New Approach to the Genetics of *Streptomyces coelicolor*

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ABSTRACT

SERMONTI, G. (Istituto Superiore di Sanità, Rome, Italy), MILENA BANDIERA, AND ISABELLA SPADA-SERMONTI. New approach to the genetics of *Streptomyces coelicolor*. J. Bacteriol. 91:384-392. 1966.—Mixed cultures of complementary auxotrophic strains of *Streptomyces coelicolor* A3(2) were preincubated on discs of cellophane on complete medium and were then transferred onto selective media. When one strain was streptomycin-resistant and the other was streptomycin-sensitive, and the transfer medium contained streptomycin, distinct minute tufts of aerial mycelium appeared on the background growth of the mixed culture. They turned out to be heterozygous clones (heteroclones) in which the streptomycin-sensitive allele was, as a rule, missing. The pattern of marker contribution of the streptomycin-sensitive parent to the zygotes was indicative of a continuous structure carrying the hereditary material. A gradual transfer of the donor genome during conjugation was suggested by the progressive completion of the zygotes obtained by increasing preincubation time.

The discovery of heterozygous clones (heteroclones) in *Streptomyces coelicolor* (12) and the development of their analysis (6) opened new prospects in the genetics of *Streptomyces*. A non-selective type of genetic analysis was devised, and some information on the structure of the single heterozygous nucleus was obtained in this genus of bacteria. However, heteroclones had to be detected among recombinant colonies from the plating of spores of mixed cultures, which involved selective conditions under which the number of growing recombinants had to be reduced to a minimum. Moreover, the heterozygous nuclei which were at the origin of the heteroclones analyzed were not the immediate products of the karyogamic process, but most likely were late derivatives which had surpassed the barrier of sporulation in the mixed culture (5). The new technique described in this paper, by which the heteroclones are detected directly on the basal mycelium of mixed cultures, shortens and simplifies the crossing procedures in *Streptomyces* and opens new possibilities to the study of the genetic system in this bacterial genus.

MATERIALS AND METHODS

All the strains used were mutant and recombinant derivatives of *S. coelicolor* A3(2).

The following three strains were most widely

adopted: 86 (*ura-1 acr-3 his-1 arg-1* R⁺), 206 (*str-1 phe-1 met-2* R⁺), and 219 (*str-1 phe-2 met-2* R⁻).

The meanings of the mutation symbols are: *ura-1*, requirement of uracil; *acr-3*, resistance to acriflavine; *his-1*, requirement of histidine; *arg-1*, requirement of arginine; *str-1*, resistance to streptomycin; *phe-1*, requirement of phenylalanine; *met-2*, requirement of methionine. The wild alleles are indicated by a plus sign followed by the mutation symbols, or simply by a plus sign when the corresponding mutation is evident. R⁺ means fertile strain; R⁻, self-sterile strain (10). The location of the markers is given in Fig. 1, where the numbers inside the circle refer to the map distances [according to Hopwood and Sermonti (5) and Sermonti and Hopwood (11)]. The two linkage groups were recently shown by Hopwood (4) to be connected as indicated in Fig. 1 to give a circular linkage map.

Media and general procedures were as described in a review paper by Hopwood and Sermonti (5). Reproductive medium 1 was enriched by the addition of yeast nucleic acid hydrolysate [3 ml per liter of a solution prepared as described by Pontecorvo (9)]. The mixed cultures were obtained by plating 10⁴ to 10⁵ viable spores of each of the two strains to be crossed on a disc of cellophane, 6 cm in diameter, placed on the surface of the reproductive medium, which gave a total of 6 to 60 microcolonies per square millimeter of the cellophane disc. It is advisable, when trying a cross for the first time, to use a range of spore concentrations of both strains.

