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Soil To Genomics: The *Streptomyces* Chromosome

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Streptomyces chromosome, bacterial conjugation, linear chromosome replication, chromosome partition

Abstract

The 8–9-Mb *Streptomyces* chromosome is linear, with a "core" containing essential genes and "arms" carrying conditionally adaptive genes that can sustain large deletions in the laboratory. Bidirectional chromosome replication from a central *oriC* is completed by "end-patching," primed from terminal proteins covalently bound to the free 5′-ends. Plasmid-mediated conjugation involves movement of double-stranded DNA by proteins resembling other bacterial motor proteins, probably via hyphal tip fusion, mediated by these transfer proteins. Circular plasmids probably transfer chromosomes by transient integration, but linear plasmids may lead the donor chromosome end-first into the recipient by noncovalent association of ends. Transfer of complete chromosomes may be the rule. The recipient mycelium is colonized by intramycelial spreading of plasmid copies, under the control of plasmid-borne "spread" genes. Chromosome partition into prespore compartments of the aerial mycelium is controlled in part by actin- and tubulin-like proteins, resembling MreB and FtsZ of other bacteria.

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INTRODUCTION

Streptomyces is one of some 120 genera in the *Actinomycetales* (E. M. H. Wellington, personal communication). From the discovery of the first actinomycetes in the 1870s right up to the late 1950s, they were commonly believed to be intermediate between bacteria and fungi, but they are in fact a major branch of the Gram-positive bacteria, distinguished by a much higher G+C content in their DNA, often over 70%, compared with less than 50% for low-G+C Gram-positives like *Staphylococcus*, *Streptococcus*, and *Bacillus*.

Streptomyces colonies, most unusually for bacteria, consist of differentiated "tissues." A resting spore produces one or more germ tubes that elongate by apical growth (31), make cross walls at intervals, and initiate side branches with new hyphal tips (**Figure 1**), allowing colony biomass to increase quasi-exponentially (22). Probably in response to a diminishing food supply, the substrate or vegetative mycelium produces branches extending into air spaces in the soil. After elongating for a while, distal regions of these aerial hyphae divide by special "sporulation septa" into compartments that become spores (**Figure 2**).

Soil-inhabiting actinomycetes are preeminent antibiotic producers—Bérdy (13) estimated 8700 antibiotics discovered in them by 2002, compared with 2900 for all other bacteria and 4900 for fungi—so *Streptomyces* genetics acquired an applied dimension right from its independent inception in six laboratories in the mid-1950s (1, 16, 17, 41, 98, 104). For some, a major aim was to develop methods for breeding superior antibiotic-producing strains, but I was motivated by the still unresolved phylogenetic relationships of the actinomycetes. The young science of bacterial genetics had revealed three bizarre gene exchange mechanisms, all transferring incomplete genomes from a donor to a recipient: transformation by naked DNA, transduction by a bacteriophage, and a novel form of mating, whereas fungal life cycles resembled those of plants and animals, with fusion of genetically complete nuclei alternating with recombination at meiosis. This made the actinomycetes a potentially happy hunting ground for novel genomic organization and behavior. Even after the true phylogeny of the actinomycetes was established, they represented a group of bacteria far removed from the few unicellular types in which genetic studies had been made, so my interest remained unabated.

Actinomycetes: microorganisms forming the high G+C branch of the Gram-positive bacteria, including antibiotic-producing soil inhabitants like *Streptomyces*, and pathogens such as *Mycobacterium*

Mycelium: a mass of interconnected, multigenomic, tubular cells (hyphae), characteristic of molds and soil-inhabiting actinomycetes

GENOME TOPOLOGY AND REPLICATION

Topology

When *Streptomyces* genetics began, cytological studies had revealed no significant information about the genome. Sporadic reports (4, 75) of chromosome behavior resembling mitosis and meiosis in higher organisms were no more firmly based than for rod-shaped bacteria (96). As in *Escherichia coli*, only genetic studies began to reveal the nature of the carriers of genetic information.

By analyzing recombinants arising from mixed cultures of two genetically marked derivatives of a strain called *Streptomyces coelicolor* A3(2), I deduced three linkage groups (42), later combining them into two (43) and then into a single, circular linkage group (44). However, although this fitted the finding of a circular chromosome in *E. coli*, linkage map circularity does not prove chromosome circularity, as Stahl & Steinberg had pointed out (110): It can result from a linear genome if viable recombinants arise only from even-numbered crossovers, because this forces inheritance of both ends of the same parental chromosome, formally equivalent to complete linkage of markers at the two ends (**Figure 3**). Since mating in *Streptomyces* was likely to yield "merozygotes" with one complete and one incomplete chromosome, as in other bacteria, there could have been just such a requirement for even-numbered crossovers, leading to a circular map even if the chromosome were linear.

I tried to resolve the dilemma using partially heterozygous colonies called heteroclones that appear when recombinants inheriting two closely linked nutritional markers *in trans* are selected from a cross (103). Few haploid recombinants can grow because they need crossing over in the short map interval between the markers, so heterozygotes with a prototrophic phenotype resulting from complementation between the selected markers are favored. As they develop into colonies, haploid recombinant genomes arise by crossing over within their duplicated regions, but most of these segregants inherit only one of the selected markers, so hyphae containing them stop growing, and the colonies expand slowly (**Figure 4**). Analysis of the spores produced by individual colonies reveals their regions of heterozygosity (54). In a large set of such heteroclones, heterozygosity occurred for any arc of the circular linkage map so, assuming this corresponded to a contiguous chromosomal segment, the chromosome lacked constant ends: It was either a circle or a circularly permuted rod (45). The ambiguity remained from 1966 until 1992, when a macrorestriction map appeared to prove circularity (72), but this conclusion was overthrown the following year when the chromosome of the closely related *Streptomyces lividans* was found to be linear with constant ends, a state soon found to apply to *S. coelicolor* also (79). [A false linkage had been caused by a repeated sequence—a terminal inverted repeat (TIR)—on the chromosome ends, which hybridized with one of the "linking clones" used to identify presumed adjacent restriction fragments.] Remarkably, viable variants arose, or could be engineered, with the ends fused, but the resulting circularity was not the normal chromosome topology (79).

The hypothesis that map circularity deduced from haploid recombinant frequencies might be due to recovering only even-numbered crossover progeny was strikingly confirmed by a recent physical analysis of crosses between *S. coelicolor* and *S. lividans*, which have recognizably different chromosomal end sequences: Nearly all recombinants inherited both ends from the same parent (114). The results of the heteroclone experiment required a different explanation. A revealing microscopical study suggested one

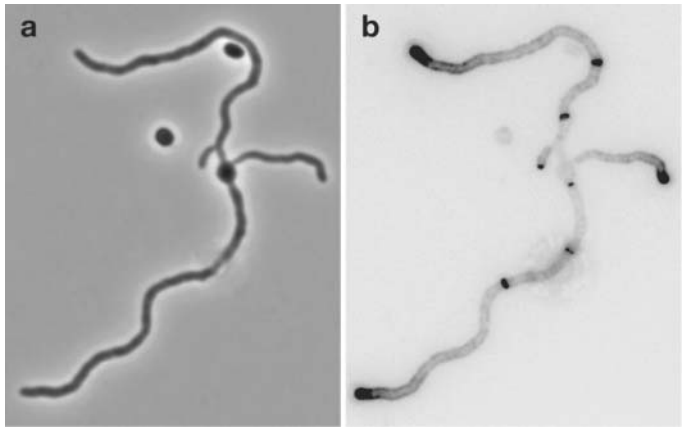

Figure 1

A spore of *S. coelicolor* has produced three germ tubes that are developing into young vegetative mycelium (two ungerminated spores are also seen). (*a*) Phase-contrast; (*b*) stained with a dye coupled to vancomycin, which binds to hyphal tips and developing cross walls, where new wall material is being made (Klas Flärdh, University of Lund).

