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David Hopwood and the emergence of *Streptomyces* genetics

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Summary *Streptomyces* spp. are unusual among bacteria in growing as mycelial colonies with sporulating aerial hyphae. They are very important as the source of most of the major antibiotics. Pioneering work by David Hopwood in the 1950s and 1960s established *Streptomyces coelicolor* A3(2) as the model system for the genus. Since then he has led successive key phases of research on this organism. In the 1970s, plasmids were discovered and characterised, and used both to establish conditions for transformation and in the subsequent development of cloning vectors. Protoplasts were exploited in both transformation and highly efficient cell fusion. In the 1980s, the early cloning of resistance genes from antibiotic-producing strains was followed by the cloning of antibiotic biosynthetic gene clusters, and the development of general methods and probes for the cloning of such clusters from diverse species. Analysis of these gene sets led to wide-ranging inferences about the biosynthesis of the important polyketide class of antibiotics, and to the production of hybrid antibiotics, and then, in the last decade, to more sophisticated combinatorial biosynthesis of designer molecules. In parallel, David Hopwood's work has also provided a crucial platform for studies of the regulation of the morphological and physiological differentiation that is manifested by sporulating antibiotic-producing colonies. Most recently, his involvement in the physical mapping of the entire 8 Mb genome of *S. coelicolor* A3(2) has culminated in its complete DNA sequencing: a project that should be completed under his management during the year 2000.

Key words *Streptomyces coelicolor* · David Hopwood · Linkage analysis · Actinorhodin · Polyketide pathway

Introduction

In the world of academic science, very few bacteria become truly international currency, and fewer still are the subjects of concerted attempts to penetrate all aspects of their biology. Of course, thanks ultimately to the pioneering work of Lederberg and Tatum, *Escherichia coli* K-12 (supplemented by the organism formerly known as *Salmonella typhimurium* LT2) has provided a marvellous model of most of the basic mechanisms of growth: metabolic pathways, gene expression, DNA replication, macromolecular synthesis, regulatory circuitry, interaction with the environment, cell division, mobility, plasmid and phage biology, and the simplest kind of cellular differentiation [67]. *Bacillus subtilis* 168, with its twin bonuses of efficient natural competence for transformation (the discovery of Spizizen in 1959) and comparatively complex cellular differentiation (first emphasized as a subject for genetic analysis by Schaeffer in 1960), has been almost as extensively studied [76]. It has provided harmony and counterpoint to many of the themes played by *E. coli*, and has added several of its own, most conspicuously in the fields of phosphorylation cascades, developmental decision-making, sigma factor evolution and diversity, partner-switching models of regulation by anti-sigma factors, morphological coupling of gene expression, chromosome partitioning, and entry into, maintenance of, and exit from, extreme dormancy. No one person dominates

the history of *E. coli* or *B. subtilis* genetics—our extraordinary knowledge of them is the result of the communal efforts of many outstanding researchers. A number of other unicellular bacteria, sometimes more closely associated with one or a few major contributors (a notable coupling being *Pseudomonas aeruginosa* and Bruce Holloway [24]), have been subjected to sustained analysis, but on narrower fronts, providing windows onto various other aspects of microbial life such as catabolic versatility, nitrogen fixation, symbiosis, photosynthesis, pathogenesis, cell–cell interactions, and developmental differences between siblings, and still more have been studied because of their direct relevance to human medicine, food, agriculture or industry. However, the mycelial organism *Streptomyces coelicolor* A3(2) perhaps comes closest to rivaling *E. coli* K-12 and *B. subtilis* 168 as a subject for global study. The discovery of recombination in *S. coelicolor* was in 1955. In 2000, its genome (nearly equal in size to the sum of the *E. coli* and *B. subtilis* genomes) will have been completely sequenced. In between, an enormous range of aspects of its biology have been studied, not least the multicellular differentiation of its mycelial colonies and the spectacular biochemical genetics and regulation of its secondary metabolism. Remarkably, much of this work was inspired and initiated by David A. Hopwood, whose sustained influence on the development of studies of a particular organism over more than four decades has few parallels in the history of prokaryotic biology. This article was written to mark the occasion of David Hopwood's formal retirement in 1998.