The adopted procedure required one streptomycin-

FIG. 1. Map of *Streptomyces coelicolor* A3(2) (from 3, 4, 5).

resistant, auxotrophic strain, and the other, a streptomycin-sensitive strain, usually auxotrophic. After about 1 day of incubation, the cellophane disc with the growing mixed culture was transferred onto a minimal agar medium containing streptomycin. After another 2 days of incubation, minute tufts of aerial mycelium, hardly visible without a microscope, appeared on the surface of the mixed culture. They could be picked up immediately or the day after, and turned out to be heteroclones (Fig. 2-4).

A variant of this procedure required the use of two complementary auxotrophic strains and the omission of streptomycin from the minimal transfer medium. In this case, large tufts of aerial mycelium appeared on the surface of the mixed culture, in the zones where different colonies overlapped (Fig. 5). Minute tufts could also be obtained under these conditions by shortening the preincubation time.

The minute tufts were collected for genetic analysis by use of a very fine needle and a dissecting microscope. A substantial improvement in the isolation procedure was attained by transferring the cellophane disc, just before collecting the minute tufts, to the inner part of the cover of the petri dish. A moderate drying of the culture, obtained in a few minutes if the cover was inverted in the open air, made the tufts firmer and much easier to sample.

Every tuft picked was transferred into a drop of water containing sodium lauryl sulfonate (0.01%), on the surface of complete medium. The drop was then thoroughly spread over the agar surface by means of a glass rod, and the same unsterilized glass rod was streaked again on the surface of a second, and some-

times even on a third, dish, to obtain less crowded dishes in case the first appeared overcrowded. After 2 to 3 days, colonies could be systematically transferred on master plates, to be subjected to phenotype analysis by the standard procedures (5).

A simplified procedure adopted in special cases involved the transfer of each tuft into a drop of sodium lauryl sulfonate solution on complete agar medium, followed by careful streaking of the tuft within the drop to produce a spotlike culture. As many as twenty spot cultures could be thus placed on a plate. After 2 to 3 days of incubation, the spots could be replica-plated on diagnostic media, to be classified on the basis of the possible presence in them of selective or visible markers. The presence of leaky nutritional mutants in a spot culture was usually easy to detect.

RESULTS

Appearance of the minute tufts. The mixed cultures on the cellophane discs were repeatedly observed and photographed before transfer on the selective media, and no trace of overgrowth between overlapping colonies was ever detected within 24 hr of incubation, when the colony density was less than 50 per square millimeter. After transfer onto selective media containing streptomycin and a further 48 hr of incubation, minute tufts, in the shape of bundles of diverging aerial hyphae (Fig. 2), appeared in the overlapping zones between different microcolonies. After another 24 hr, tufts had taken the appearance of bright white fluffs (Fig. 3), with a little bulb of basal mycelium at the bottom of each tuft. Later, they started extending radially over the background mycelium.

The minute tufts appeared very distinct on a flat background of poorly developed basal mycelium when the selective conditions were efficient in stopping the parental growth rapidly and completely. When this was not achieved, the aerial mycelium developing on the parental colonies—usually the streptomycin-sensitive parent—confused the picture; yet, the heteroclones often remained detectable as compact round-shaped tufts, and their isolation was possible. The great majority of the isolated tufts turned out to be genetically heterogeneous, and, in all those isolated on streptomycin-containing media, the allele *str* was in hemizygous condition. Many of the heteroclones (Table 1) gave rise to various classes of segregants with relative frequencies in fair accordance with the order of markers given in Fig. 1.

Heteroclones on different selective media. Minute tufts were isolated from a large range of transfer media, as a rule supplemented with streptomycin and lacking at least one substance required by the *str* parent. For the isolation of heteroclones, any transfer medium could be used

FIG. 2. *Heterozygous tufts on the surface of a mixed culture* (39 *his-1* R^+ $\times$ 137 *met-2 arg-1 ura-1 str-1* R^-). Grown for about 24 hr on complete medium, and 48 hr on minimal medium with arginine and streptomycin. Transmitted light; $\times 200$.

on which the growth of the parental colonies and of the heterokaryon between them was inhibited. A remarkable variability was observed among heteroclones detected on the same mixed culture after transfer onto different media, and also among heteroclones deriving from the same medium

