydroxylation, epoxidation and related reactions in organisms representing all branches of the tree of life. Streptomycete examples are already used in industrially and environmentally important transformations; and they often catalyse steps in antibiotic biosynthesis. Most bacteria have rather few such enzymes (*E. coli* has none), but the genome sequence of *S. coelicolor* revealed no fewer than 18. All have been

cloned and expressed in *E. coli* [13], along with the six ferredoxins and three ferredoxin reductases that would, in combinations to be determined, supply them with reducing power (D.C. Lamb, personal communication). Such enzymes have great potential for the generation of useful unnatural products.

The successful harvesting of an unnatural natural product from a recombinant host such as *S. coelicolor* also requires genetic engineering of the host to optimise what may at first be a very low titre of the desired product. This is genetically a much more complex undertaking than the engineering of the biosynthetic pathway itself, since it depends on innumerable host genes. They include genes for diverse functions, such as the biosynthesis of unnatural precursors for the engineered biosynthetic pathway, facilitating the uptake of a precursor added to the culture medium, export of the unnatural end product of the pathway and the supply of small-molecule co-factors for the pathway enzymes. Such genes can be added to the host's genotype if examination of the genome sequence indicates their absence. Undesired genes include those encoding pathways for biosynthesis of unwanted precursors, pathways competing for the substrates of the biosynthesis and pathways diverting biosynthetic intermediates into unwanted shunt-products. Such genes are candidates for inactivation. Much harder to deal with in the present state of knowledge is the multitude of regulatory genes which, in response to complex combinations of external factors, control expression of the pathway genes in either positive or negative ways. Attempts to optimise the genotype in respect of such genes will initially have to proceed in a partly empirical way, perhaps including the random recombination of pools of mutated hosts [9, 25], until current efforts in the functional genomics of *Streptomyces*, already making excellent progress, amass a database of genetic effects from which rational changes can confidently be predicted.

I hope I have convinced any doubters that the genetic path from Waksman to Sanger has led a long way and will reveal sunny horizons in the future. That this will include profound insights into the strategy adopted by the streptomycetes for life in the soil is, I think, self-evident. The extent to which the resulting knowledge and techniques will also realise some of the potential of genetic approaches for the discovery and development of useful microbial products is of course much harder to predict, depending as it does on many economic and societal factors in addition to the purely scientific. However, I am optimistic and, judging by the enthusiasm of the other speakers in this conference, I am certainly not alone. Little is totally new in science and it is salutary to look back to the origins of one's own studies from time to time. In doing so, I came across a far-sighted statement in another of Albert Kelner's papers that I had previously overlooked. He picked seven *Streptomyces* strains that had failed to reveal anti-microbial activity in tests against particular bacteria. After irradiating them with X-rays, at least five and possibly six of them gave rise to antibiotic-producing variants. Kelner [11] commented: ''Therefore, by genic manipulation of the cell we have a means for obtaining, in quantities sufficient for study, many of the metabolic products of the living organism that would otherwise be undetectable''. We can now interpret this prophetic statement in terms of the genetic potential of any randomly chosen streptomycete to make far more secondary metabolites than we detect by typical anti-microbial tests and the complex regulatory circuits that keep many of the secondary metabolic pathways ''silent'' until they are activated in response to specific signals or de-repressed by mutation. The challenge now is to achieve such changes in rational and predictable ways.

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