Merozygote: an incompletely diploid zygote resulting from transfer of a partial genome from a donor to a recipient by transformation, transduction, or conjugation

Heteroclones: partially heterozygous *Streptomyces* colonies that develop from a mating after selection for two closely linked nutritional markers *in trans*

TIR: terminal inverted repeat (an identical DNA sequence in opposite orientation on the two ends of a replicon or other genetic element)

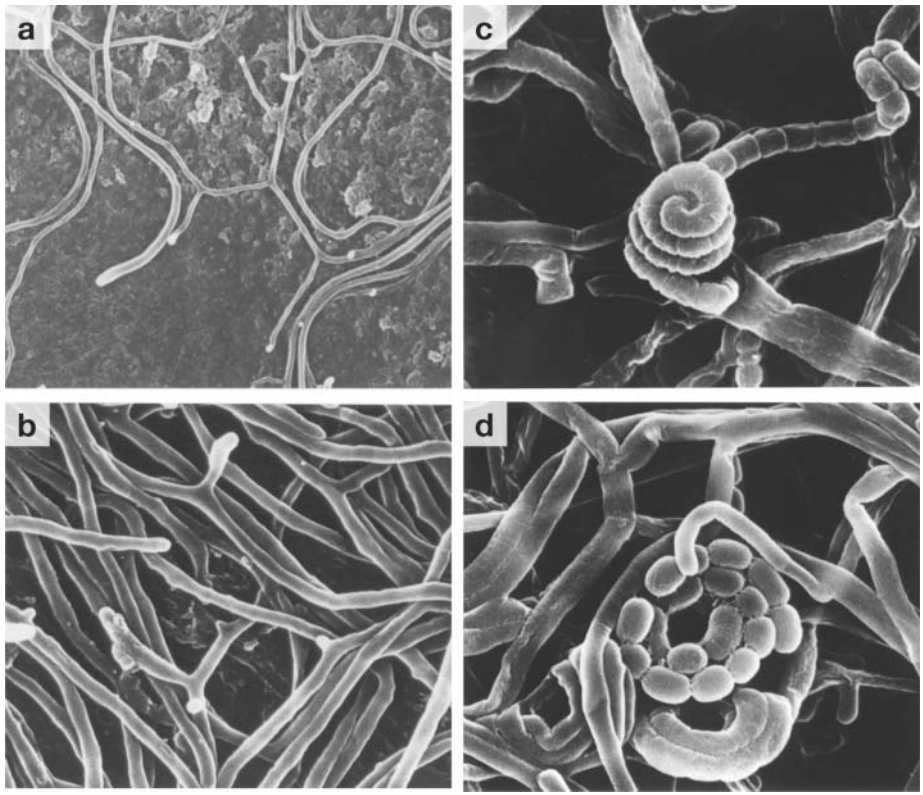

Figure 2

Scanning electron micrographs showing four stages in colony development in *S. lividans*. (*a*) Young vegetative mycelium at a colony margin; (*b*) mature vegetative mycelium producing aerial branches; (*c*) aerial hypha developing into prespore compartments (spore chains are helical in this species); (*d*) chain of mature spores. (Jeremy Burgess, John Innes Centre).

(124). Different regions of the *S. coelicolor* chromosome were tagged with fluorescently labeled probes, whereupon the two ends were nearly always seen adjacent in the vegetative mycelium. The conclusion was a (noncovalent) joining of the two chromosomal ends to give a circle, which could perhaps generate heteroclones with arcs of heterozygosity bridging the ends. Such an association of the ends of linear genomes carrying TIRs, including those of *Streptomyces* plasmids, had been proposed by Sakaguchi (99), who dubbed the genomes "invertrons" and suggested they form a "racket-frame" with the ends held together to form the "handle" by proteins binding to the TIRs and to each other.

Replication

Streptomyces chromosomes and their linear plasmids have a special mode of replication. Like other linear genomes, including those of *Bacillus* phage Φ29 and adenoviruses, they have a terminal protein (TP) bound to their free 5′-ends. However, whereas these other genomes initiate replication from the TP as a single strand from either end of the molecule (100), *Streptomyces* chromosomes replicate bidirectionally from a typical, centrally placed *oriC* (65). The two replication forks of circular bacterial chromosomes meet to complete the daughter molecule, but linear replicons face an "end problem": the leading strand is synthesized right to the chromosome ends, but removal of the RNA primer for the last Okazaki fragment of the lagging strand leaves a single-stranded 3′-gap, which is "end-patched" by DNA synthesis primed from the TP [the gap was estimated as ~280 nt for pSLA2(21) and for some other *Streptomyces* replicons (C-H. Huang & C. W. Chen, personal communication)]. The TP is encoded

Plasmid: a bacterial replicon separate from the main chromosome: *Streptomyces* plasmids can be circular or linear

TP: terminal protein attached to the free 5′ end of a linear plasmid or chromosome to prime the "end-patching" DNA synthesis that completes replication

by a gene on the *Streptomyces* chromosome and on some linear plasmids, the remainder relying on the host chromosomal gene (5, 123).

Streptomyces TIRs vary from <1 kb to >550 kb. They contain some seven tightly packed palindromic sequences (57), which would lie in the single-stranded region and might allow it to fold back on itself to provide structured recognition sites for the TP (20, 23, 93). While this is still a possibility, recent studies show the palindromes to be targets for a telomere-associated protein (Tap), encoded by an ORF upstream of the TP gene, responsible for recruiting TP to the chromosome end (6). Tap and TP are candidates for binding the chromosome ends together, and other proteins may remain to be identified.

What is the adaptive value of chromosome linearity? Presumably there is an advantage to compensate for the end problem. With many chromosomal copies in each mycelial compartment, interlinking of circular chromosomes during DNA replication and recombination might hinder their proper distribution into hyphal branches and spores. This cannot be fatal, because the variants of *S. lividans* with circular chromosomes grow normally, and fast-growing *E. coli* cells contain multiple chromosomes that are decatenated efficiently by topisomerase IV (69), but perhaps the need to constantly decatenate *Streptomyces* circular chromosomes might impede colony growth just enough for circularity to be disfavored.