Table 2 shows the different types of heteroclones selected as minute tufts from the same cross, on three different selective media. The heteroclones were completely analyzed (see, for instance, that reported in Table 1), and are

FIG. 3. *Heterozygous tufts on the surface of a mixed culture* (86 *acr-3 his-1 arg-1 ura-1* $\times$ 216 *met-2 phe-1 str-1*). Culture was grown for 21 hr on complete medium and 48 hr on minimal medium with streptomycin. Oblique light; $\times 100$.

classified in Table 2 according to the markers contributed by the streptomycin-sensitive (86) parent. The streptomycin-resistant (206) parent usually contributed all its markers. When the $+met-2$ marker of the 86 parent was selected in the transfer medium (minimal medium plus arginine, uracil, phenylalanine, and streptomycin) it

TABLE 1. Frequency of segregant genotypes from minute tufts grown on a transfer medium supplemented by methionine, histidine, uracil, and streptomycin

Cross*:	86	+	<i>ura-1</i>	<i>acr-3</i>	+	<i>his-1</i>	<i>arg-1</i>	+
206	<i>phe-1</i>	+	+	+	<i>met-2</i>	+	+	<i>str-1</i>
Allele ratios:	285	284	281	236	226	149	0	
Regions:	4	1	5	2	8	3	53	63
								140
								289

Genotypes†	No.	Single c.o. in region	Genotypes	No.	Multiple c.o. in regions
++ + m + + s	1	1	p u a c m + + s	1	1,3
+ u + m + + s	7	2	p u a c + h + s	1	1,5
+ u a c m + + s	41	3	p u a c + h a s	1	1,6
+ u a c + + + s	9	4	p + a c m + + s	1	2,3
+ u a c + h + s	75	5	+ + a c + h + s	2	2,5
+ u a c + h a s	147	6	+ + a c m + + s	1	1,2,3
—	280		+ u a c m + a s	1	3,5,6
			+ u a c + + a s	1	4,5,6
			—	—	—
				9	

* The selected markers (given in boldface type) are put at the extreme ends of the map.

† Simplified marker symbols: u, *ura-1*; ac, *acr-3*; h, *his-1*; a, *arg-1*; s, *str-1*; p, *phe-1*; m, *met-2*; + indicates the wild allele of the mutant alleles reported in the same column. Note that all segregants are streptomycin-resistant (s).

was always present in all the tufts. It could be accompanied by markers on either side, but a distant marker was never present if all the markers between the latter and +*met-2* were not also present. For instance, *ura-1* never occurred when *acr-3* was lacking, and when +*phe-1* was present it was always accompanied by both *ura-1* and *acr-3*. The sequence of the 86 markers never bridged the +*str* allele, which was selected against in all media, although in two heteroclones a few +*str* recombinants were detected (see footnotes to Table 2). When +*phe-1*, which was adjacent to *str-1*, was the selected marker (minimal medium plus methionine, histidine, arginine, uracil, and streptomycin), only the 86 markers distal to it (in respect to *str 1*) appeared among the minute tufts (Table 2). Finally, when both +*phe-1* and +*met-2* were selected (minimal medium plus arginine, uracil, and streptomycin), *ura-1* and *acr-3*, which were located between them, were never missing. Very few exceptions to this rule were encountered (3 cases besides the 57 heteroclones reported in Table 2), due always to one or two unexpected segregants in one heteroclone.

Heteroclones a.ter different preincubation times. The time of preincubation of the mixed culture on complete medium could be varied with dramatic effects both as to the number and the genetic structure of the detectable heteroclones. The

latter made their first rare appearance on the transfer media from the mixed cultures preincubated for about 20 hr. When preincubation time was protracted for another 2 to 3 hr, hundreds of minute tufts appeared on the selective media. With still longer preincubation, they reached the thousands (Fig. 4), and were easily perceptible to the naked eye after 3 days of growth on the transfer medium, if the surface of the culture was strongly illuminated by oblique light.