Mapping out the territory

David Hopwood began to study *Streptomyces* genetics as a Ph.D. student in the School of Botany, Cambridge, UK, in the mid 1950s, at a time when the *E. coli* genetic map was first beginning to make sense. His supervisor, H. L. K. Whitehouse, although a clear-sighted classical geneticist, had no knowledge of prokaryotes, and nobody else in the world was known to be working on *Streptomyces* genetics (though it turned out that Saito in Japan, Sermonti in Italy, and Bradley and Lederberg and Braendle and Szybalski in the USA were thinking along similar lines). Nevertheless, Hopwood examined several *Streptomyces* strains as potential subjects for genetic analysis, and after one false start settled on *S. coelicolor* A3(2), which was a micromanipulated single spore subculture of an agar-decomposing strain, A3, previously studied by Roger Stanier. It came to Hopwood from Dagny Erikson, preserved in sterile soil. A particular attraction of the strain was the beautiful blue pigment from which it derived its name. Hopwood saw this as a potentially valuable genetic marker at the time, little realizing that it would provide a key to understanding the pervasive problem of the programming of polyketide biosynthesis (see below). Hopwood's Ph.D. work beautifully developed the basic genetics of *S. coelicolor* A3(2), especially through his invention of the "four-on-four" method of linkage analysis [25], a procedure that was subsequently also put to good effect in revealing important aspects of the genetics of organisms as diverse as *Rhizobium leguminosarum* [4] and *Thermoactinomyces vulgaris* [31] as well as of several closer relatives of *S. coelicolor*. By the late 1960s, working entirely on his own, Hopwood had established a detailed circular linkage map of more than 100 *S. coelicolor* genes [27]. An interesting feature of this map was the clustering of essential and housekeeping genes into two arcs separated by long "silent" regions (see below). By the mid-1960s, Hopwood had become an outstanding lecturer in Genetics in Glasgow University, where he was deeply influenced by Guido Pontecorvo. In 1967 he took his wife Joyce and his children Nicholas and Jonathan (Nick and John) off to New York for a sabbatical year with Werner Maas. [Their daughter Rebecca (Becky) was born soon after their return.] Hopwood's work there on the role of arginyl tRNA synthetase in regulating arginine biosynthesis in *E. coli* [23] provided the activation energy for him to feel comfortable about biochemistry, which gave him just the start he needed to be able to move into more molecular aspects of genetics in the next decade. Elsewhere, other events were taking place that would shape Hopwood's future career. The John Innes Institute, then a fading research center with an honorable place in the history of British plant and microbial genetics, moved 100 miles to a new location in Norwich, to become associated with the recently established University of East Anglia. The new Director of the John Innes Institute was an organic chemist, virologist and inventive engineer called Roy Markham. With extraordinary prescience, Markham appointed David, who was still only 34, as Head of the Genetics Department, giving him the opportunity to establish a more substantial group, as well as to shape the Department's future. The blend of plant and bacterial genetics that Hopwood established in the Department has itself been an exceptional success, sustained over three decades, and has contributed significantly to the re-emergence of the John Innes Centre as a leading centre for plant and microbial science.

The 1970s: the adolescence of *Streptomyces* molecular genetics

In the early 1970s, Hopwood's laboratory established the existence and basic features of plasmids and phages of *S. coelicolor* [15, 73, 81]. The first genetically defined plasmid, SCP1, proved impossible to isolate by conventional