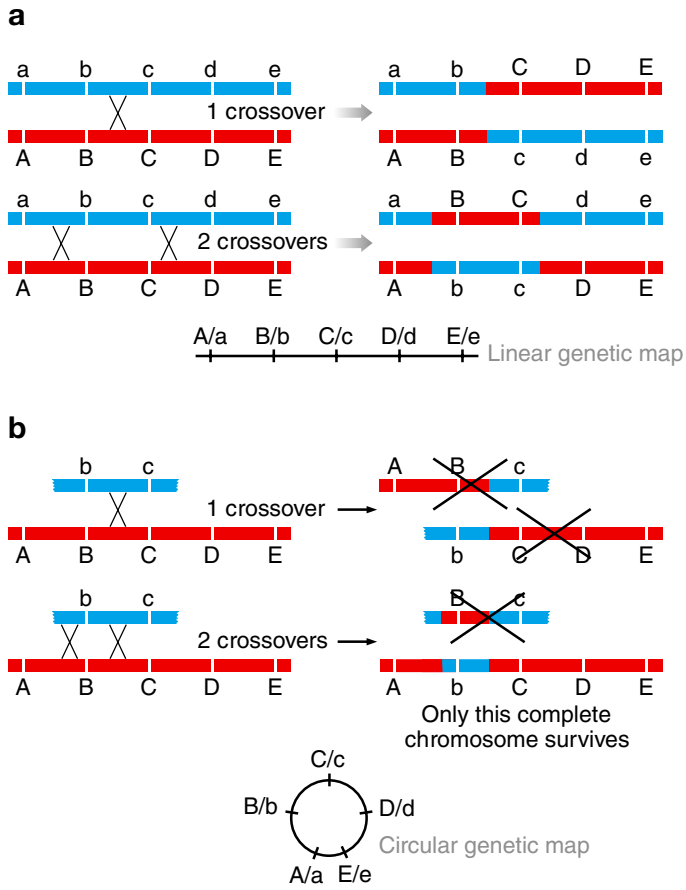

Figure 3

Consequences of crossing over between complete and incomplete chromosomes. (*a*) With two complete chromosomes, both single and double crossovers give full-length recombinant chromosomes, hence viable progeny, and the genetic map is linear; (*b*) with one of the parental chromosomes incomplete, only double crossovers give full-length recombinant chromosomes, and they inherit both ends from the same parent, which therefore appear close on the chromosome, making the map circular.

Genome Instability

Chromosome circularization, via recombination between sequences near the ends (19, 60), is only one of the structural changes undergone by *Streptomyces* chromosomes. Robinson et al. (97) described variants carrying a 6-kb sequence tandemly amplified ~300 times, and many other reports followed, some very detailed. Amplifiable units of DNA (AUDs) may generate variants by unequal crossing over between two pre-existing copies (2), and amplification is often associated with deletion of long (megabase-sized) DNA segments (113), sometimes extending from the AUD to (near) the chromosome end [see references in (118)]. Large terminal deletions can be progressive, as in *S. lividans*, in which ~0.5% of spores gave variants lacking a short terminal segment including a chloramphenicol resistance gene and, among these, up to 25% of arginine auxotrophs arose by loss of a further large

ORF: open reading frame (coding sequence of a gene)

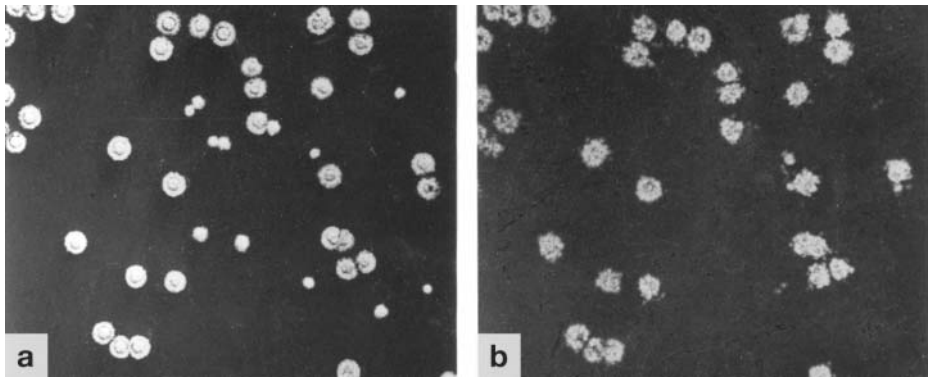

Figure 4

(*a*) Haploid *S. coelicolor* recombinants and heteroclones (*small colonies*) growing on a medium selecting for two closely linked nutritional markers *in trans*; (*b*) replica plate on the same medium, on which the haploids have grown but the heteroclones mostly have not because their spores nearly all inherit only one of the selected markers (54).

DNA segment (27). All these gross structural changes were found in laboratory-grown cultures, so it is an open question how far they are relevant in natural habitats.

Streptomyces TIRs can be plastic too. In *S. coelicolor* A3(2) strain M145 they are 22 kb long (9), but other laboratory derivatives, and presumably the soil progenitor of the A3(2) strain (47), have 1.06-Mb TIRs, containing over 1000 duplicated genes; their subculture gave strains in which recombination between copies of a transposon had shortened the TIRs (115). The longest recorded TIRs are in *Streptomyces ambofaciens* variants, which arose by end-to-end fusion of deleted chromosomes, giving genomes with 6.5-Mb TIRs and only very short unique segments (118). Unsurprisingly, these genomes were highly unstable, perhaps because they contained two competing *oriC* regions. Crosses between *S. coelicolor* and *S. lividans* generated rare recombinants stably inheriting one TIR from each parent and so differing except for a short terminal sequence (114), while perhaps *S. avermitilis*, with TIRs of only 168 bp (59), is such a naturally occurring hybrid (24). Likewise, *S. lividans* plasmid SLP2 is presumed to have originated by recombination between a progenitor plasmid and the *S. lividans* chromosome (58).

SCP1: 356-kb linear plasmid of *S. coelicolor*

DNA TRANSFER BY MATING

Plasmid-mediated DNA Transfer

The first *S. coelicolor* linkage mapping proved gene transfer by some kind of mating, rather than by transformation or transduction, because recombinants often inherited distant markers from each parent, not just closely linked genes (41). The only precedent for bacterial mating was F-mediated conjugation in *E. coli*, so it was natural to compare the two processes. The comparison was strengthened when strains derived from the *S. coelicolor* A3(2) wild type by successive mutagenesis and recombination were deduced to contain, or lack, an autonomous plasmid, SCP1, or to have it integrated into the chromosome, dramatically affecting recombination frequencies (49), and so at least superficially resembling the F^+, F^-, or Hfr states in *E. coli* K-12. It is now clear, however, that the iconic single-stranded, progressive DNA transfer represented by F-mediated conjugation does not apply to streptomycetes, which seem to transfer plasmids and chromosomes by mechanisms related to those promoting DNA movement during normal bacterial development.

The first indication that *Streptomyces* plasmid transfer, and hence chromosome

mobilization, differs fundamentally from mating in Gram-negative bacteria was the finding that the transfer region of a multicopy plasmid, pIJ101, was no more than 2.1 kb long (71), and the complete sequence later revealed a single *tra* gene (70), in contrast to more than 30 on F (119). This is typical of (circular) *Streptomyces* plasmids, as shown by studies of the multicopy pSN22 (68) and pSG5 (88), the low-copy-number SCP2 (18), and an integrating plasmid, pSAM2 (39).

Separate from the transfer gene, "spread" genes (*spd*) were postulated to explain plasmid movement from the point of initial transfer to colonize the recipient mycelium (71). They were revealed by the appearance of "pocks," circular zones within an initially plasmid-free culture surrounding a coinoculated plasmid-containing spore (**Figure 5**) and reflecting plasmid colonization of the recipient mycelium, assuming this led to growth retardation or perhaps, as originally proposed, even death of some hyphae (14, 15). Small-pock mutations identified the *spd* genes, which were postulated to promote migration of plasmid copies inside the recipient mycelium (71), a hypothesis that has been universally adopted but not proven.