In the experiment reported in Table 3, the selective conditions permitting the occurrence of heteroclones carrying +*phe-1* and donor markers at its right were adopted (see Table 2, selection on minimal medium plus methionine, histidine, arginine, uracil, and streptomycin). The *met-2 phe-1 str-1* strain used was an R⁻ derivative of strain 216, i.e., strain 219, isolated for its high fertility in crosses with R⁺ strains. Minute tufts were picked up after different preincubation times, and were tested as spot cultures for the occurrence of three selective donor markers, +*phe-1*, *acr-3*, and +*met-2*, as well as for the occurrence of the *ura-1* donor marker, easily scorable as it produced minute colonies on media lacking uracil.

From the data of Table 3, it is obvious that heteroclones appeared progressively more complete as preincubation time was increased. Heteroclones containing only the first marker

TABLE 2. *Markers contributed by the streptomycin-sensitive (86) parent to different heteroclones selected as minute tufts on various transfer media containing streptomycin*

Cross ^a :	86	+	<i>ura-1</i>	<i>acr-3</i>	+	<i>his-1</i>	<i>arg-1</i>	+
	206	<i>phe-1</i>	+	+	<i>met-2</i>	+	+	<i>str-1</i>
Selective transfer medium ^b	Selected 86 markers				Markers ^c of the 86 parent detected in the heteroclone ^c			Total heteroclones of given types
Arginine, uracil, phenylalanine, streptomycin	+ <i>met-2</i>				+			6
					+ h			7
					ac + h			4
					ac + h a			2
					u ac + h			2
					u ac + h a			2 ^d
					+ u ac + h			1
					+ u ac + h a			2
Methionine, histidine, arginine, uracil, streptomycin	+ <i>phe-1</i>				+			2
					+ u			4
					+ u ac			6
					+ u ac + h a			1 ^e
Arginine, uracil, streptomycin	+ <i>met-2</i> + <i>phe-1</i>				+ u ac +			3
					+ u ac + h			7 ^d
					+ u ac + h a			8

^a See footnotes to Table 1.^b Minimal medium with indicated additions.^c The genotype of at least 50 segregants from each heteroclone has been classified.^d One heteroclone carried some +s recombinants.^e See Table 1 for complete analysis.

(+ *phe-1*), which amounted almost to the totality after 19.5 hr of preincubation, disappeared after the 25th hr to be replaced by heteroclones carrying two, three, or all four of the scorable donor markers. Heteroclones carrying *ura-1*, *acr-3*, or + *met-2* successively made their first appearance (after 19.5, 21, and 25.25 hr, respectively, of preincubation), and their frequency gradually increased with time. This is clearly evident from Fig. 5, derived from the data of Table 3.

These results were confirmed by several experiments. Care should be taken not to pick up minute tufts from exceptional zones of the mixed culture, showing anomalous growth conditions due to ridges on the cellophane, occasional contaminations, uneven distribution of the seeded spores, location at the edge of the cellophane disc, etc.

Tufts appearing on selective media without streptomycin. If the transfer medium did not contain streptomycin and the growth of both strains was blocked after transfer because of the lack of required substances, the overgrowing aerial mycelium usually presented a different aspect. Large tufts appeared in the zones of overlapping

between different colonies (Fig. 6). A shorter preincubation time was required for the formation of these tufts than for the appearance of the tiny tufts occurring in the presence of streptomycin. This was probably due to some residual of growth after the transfer on unsupplemented media without streptomycin. By plating spores formed on the large tufts on the complete medium, as a rule, one obtains a large excess of the parental types, among which occasional recombinant types (rarely exceeding 2%) may occur. The large tufts may represent either a syntrophic growth or a true heterokaryotic mycelium, carrying in the same continuous cytoplasm nuclei of different types (1). The latter alternative appears more likely, since typical large tufts were obtained in the zones of overlapping between colonies of types whose complementation involved the exchange of intracellular intermediates. This is the case in many pairs of histidineless mutants accumulating products which appear not to be diffusible through the cytoplasmic membrane (2). When transfer to selective media without streptomycin was done early (18 to 21 hr), several minute tufts appeared—in addition to the elongated

FIG. 4. Heterozygous clones appearing as minute tufts on the surface of a mixed culture (86 *acr-3 his-1 arg-1 ura-1* R^+ $\times$ 219 *met-2 phe-1 str-1* R^-) growing on a cellophane disc. Cultural conditions as in Fig. 3. Oblique light; $\times 6$.

tufts—and turned out to be heteroclonal. When transfer was made still earlier, the minute tufts sometimes represented the great majority.