procedures [82], and it was many years before it was revealed as a huge linear molecule of some 350 kb [52]. The large size may go some way towards explaining one of the two "silent" arcs in the linkage map (that at "9 o'clock"), since many of the strains used in linkage mapping contained an integrated copy of SCP1 exactly at the position of this arc [22, 36, 37]. Not all plasmids proved so difficult, and biochemical work by Hildgund Schrempf in Werner Goebel's laboratory revealed a cryptic covalently closed circular plasmid, SCP2, in *S. coelicolor.* This work was completed during a visit to Norwich by Dr. Schrempf during 1975 [73]. In the later 1970s, Hopwood's Ph.D. student Mervyn Bibb showed that SCP2 was an important mediator of chromosome transfer [6]. Mervyn also discovered that SCP2 transfer was associated with transient growth inhibition which could in some circumstances be seen as pocks on growing lawns of a suitable recipient strain. The plasmid studies and protoplast technology were then combined in the first successful protoplast transformation experiments, in which it was shown that polyethylene glycol could stimulate SCP2 DNA uptake to the extent that up to 10^7 pock-forming transformants could be obtained with 1 µg DNA [8]. At about the same time, Hopwood's laboratory demonstrated protoplast fusion and explored its genetic consequences and possibilities in a few particularly penetrating papers [28, 34, 40]. Interestingly, much of this work was going on when Stanley Cohen, a world leader in plasmid biology and pioneer of recombinant DNA methods, was in Hopwood's laboratory on sabbatical leave, so it was no surprise that the transformation technology, allied to further molecular studies of plasmids [5, 6, 9, 50] led to recombinant DNA experiments in *Streptomyces,* the first cloning in *Streptomyces* of a *Streptomyces* gene (for methylenomycin resistance) by Mervyn Bibb (by then in Stanley Cohen's lab, [7]), with almost simultaneous success by Charles Thompson in Hopwood's own lab [80].

Breaking through from the primary to the secondary phase: genes for antibiotic production

Soon, the pioneer cloning experiments were followed by the first cloning of antibiotic production genes by the Norwich group, following on from conventional genetic analyses of production of three distinct antibiotics of *S. coelicolor,* which Hopwood had initiated in the 1970s, and which had revealed that although two sets of genes were chromosomically located, one set (for methylenomycin) was located on the SCP1 plasmid [53, 54, 71, 72, 85]. A key feature of this time was the influx of Spanish post-doctoral scientists into the group; this has continued almost as a tradition at Norwich, with at least 20 such visitors in the last 20 years (Fig. 1). Among the early visitors, Francisco Malpartida came from Antonio Jiménez's laboratory and cloned the *act* genes for production of the famous blue pigment, actinorhodin, using high capacity low copy number vectors based on SCP2 [60]. Malpartida evidently liked color: later he cloned the even bigger entire set of *red* genes for biosynthesis of the red antibiotic undecylprodigiosin [62], following the first cloning of some *red* genes at Norwich by Jerald Feitelson [16, 17]. José Antonio Gil came from Juan-Francisco Martín's laboratory in León and cloned *pabA* from a candicin-producing strain of *S. griseus* [21]: a gene that proved to encode the first step in candicidin biosynthesis, and that eventually provided access to linked DNA encoding the polyene biosynthetic machinery [41]. Alfredo Aguilar, now influencing the progress of science in a different way through his work in Brussels, discovered that the methylenomycin production genes of a second producing organism were located on a plasmid (pSV1) as in *S. coelicolor* A3(2), but in this case the plasmid was circular instead of being linear as in SCP1 [1]. Two visitors to Norwich from Carlos Hardisson's group in Oviedo, Juan Evaristo Suárez and M. Rosario Rodicio [70, 77, 78] developed *Streptomyces* vectors from the temperate phage ϕC31, discovered in Moscow by Natalia Lomovskaya [58]. These were then used in a novel strategy to isolate and analyze a large segment of the *mmy* gene cluster for methylenomycin biosynthesis from *S. coelicolor* [11, 12]. The strategy used depended on earlier papers from Hopwood's group: first, the demonstration that the *mmy* genes were located on SCP1 [53, 54]; and second that the physically unisolable SCP1 could be transferred between *Streptomyces* species [32, 33]. SCP1 DNA provided the only extensive DNA homology between *S. coelicolor* $SCP1^+$ and *S. parvulus* $SCP1^+$ strains, and it was this that made the *mmy* cloning possible. During this time, the first discovery of cryptic antibiotic production genes was made when George Jones found that *Streptomyces lividans*, despite being a non-producer of actinomycin D, contained a gene for the final step in actinomycin D biosynthesis that could be activated by certain cloned DNA fragments from another species [42, 43]. The use of cloned heterologous DNA to activate the normally silent actinorhodin genes of *S. lividans* has proved a fertile approach to the isolation of regulatory genes for secondary metabolism [19].