A crucial issue has been the strandedness of the transferred DNA, since this bore directly on the relevance of the *E. coli* paradigm. Several F-borne transfer genes encode a "relaxosome" that nicks the starting plasmid before it transfers one of its strands to the recipient during rolling-circle replication (119). *Streptomyces* plasmids lack such genes, providing initial evidence for double-stranded transfer. Later, an important experiment convincingly demonstrated this for pSAM2 (92): Transconjugant frequencies were dramatically reduced when the recipient contained a restriction system cutting only double-stranded DNA. A control conjugation from *E. coli* into *Streptomyces* (84) was unaffected by the restriction system (92), as expected for single-stranded transfer.

There are no known examples of double-stranded plasmid transfer in Gram-negatives,

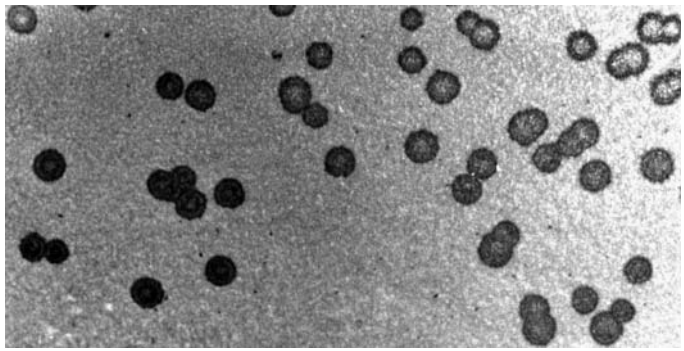

Figure 5

Pocks produced by plasmid SCP2* on a lawn of an SCP2$^-$ *S. coelicolor* strain.

and in unicellular, low-G+C Gram-positive genera like *Staphylococcus*, *Streptococcus*, *Enterococcus*, and *Lactococcus*, DNA sequencing showed plasmid-mediated conjugation to resemble the Gram-negative paradigm in transfer of single-stranded DNA after nicking of the donor molecule at an *oriT* by a relaxase, even if they differ from *E. coli* in lacking pilus-like appendages to bring the mating partners together (38). Conversely, there are no homologues of the *Streptomyces tra* genes on low-G+C Gram-positive plasmids, again suggesting different processes. Such homologues do, however, exist in other bacteria—an extremely illuminating finding.

These genes include *ftsK*, essential for cell division in *E. coli* (and other bacteria) (8), and a sporulation gene of *Bacillus subtilis*, *spoIIIE* (120). Their products are motor proteins that move circular, double-stranded DNA. FtsK, as part of the developing septum, helps chromosome copies enter daughter cells when *E. coli* divides, whereas SpoIIIE catalyzes chromosome movement into the developing *Bacillus* spore through a closing asymmetrically placed septum in the sporangium. Homology between SpoIIIE and *Streptomyces* Tra proteins helped develop the idea of conjugation-like DNA movement during *Bacillus* sporulation (28, 120); conversely, detailed studies of SpoIIIE and FtsK are now helping to illuminate the molecular details of *Streptomyces* conjugation. Both proteins form multimeric

SCP2: 31-kb circular plasmid of *S. coelicolor*

Pocks: circular zones of growth retardation in a lawn of a plasmid-free *Streptomyces* culture caused by colonization of the recipient lawn by a plasmid transferred into it in a mating

Tra: transfer proteins encoded by *Streptomyces* plasmids, acting as motors to move DNA into a recipient during mating

rings with a hole that, at least for FtsK, would accommodate two DNA double helices (as needed to transport circular DNA as a loop), and both use ATP hydrolysis to pump DNA (3, 106). Recently, Reuther et al. (95a) showed that purified Tra protein of the *Streptomyces venezuelae* plasmid pSVH1 hydrolyzes ATP, as does pSN22 Tra (77), supporting a mode of action similar to that of the other DNA-translocating proteins.

While all DNA transfer during *Streptomyces* mating seems to depend on plasmid-borne *tra* genes (52), a key question is whether *Streptomyces* plasmids promote hyphal fusion, or whether they use pre-existing, "vegetative" fusions. It is relevant that multiple fusions occur in filamentous fungi during vegetative growth, even of a haploid, genetically uniform hyphal mass, giving an interconnected network with a selective advantage attributed to "intrahyphal communication, translocation of water and nutrients, and general homeostasis within a colony" (36). Although we cannot rule out such "spontaneous" fusion of *Streptomyces* hyphae, evidence suggests that their plasmids do indeed promote fusion. Thus, some *Streptomyces* plasmids, such as the linear SLP2 of *S. lividans* (58), carry a gene perhaps involved in breaking and remaking linkages in peptodoglycan, like the muramidases of certain Gram-negative plasmids (7).

As well as wall fusion, membrane coalescence is needed for confluence of donor and recipient cytoplasm. Biological membranes do not fuse spontaneously: Energy is needed to surmount repulsion between them and to disrupt and remodel the bilayers of the two membranes into a channel between fusing cells or organelles (62, 111). A class of SNARE proteins promotes these functions in eukaryotes, but have not been described in bacteria, perhaps because membrane fusion is not so widespread in prokaryotic life cycles. But it needs to occur when growing septa close, so the finding that FtzK and SpoIIIE have a membrane-spanning N-terminal domain, separate from the C-terminal DNA motor domain, is highly significant. It promotes membrane fusion, to either complete the new membranes separating daughter cells during vegetative growth or enclose the developing *Bacillus* spore inside the mother cell (107). *Streptomyces* plasmid Tra proteins have such a domain (82), supporting the idea that the intermycelial fusions leading to plasmid transfer are indeed plasmid-determined.

In fungi, it is hyphal tips that fuse, and this seems most likely in *Streptomyces*, since tips are where new wall material is laid down, with the breaking and remaking of peptidoglycan bonds. The recent work of Reuther et al. (95a) provides strong evidence for mating at hyphal tips because these authors found fluorescence-tagged *Streptomyces ghanaensis* pSG5 Tra protein preferentially in these positions.

For DNA transfer, *Streptomyces* plasmids, like those of other bacteria, would need sites for the transfer proteins to interact. Such a *cis*-acting site, named *clt*, was identified on pIJ101 and another circular *Streptomyces* plasmid, pJV1 (91, 105). It conferred transferability by a mobilizing plasmid when cloned onto a nonself-transferable plasmid, but no association was proven between *clt* and Tra (26). In contrast, Reuther et al. (95a) recently identified a 50-bp noncoding sequence on pSVH1 immediately downstream of *tra*, containing inverted and direct repeats, to which purified Tra protein binds, probably as multimers. Different sequences, but with similar features, occurred on other plasmids in corresponding positions, probably ensuring specific interactions between each Tra protein and its binding site.

What about the *spd* genes of *Streptomyces* plasmids? Their products, small hydrophobic proteins, show no revealing resemblances to other proteins, yet surely they are candidates for moving DNA, in this case long distances in molecular terms because pocks can have a radius of one or more millimeters, and the plasmids must cross septa a few tens of micrometers apart in the vegetative mycelium, although the same plasmid molecule may not travel so far. The *spd* genes have not been studied in the absence of the *tra* gene, but

their products may work together with Tra to form pores in the cross walls for DNA to pass (38).

Transfer of Chromosomal Genes

Chromosomal gene transfer during F-mediated mating was originally attributed entirely to stable, covalent plasmid integration into the *E. coli* chromosome to establish Hfr strains, which transfer host genes as a simple extension of plasmid transfer starting from *oriT*. Later, Hfr clones within the F^+ culture were suggested to account for only 15%–20% of recombinants in $F^+ \times F^-$ crosses (25), but the remaining 80%–85% has never been explained comprehensively. As recently as 2005, K. Brooks Low (personal communication) wrote that these donors "seem to be unstable and may represent cells where either the F factor integration event is incomplete and aborted (but still far enough along to join one strand of F to one strand of the chromosome and thus get chromosomal transfer) or else perhaps integrated into essential genes which thus prevents viability of the transient "Hfr" cells." Most classical Hfr strains arose by homologous recombination between identical copies of insertion sequences (IS) on the chromosome and on F (80), but many other sequences can promote cointegrate formation and hence chromosomal mobilization in Gram-negative bacteria (95).

In the first example of stable cointegrate formation in *Streptomyces*, the SCP1 plasmid was inserted into the *S. coelicolor* chromosome to yield a donor with fertility properties designated NF (51). Recombinants arose from matings between NF and $SCP1^-$ strains with frequencies approaching the 100% transfer shown by the free plasmid. Recombinants inherited donor markers with frequencies inversely proportional to their distances from the plasmid integration point, on either side of it ("bidirectional" transfer), and all inherited NF fertility. This differed sharply from the Hfr mechanism, with its high-frequency transfer of markers on only one side of the plasmid integration point and only a few recombinants being high-frequency donors, because mating unions nearly always rupture before complete chromosome transfer, so markers on the other side of the insertion point, and the distal region of F, rarely enter the recipient. Subsequent physical analysis showed SCP1 to be a 356-kb linear molecule (10, 74) and confirmed that the NF state arose by stable integration of SCP1 into the chromosome by crossing over between ISs, with loss of the plasmid ends (and some host genes adjacent to the integration site), thereby avoiding introduction of adventitious termini into the chromosome (122).

NF: the high level of fertility (chromosomal recombinant formation) shown by a donor strain of *Streptomyces coelicolor* with plasmid SCP1 integrated near the center of the linear chromosome when mated with a plasmid-free recipient

Other stable donors arising from SCP1 interaction with the chromosome were "unidirectional" because they donated chromosomal genes at high frequency on only one side of the presumed plasmid integration point, as in Hfr strains (112), but again recombinants inherited donor behavior. One, called 2106 (55), was recently analyzed physically. As previously reported in *Streptomyces rimosus* (90), a crossover between linear plasmid and linear chromosome was deduced to have generated two replicons, each with one chromosomal and one plasmid end [(121); these authors stated that the strain had been interpreted as an SCP1-prime strain with the *cysD* chromosomal gene on the plasmid, but this had been specifically excluded by Hopwood & Wright (55)]. The strain was stable because both replicons carried essential genes, so loss of either would be fatal.

Chen (23) proposed that *Streptomyces* linear plasmids might mobilize linear chromosomes end-first, independently of cointegrate formation, even suggesting that DNA replication primed by TP might displace the 5′ strand for transfer. Although this would be incompatible with transfer of pre-existing, double-stranded molecules discussed above, end-first transfer is still an attractive hypothesis. Perhaps the proteins postulated to hold the ends of the chromosome together can bind chromosome and plasmid ends, allowing such mobilization.

What about chromosome transfer by circular *Streptomyces* plasmids? For SCP2 of *S. coelicolor*, the most studied, an extensive search in the late 1970s gave no evidence for stable integration to generate highly fertile strains (J. A. Ewing, personal communication). The published sequence (40) is of a variant called SCP2*, with a 1000-fold enhanced capacity to mobilize chromosomal genes (14). The basis of the difference is unknown. If it is due to gain of a sequence promoting cointegrate formation with the chromosome, this must be too short to have been identified by restriction analysis, and is nonspecific, since SCP2* promotes generalized gene transfer: The wild-type plasmid should be sequenced to find out. But (transient) cointegrate formation is strongly suggested by an experiment in which donors containing artificial SCP2*-prime plasmids carrying chromosomal DNA were mated with an SCP2$^-$ recipient (53). They acted as bidirectional donors for markers on both sides of the corresponding chromosomal locus, with higher frequencies for the longer inserts. Significantly, the same marker gradient was found irrespective of the orientation of the cloned fragment in the plasmid, and therefore of the integrated plasmid after presumed homologous crossing-over with the chromosome. NF and a second bidirectional donor with SCP1 integrated in the opposite orientation (strain A634: 112, 122) likewise gave the same pattern of marker donation. Transfer of a loop of double-stranded DNA would neatly fit these observations.

Merozygosity or Complete Chromosome Transfer?

The concept of merozygosity (incomplete diploidy) resulting from DNA transfer is central to bacterial genetics. In transformation and transduction, merozygosity results from the fragmentary nature of the donor genetic material, as cell-free DNA or genome fragments encapsulated in bacteriophage heads, whereas for conjugation in *E. coli*, it reflects transfer of a fragile, single-stranded molecule via an easily ruptured mating union. Thus, merozygosity was the default hypothesis in the early days of *S. coelicolor* genetics, and it seemed to fit the facts. For example, the likelihood of an unselected crossover per unit map length was on average five times higher in an interval adjacent to one in which a crossover had been selected than in an interval diametrically opposite on the map (46, especially Table 6). Although not proving merozygosity, this result agreed with it, since two crossovers could occur only in a contiguous region of diploidy. Heteroclones seemed to support the idea of merozygosity in being heterozygous for incomplete sets of markers, but the genomes of heteroclones might not have corresponded to those of the primary zygotes from which they arose, so this did not prove incomplete diploidy of the zygotes themselves.

In revisiting the hypothesis of merozygosity I quote a discussion of what we knew about conjugative *Streptomyces* plasmids 20 years ago (53): "An attractive, alternative model [to the unidirectional single-stranded Hfr paradigm] . . . involves the possible transfer of *Streptomyces* plasmids as double-stranded circular molecules. Transfer of a plasmid integrated into the chromosome would then result in the passive conduction of the whole chromosome." This conclusion was based on several observations.

1. As described above, in a mating between an NF strain, with SCP1 integrated near the center of the chromosome, and an SCP1$^-$ strain, all recombinant progeny inherited donor fertility, and there was a bidirectional gradient of donor marker inheritance from very high frequencies closest to and on either side of the integration point, falling to very low values for markers near what we now know are the chromosome ends. When the same two strains were combined by protoplast fusion, integrated SCP1 was still inherited by all progeny, but the marker

gradient was replaced by equal inheritance of donor and recipient alleles (barring a slight excess of a donor marker nearest the plasmid integration point, presumably reflecting physical coupling of the plasmid with the marker) (53). Instead, when a strain carrying integrated (NF) SCP1 was either mated or fused with a strain containing autonomous ($SCP1^+$) SCP1, there was equal segregation of the two forms among the progeny.

2. For the circular plasmids SCP2* (14) and pIJ101 (71), pocks produced by a plasmid-carrying individual in a plasmid-free lawn contained the plasmid throughout the area of the pock, so (copies of) the plasmids really do travel far within a plasmid-free recipient. In the center of pocks, chromosomal recombinants were much more frequent than in a mass mating (14). Further, a particular nonpock-forming deletion variant of SCP2* (pIJ916), deduced to lack spread genes because it was transferred into an $SCP2^-$ strain in a mass mating much less frequently than the wild type, showed very little reduction in chromosomal recombination, so recombination per transfer approached 100% (81).