The programming of polyketide biosynthesis

By the mid-1980s, and almost entirely because of Hopwood's work, *Streptomyces* genetics research groups were springing up all over the world, most notably in North America, Europe and Japan (augmenting a long-standing interest in

Fig. 1 David Hopwood with some of his Spanish collaborators. The picture was taken at Hopwood's retirement celebration at the John Innes Centre, September 11, 1998. Rear, left to right: J. A. Salas, A. M. Cerdeño, J. L. Caballero, J. A. Gil, D. A. Hopwood, C. Vallin (Cuba), A. Jiménez, J. A. Soliveri, J. A. Aínsa, F. S. Malpartida. Front, J. E. Suárez, M. A. Fernández-Abalos, M. L. Villalobos, R. Santamaría

Streptomyces emanating from the successful Japanese pharmaceutical industry), and there was a sea-change in the activities of industrial *Streptomyces* research scientists with excellent contributions to *Streptomyces* genetics coming particularly from the laboratories at Eli Lilly. Hopwood then continued to establish general methodologies and tools for cloning antibiotic production genes, and to begin to use them to study the mechanisms by which one simple biosynthetic route, the polyketide pathway, could lead to an astonishing range of end products, serving diverse roles, across the whole of the prokaryotic and eukaryotic kingdoms. A string of widely used plasmid vectors was constructed [3, 45, 59, 79] and Hopwood organized a series of EMBO practical workshops on *Streptomyces* genetics, plus a similar course for Asia held in Wuhan, China in 1989, that ensured that a whole generation of young scientists was brought together in an international network of *Streptomyces* researchers that has continued to flourish throughout the 1990s. An important offshoot from this venture was the practical manual [35] that came out of the needs of the early courses. This now aging text, which is still in use throughout the world, is soon to be replaced [49].

Meanwhile, the first production of a hybrid antibiotic by genetic engineering was achieved [38]. This involved the introduction of segments of the actinorhodin gene cluster into other *Streptomyces* spp. that made different, but related, polycyclic aromatic polyketide antibiotics. A feature of these experiments that harked back to Hopwood's early choice of *S. coelicolor* A3(2) as an experimental organism was the exploitation of pigments—all the antibiotics involved, and the resulting hybrid compounds, could be recognized by their characteristic color. Another feature of the work was the international collaboration between researchers in Spain, Japan, the USA and the UK, which is typical of Hopwood's work in the last dozen years. For example, reference 44 was the sixth co-publication with Prof. S. Omura. The ground-breaking hybrid antibiotic paper opened up two research directions: one, the use of hybrid pathways to elucidate the roles of genes in clusters such as *act* [20, 46, 47, 51, 63–65, 74]; and the other, the microsurgical construction of hybrid

polyketide biosynthetic gene sets in the "combinatorial biosynthesis" of designer antibiotics [2, 44, 55, 66]. Much of this work was made possible by the discovery that conservation of equivalent genes from different but related pathways was often high enough to permit cloning by hybridization using probe genes initially derived from the *act* cluster [61, 75]. The use of this route, and a companion route based on the often-observed linkage of cognate resistance genes to biosynthetic clusters [12], permitted the cloning of many of the gene sets now in use to make hybrid antibiotics. In the case of polycyclic aromatic polyketides such as actinorhodin, it has been found that each of the several iterative additions of C2 units is done by the same condensing enzyme, and that nearly all the modifications and shaping of the resulting chain are done by separate enzymes (typically about 20 in a pathway). The situation for macrocyclic polyketides and polyenes has proved interestingly different [29, 41].

Threading through this remarkable flood of work has been a continual interest in the regulation of antibiotic biosynthesis. Early on, it was deduced that one class of *act* mutants (*actII)* might lack a positively acting regulatory element, because they neither secreted nor converted biosynthetic precursors of actinorhodin; and indeed *actII*-ORF4 proved to encode a novel class of transcription factor, that is widespread in diverse antibiotic biosynthetic gene clusters [18, 83]. The realization of the significance of *actII*-ORF4 for properly timed expression of genes for secondary metabolism led to the design of an expression system by Chaitan Khosla's group, in association with David, which was subsequently used to analyze and exploit diverse gene sets for polyketide synthesis [64].