These findings prompted the idea of repeated "backcrossing" of recombinant chromosomes with recipient genomes. The likelihood of chromosomal marker donation would be high during primary intermycelial transfer—as in the center of a wild-type pock or in a mass mating when subsequent intramycelial migration was blocked by deletion of the spread genes in pIJ916—but donor markers would be diluted by recombination with recipient genomes during intramycelial spreading. Favored inheritance of a point on the donor chromosome, like the integrated SCP1 in a mating of an NF with an $SCP1^-$ strain, would generate the observed high-frequency inheritance of markers close to it, decreasing with increasing distance from the integrated plasmid because of crossing over with recipient genomes, even if the complete donor chromosome had been transferred. Protoplast fusion brings complete parental genomes together in a fusion sac in which rounds of recombination occur (56), so there would be no preferred backcrossing with recipient genomes, and therefore no marker gradient.

The obligate inheritance of integrated SCP1 in NF x $SCP1^-$ crosses required a special explanation. As we speculated (53), "... it may result in part from the efficient spreading of plasmid copies, including those integrated into the chromosome, through the substrate mycelium, combined, perhaps, with an efficient mechanism that tends to ensure the inclusion of a plasmid copy in every spore. Possibly the development of mycelium and/or spores is inhibited in regions in which too few copies of SCP1 exist to allow their inheritance by every cellular compartment or spore, a phenomenon that would be analogous to the coupling of host-cell division to plasmid proliferation exhibited by F (89). When both variant [integrated] and normal [autonomous] forms of SCP1 are present together, a segregation of plasmid copies would result in the observed inheritance of either plasmid form." This hypothesis has recently been supported by the discovery of plasmid partitioning functions that ensure inheritance of plasmid copies by the spores (see below).

While transfer of the complete chromosome as a loop centered on the point of plasmid integration (transient for SCP2*) seems to account for most of the known facts, there remain at least two phenomena to explain. One is donation of chromosomal markers in a unidirectional gradient by certain SCP1 variants. As described above, one of these, strain 2106, contains two chimeric chromosomes, each consisting of parts of the chromosome and of SCP1 (121). However, the evidence for unidirectional donation by this strain is, in retrospect, inconclusive because there were no markers anticlockwise between the donation

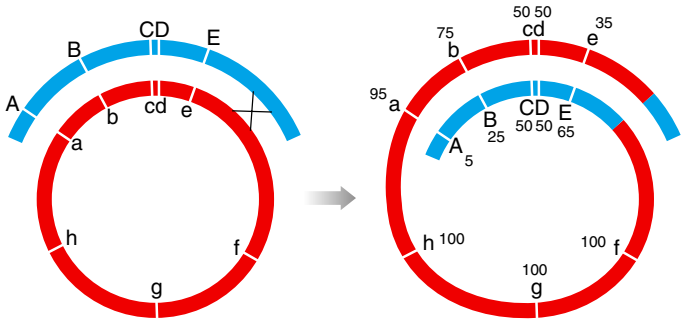

Figure 6

Hypothesis of Hopwood (46) in which heteroclone genomes are terminally redundant linear structures produced by crossing over between a complete circular chromosome and a linear fragment. Numbers against allele symbols represent the gradient of marker inheritance by haploid recombinants arising when the partially heterozygous genomes develop into colonies.

point and what we now know to be a chromosome end; the closest marker (on the circular map) was *argA*, on the other end of the chromosome. Strain A608 (112) behaved as a true unidirectional donor, but its physical structure has not been established, so it is too early to interpret unidirectional donation in physical terms.

Another problem requiring a new explanation in light of the linearity of the chromosome is the nature of heteroclone genomes. I proposed (46) that they might be terminally redundant linear structures arising by odd numbers of crossovers between a donor chromosome fragment and a circular recipient chromosome (**Figure 6**), whereas haploid recombinants would result from even-numbered crossovers. This fitted the finding of heteroclones and haploids with comparable frequencies, rather than heteroclones being vanishingly rare and so requiring a special explanation, but no mechanism was known to explain replication of a linear bacterial replicon. This problem could be circumvented by postulating (46) that heteroclone genomes were circular with long tandem duplications, which would have given the same pattern of haploid recombinants by crossing over between the duplicated segments as in a terminally redundant linear genome, but generation of such genomes would have needed fusion of broken chromosome ends by an unknown mechanism.

We now know how linear chromosomes (with intact telomeres) can replicate, but we need a new hypothesis to explain how partially heterozygous genomes with random arcs of heterozygosity arise in matings between linear genomes with fixed ends. In the original model of heteroclone chromosome structure, I assumed that its genome consisted of a single physical entity; otherwise, how could two alleles at heterozygous loci be inherited together? However, arguing from the structure of the genome of donor 2106, we might now postulate that heteroclone genomes actually contain two replicons. In strain 2106, the two replicons are coinherited because each contains an incomplete gene set. In heteroclones the experimenter imposes inheritance of a region of both parental genomes because heteroclones are observed only when selection is made for wild-type alleles at two closely linked loci *in trans*. Could the two genomes be complete?

Figure 7 shows the constitution of the two parental genomes in my 1966 experiment

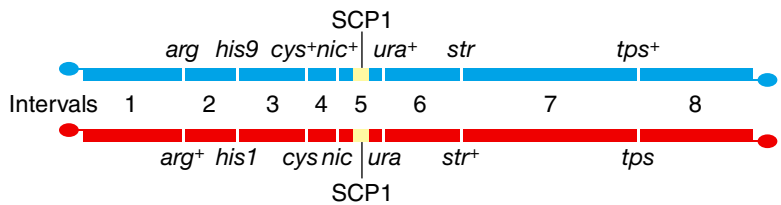

Figure 7

Arrangement of markers in the experiment of Hopwood (45) in which a population of heteroclones was analyzed for their regions of heterozygosity.

(45), using marker positions derived from the genome sequence (sometimes presuming the assignment of the marker to an ORF in the sequence). Hypothesizing that heteroclones contain two genomes drawn at random from a pool of complete linear parental and recombinant chromosomes, one carrying *hisA1* and the other *hisC9* (the mutations *in trans* that select the heteroclones), we could calculate the expected composition of a population of heteroclones, assuming various values for the average number of crossovers per chromosome, and compare the frequencies with the observed population. The data are incompatible with this simple hypothesis. For example, since map interval 7 is the longest, genotypes arising by a crossover in this interval should be among the most frequent recombinant chromosomes. Combining such a genotype with the parental genome carrying the alternative *his* marker (also very abundant) would give heterozygosity at all loci except *tps*, yet this class of heteroclones was one of the least frequent in the experiment (3 out of 447 heteroclones), compared with the most abundant class arising by crossing over in the much shorter interval 3 (132 out of 447). More thought and experiment are obviously needed.

Summarizing this section, DNA transfer during *Streptomyces* mating occurs by very different mechanisms from those familiar from *E. coli*. At least for circular plasmids, motor proteins transfer DNA as double strands. Transient recombination of the plasmid with the chromosome would lead to chromosome transfer, probably as a loop, but whether transfer is normally complete or partial is unresolved. Wang et al. (114) found both the unselected genetic markers and the telomeres of the recipient to be preferentially inherited in such crosses, but this does not prove incomplete transfer since backcrossing of recombinant genomes with recipient chromosomes during intramycelial spreading would lead to the same result.