Getting studies of aerial mycelium off the ground

Early in his career, Hopwood carried out a number of cytological studies of the growth and sporulation of *S. coelicolor,* with the participation of some highly skilled and experienced microscopists, especially Audrey Glauert [e.g. 30, 84]. This revealed the occurrence in aerial hyphae of a kind of septum specific to sporulation-associated cell division—a topic that continues to be explored in several laboratories. Hopwood's interest in development led to the isolation of the first aerial mycelium-less "bald" mutants. Several of these, including S48 [27], later proved to be defective in the gene (*bldA*) for the tRNA specific for the rare TTA (UUA) leucine codon [56]. The presence of a TTA codon in the *actII*-ORF4 regulatory gene was eventually found to account for the failure of such *bldA* mutants to make actinorhodin [18]. The Hopwood group also isolated and did some initial characterization of about 250 mutants with white, brown or green, instead of grey, aerial mycelium [39]. Many of these mutants, which are still extensively studied today [10], were defective in various aspects of sporulation in the aerial hyphae. These early studies stimulated the emergence of *S. coelicolor* A3(2) as one of the handful of bacterial systems studied in detail for their developmental biology. Hopwood is still making contributions in this area, particularly in relation to the compartmentalization of polyketide-related gene expression between different cell-types, with the demonstration that homologous genes from the *act* and *whiE* spore pigment gene clusters are capable of functioning in each other's place, but only if ectopically expressed [86]. On the other hand, just one malonyltransferase in *S. coelicolor* appears to provide the last pathway-non-specific step in biosynthesis of these two polyketides [69].

Back to the future: sequencing the *Streptomyces coelicolor* chromosome

The isolation of mutants and the mapping of genes to the chromosome has continued to underpin all of these investigations of *Streptomyces* biology, though the methodologies and resolution have evolved considerably. Remarkably, almost no inconsistencies have been revealed between the early linkage maps [36] and the later maps based on hybridization of cloned DNA first to macrorestriction fragments of the whole 8 Mb chromosome [48], and then to an ordered cosmid library [68]. The latter library of ~320 cosmids is providing the foundation for the sequencing of the entire *S. coelicolor* genome at the Sanger Centre, UK (http://www.sanger.ac.uk/Projects/S_coelicolor/), a project inaugurated and managed by David. Typical of Hopwood's *modus operandi*, the whole *Streptomyces* and microbiological community is benefitting from the immediate availability of sequence information on the website.

The physical analysis of the genome yielded one major surprise that had not been foreseen from linkage mapping: the chromosome was not a circle, as expected both from genetic mapping and by analogy with other bacterial chromosomes, but a linear structure with long terminal repeats attached to a protein [13, 14, 57]. Prophetically, Hopwood had earlier pointed out that a circular genetic map did not necessarily mean physical circularity of the genome [26]. The second of the long silent regions in the original linkage map, that at "3 o'clock", has proved to consist of the two chromosome ends—more than 1 Mb at each end contains no housekeeping or essential genes, instead comprising various genes with more "ecological" roles, or integrated genetic elements of diverse kinds.

Coda

This outline of Hopwood's research career is incomplete. It does not mention the enormous influence he has had on the programmes of innumerable congresses, international symposia and other scientific meetings, nor his long and highly influential role in the funding and direction of microbial science in the UK. One measure of his significance is the 10-fold increase in the proportion of *Streptomyces* papers in leading microbiological journals, from about 0.4% to 4% over the last 30 years. It is in recognition of the sum total of these achievements that he has been internationally honoured by many national academies, microbiological societies and institutions, including Honorary Membership of the Spanish Society for Microbiology in 1985. In the UK, he is a Fellow of the Royal Society, and has received public recognition by the rare award (to a scientist) of a Knighthood.

David's energies have not diminished as he enters formal retirement. His coordination of the *S. coelicolor* genome project and continuing engagement in the problems and possibilities of combinatorial biosynthesis of antibiotics make sure of that. It has been a great privilege to work with him for the last 30 years, and he continues to be a friend and an outstanding example to me and many others.

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