Chromosome transfer by autonomous linear plasmids is especially intriguing, with end-first transfer an attractive hypothesis. For the two examples studied (114), recombinants in interspecific (*S. coelicolor* × *S. lividans*) crosses, unexpectedly, tended to inherit telomeres and nonselected markers from the donor. This is compatible with complete chromosome transfer, but the authors needed a special explanation for a failure to inherit recipient DNA, attributing it to active destruction of resident genomes, perhaps through invasion by transposons from the incoming genome released from repression on entering a naïve cytoplasm. Alternatively, if both ends of the plasmid were bound to both donor chromosomal ends, leading to chromosomal transfer as a loop, and if the binding tended to be maintained during post-transfer, intramycelial spreading through the recipient mycelium, preferential inheritance of the donor chromosome ends could perhaps result.

When the linear plasmid is covalently integrated, chromosomal transfer as a loop is again the most attractive hypothesis, at least for the SCP1 bidirectional donors, and again we cannot yet choose unambiguously between complete and incomplete transfer. A satisfactory explanation for the heteroclone genomes arising in matings between pairs of such NF donor strains, the only situation in which they have been studied, is also still elusive. The time is ripe for application of current molecular techniques to these intriguing problems.

CHROMOSOME MOVEMENT DURING COLONY GROWTH AND DEVELOPMENT

In eukaryotes the mitotic spindle coordinates chromosome replication with cell division, ensuring that daughter cells inherit a full gene set. The spindle microtubules are bound to the centromere region of the chromosomes by a protein complex, the kinetochore, and polymerization and depolymerization of the spindle tubulin leads complete sets of sister chromatids apart (76). In the first bacteria studied, having a single chromosome kept complete

sets of genes together, but without a spindle an alternative mechanism was needed to link chromosome replication to cell division. This was provided in the replicon hypothesis (61) by attachment of the chromosome to the inner face of the plasma membrane. Daughter chromosomes would remain attached at adjacent positions and be moved passively apart by deposition of new membrane and wall material between the two attachment sites, with eventual synthesis of a division septum. Plasmids would cosegregate with chromosomes by having their own attachment sites.

In the subsequent 40 years the replicon hypothesis has needed major revision, especially because bacterial walls were found not to grow in positions required by the hypothesis, but some of its basic concepts have survived, notably chromosome attachment via specific sequences to "capture sites" that position daughter chromosomes appropriately in the cells. Meanwhile, bacterial chromosome segregation has been revealed as an active process with analogies to mitosis. Much of the new knowledge has come from fluorescence microscopy (29). Bacteria have turned out to resemble eukaryotes in their cell biology more than had been thought, notably in having homologues of all the major components of the eukaryotic cytoskeleton: Tubulin and actin homologues are widespread in bacteria, whereas intermediate filament homologues are restricted in distribution (34).

Rod-shaped bacteria like *E. coli* face a relatively simple challenge in ensuring inheritance of a chromosome copy by two equivalent daughter cells during binary fission. Other bacteria have more complex segregation scenarios: *B. subtilis* and *Caulobacter crescentus* use asymmetric septum formation in differentiation, initiating endospores in the former and in the latter generating swarmer and stalked cells with different developmental prospects. These two bacteria have a centromere-like element represented by sequences extending for several hundred base pairs around *oriC*, together with proteins—RacA in *B. subtilis* and MreB in *C. crescentus*—that bind to it and help anchor chromosome copies to the cell poles (11, 12, 35).

The filamentous streptomycetes face multiple challenges in coordinating chromosome replication with septum formation. In vegetative hyphae, septation at distant intervals produces compartments with around a dozen genomes, so there is no obligate coupling of DNA replication with septation, although chromosomes are presumably coordinated with new tip and branch formation. In contrast, metamorphosis of distal compartments of the aerial hyphae into chains of uni-genomic spores requires precise septum placement and segregation of a chromosome into each developing spore (37). Hopwood & Glauert (50) argued, from the disposition of stained DNA in sporulating aerial hyphae, that this involved separation of pre-existing genome copies rather than formation of new genomes in step with sporulation septum formation, because rod-like nucleoids apparently subdivided via units of varying length to give the individual genomes for about ten adjacent spores (**Figure 8**), a view reinforced by recent molecular studies.

In bacteria, the tubulin homologue FtsZ plays a key role in septation, forming rings at division sites that recruit other proteins to complete cell division (83, 116). Essential in other bacteria, FtsZ is dispensable in *Streptomyces*: an *ftsZ* mutant failed to form any septa, but grew surprisingly well, considering the whole colony presumably consisted of a single giant cell (86). But *ftsZ* mutants cannot sporulate, consistent with a role for FtsZ in forming ladder-like assemblies at positions where sporulation septa will occur (102). In other bacteria, FtsZ ring placement depends on exclusion from inappropriate sites, notably positions occupied by nucleoids, but this does not appear to be true for *S. coelicolor* sporulation, since developing septa are seen apparently closing in positions still occupied by DNA (**Figure 8**) (31, 37).

For low-copy-number plasmids, partitioning loci (*par*) ensure that daughter cells inherit plasmid copies (32). Many, but not all,

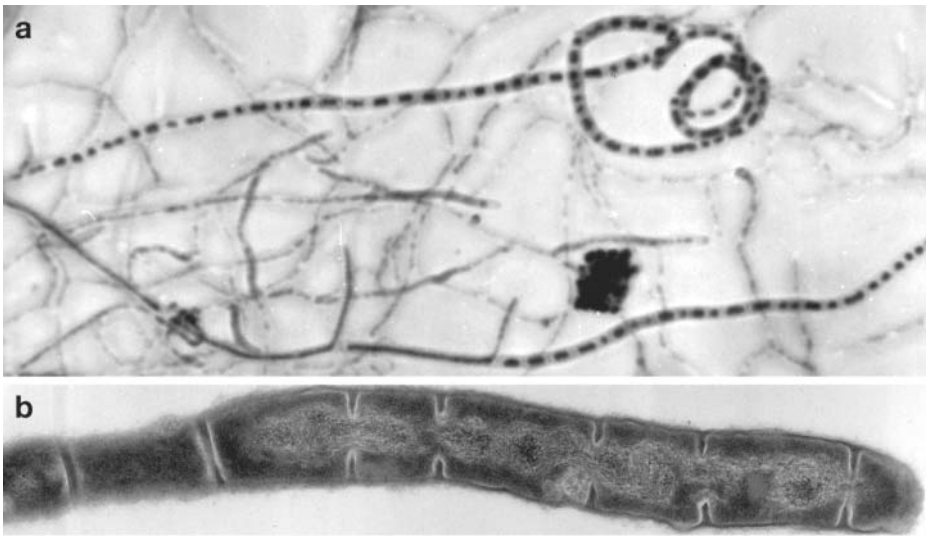

Figure 8

(*a*) Light micrograph of *S. coelicolor* aerial hyphae stained for nucleoids; the lower hypha shows a gradient of maturity from left to right, from rods of DNA in basal regions, via bodies of intermediate length, to uni-genomic spore compartments near the tip, while the upper hypha shows more synchronized genome segregation (50). (*b*) Transmission electron micrograph of a thin section of a sporulating aerial hypha with sporulation septa closing in positions still occupied by nucleoids (Mark Buttner & Kim Findlay, John Innes Centre).

bacteria have chromosomal equivalents of the *par* genes. Perhaps they are required to coordinate cell division and chromosome movement in situations more complex than simple binary fission. Thus, *E. coli* lacks a *par* system (33), whereas in *C. crescentus*, which depends on asymmetric cell division for growth, it is essential (87); and in *B. subtilis* disruption of *parB* prevents sporulation while scarcely affecting DNA partitioning during vegetative septation (78). *S. coelicolor par* mutants grew apparently normally, but at least 13% of spore compartments lacked a proper DNA allocation (73). While ParA is an ATPase, ParB binds to a set of 20 *parS* sites within 200 kb of *S. coelicolor oriC* to form a massive nucleoprotein complex (63), and such foci showed interesting locations (64). In aerial hyphae they were spaced regularly at intervals characteristic of prespore compartments before sporulation septa appeared, clearly indicating a role in accurately partitioning chromosome copies into spores. In vegetative hyphae the most obvious foci were associated with the apices (though interestingly about 1 μm behind the tip), raising the possibility that, as new tips form, they might be connected to *oriC* so that genomes are dragged into side branches. Perhaps a link in such coupling is an essential protein called DivIVA, named after its homologue in *B. subtilis* where it interacts with the centromere-binding protein RacA during sporulation: in *S. coelicolor* DivIVA is seen at vegetative hyphal tips (30).

In the aerial hyphae, such a link may be the newly discovered family of SsgA-like proteins (SALPs), unique to sporulating actinomycetes. Several of the seven members of this family in *S. coelicolor* play important roles in coordinating DNA segregation and sporulation septum synthesis. In the absence of SsgG the DNA segregated normally, but often without a septum, producing spores with up to five well-defined chromosomes. Evidently,

septum formation and DNA segregation can be uncoupled (88a).

A further important player is MreB, an actin-like protein of rod-shaped bacteria but not cocci, suggesting a role in determining cell shape (66), but it seems to be involved also in chromosome segregation as a component of a "mitotic machinery" (32). Among actinomycetes, *mreB* occurs only in genera that sporulate within an aerial mycelium, and in *S. coelicolor* MreB was found on sporulation septa and the inner face of developing spore walls (85). It is not essential for vegetative growth but its deletion caused gross changes in the formation and physical properties of spores. A role in chromosome segregation into spores, as in vegetative segregation in *B. subtilis* (109) and *C. crescentus* (35), was suggested by the aberrant appearance of nuclear material in the *S, coelicolor* mutant.

Summarizing, tubulin and actin homologues play roles in chromosome segregation in various bacteria, with an interesting reversal of their functions compared with eukaryotes: In the latter, actin is part of the cytokinetic ring and tubulin forms the mitotic spindle fibres, whereas in bacteria, where implicated, the proteins tend to play reciprocal roles (32). The situation in *Streptomyces* is complicated, reflecting the varied challenges faced by the chromosome at different stages in the life cycle, but the jigsaw is beginning to be assembled. The next few years will offer exciting opportunities for revealing the picture.

GENETIC ENDOWMENT OF THE *STREPTOMYCES* CHROMOSOME

Completion of the *S. coelicolor* A3(2) genome sequence (9) was a milestone in understanding the genetic endowment of this model streptomycete and its chromosome organization. At 8667,507 bp it was the biggest finished bacterial genome, with a correspondingly large number of genes: 7825 coding sequences. Until the sequence was determined, it had been possible that the "arm" regions, in which large deletions occur without loss of viability under laboratory conditions, might have been poorly endowed with coding sequences, but this turned out not to be the case: They contain just as many ORFs per unit length as the chromosomal "core," but with an interesting difference between the classes of genes in the 4.9-Mb core compared with the arms (1.5-Mb left and 2.3-Mb right). Unconditionally essential genes controlling DNA replication, transcription, translation, and central metabolism occur only in the core, while the arms are disproportionately endowed with genes presumed to be "conditionally adaptive," such as those encoding cellulases, chitinases and, to some extent, secondary metabolism (9). Reflecting this difference, DNA microarray analysis showed genes in the core to be more highly expressed under nonchallenging growth conditions than arm genes, whereas gene expression under various stresses was more uniform (67).

Recognizable synteny was found between the whole 4.4-Mb, circular genome of *Mycobacterium tuberculosis* (also an actinomycete) and just the core of the *S. coelicolor* chromosome (9). Also, orthologues between the *S. coelicolor* and *S. avermitilis* genomes tend to occupy the core, whereas the arms contain more nonorthologous genes (59). These findings all support the idea that, in descending from a common actinomycete ancestor, probably with a ~4-Mb circular genome, streptomycetes acquired different sets of new genes by horizontal transfer, primarily to form the chromosome arms, a view supported by a preponderance of transposons and transposon relics toward the ends (24). More recent horizontal transfer is indicated by the finding of numerous "islands" carrying from just a few genes to nearly 150, which differentiate *S. coelicolor* A3(2) even from its close relatives (C. M. Kao, personal communication). All these "extra" genes would help the organisms deal flexibly with the myriad challenges they meet in the soil, probably with some specialization

Synteny: a conserved order of corresponding genes on the chromosomes of organisms descended from a (recent) common ancestor

of different streptomycetes toward particular habitats.

Extreme flexibility in expression of the *Streptomyces* genome is also indicated by the abundance of genes encoding transcriptional regulators (an unprecedented 13% of all genes), as well as the numerous examples of two or more paralogues of the same genes (9). It is an attractive hypothesis that the latter may often be "tissue-specific" in expression, as already proven for some glycogen biosynthetic genes, which are expressed either in mature vegetative mycelium or in immature spores (101).

CONCLUSION

As I hope I have shown, understanding the architecture and behavior of *Streptomyces* chromosomes has come a long way in the past 50 years, but there are still plenty of fascinating things to find out. Genetic manipulation of antibiotic biosynthesis has been another strong interest of mine, and this subject, especially the harnessing of genetic knowledge to make "unnatural natural products," has caught the imagination of many scientists (94, 108, 117). My book (48) describes these and other aspects of *Streptomyces* genetics from an autobiographical perspective.

SUMMARY POINTS

1. *Streptomyces* chromosomes are novel in being large and linear, with a unique mechanism of "end-patching" to overcome the "end problem" in replicating linear DNA molecules.
2. Mating differs from the single-stranded, rolling circle paradigm of Gram-negative plasmids in the transfer of double-stranded DNA by motor proteins, which probably mediate fusion of hyphal tips.
3. While circular plasmids probably transfer the chromosome as a loop via (transient) integration, linear plasmids may well lead the chromosome end-first into the recipient through protein-protein interactions between terminally bound proteins.
4. Transferred plasmids colonize the recipient mycelium by migration, believed to be intramycelial, via a special mechanism still to be elucidated.
5. Heteroclones are partially heterozygous colonies arising in matings whose genome structure is in need of determination.
6. Vegetative hyphae consist of hyphal compartments containing multiple genomes, so cross wall formation is uncoupled from chromosome replication, but a mechanism is needed to lead chromosomes into hyphal branches.
7. During sporulation, regular placement of chromosomes precedes spore formation, promoted in part by actin and tubulin homologues.
8. *Streptomyces* chromosomes consist of a "core" containing essential housekeeping genes and "arms," often dispensable under laboratory conditions, rich in conditionally adaptive genes, and probably acquired by horizontal transfer since present-day actinomycetes diverged from a common ancestor.

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