Heteroclonal in heterosexual crosses. A sexual polarity system in *S. coelicolor* A3(2) was described by Sermoniti and Hopwood (11). This im-

plies a group of intersterile strains designated as R^- , and a group of strains fertile in any combination, or R^+ . A larger contribution of the R^- markers to the progeny was shown in $R^+ \times R^-$ crosses (10), and in $R^+ \times R^-$ heteroclonal (Bandiera, Doctoral Thesis, University, Rome, Italy, 1965).

Mixed cultures on cellophane discs were prepared by use of the following strain combinations: (A) R^- *met-2 arg-1 ura-1 str-1* $\times$ R^+ *his-1*; (B) R^+ *met-2 arg-1 ura-1 str-1* $\times$ R^- *his-1*; and (C) R^- *met-2 arg-1 ura-1 str-1* $\times$ R^- *his-1*.

Large heterokaryotic tufts appeared after transfer onto unsupplemented minimal medium from all mixed cultures. After transfer onto minimal medium plus arginine, uracil, and streptomycin, culture A showed a very great number of minute tufts; culture B showed a reduced number of minute tufts, from 1 to 10% of those observed on culture A. No tufts were detectable on culture C. Best conditions for the growth of the minute tufts were, therefore, when streptomycin resistance was on the recipient (R^-) side, the other strain being of the donor (R^+) type.

DISCUSSION

The cellophane transfer technique, as applied to mixed cultures of *S. coelicolor*, provides heterozygous clones stemming directly from the basal mycelium of the mixed culture. Such heterozygous clones show up only under special selective conditions. Otherwise they are concealed in the aerial mycelium of the mixed cultures. When the young mixed culture is transferred on a partially supplemented or unsupplemented minimal medium without streptomycin, the growth of the auxotrophic parental colonies is stopped, but the heteroclonal still remain concealed by the overgrowing heterokaryotic mycelium. However, when streptomycin is present and only one parent is streptomycin-resistant, any heterokaryotic growth is also inhibited, since it involves cooperation between complete nuclei, and a mycelium carrying both the *str* allele and its wild counterpart is sensitive and unable to grow in the presence of streptomycin (5). Only streptomycin-resistant recombinants or incomplete zygotes (merozygotes) can develop, the latter being hemizygous for a region comprising the allele *str*. Thus, for the emergence of the heteroclonal on the transfer medium, we only need to select against the parental phenotypes and the heterokaryon between them. No selection is required, as in the former method (6), against the

TABLE 3. Frequency of heteroclones of different composition^a isolated on cellophane discs, as minute tufts, after different times of preincubation of the mixed cultures on complete medium^b

Cross ^c :	86	+	<i>ura-1</i>	<i>acr-3</i>	+	(<i>his-1</i>) ^d	(<i>arg-1</i>)	+	R ⁺
	219	<i>phe-1</i>	+	+	<i>met-2</i>	+	+	<i>str-1</i>	R ⁻
<hr/>									
Preincubation time (hr)	Markers ^c contributed by the donor (86) parent							Total heteroclones tested after different preincubation times	
	+p	+p u	+p u ac	+p u ac +m					
19.5	21	1	0	0			22		
20.25	34	6	0	0			40		
21	26	5	2	0			33		
23	14	11	5	0			30		
25.25	0	8	19	1			28		
27.5	0	6	17	7			30		
Total heteroclones of given types	95	37	43	8			183		

^a The composition of the heteroclones is given with respect to the markers contributed by the donor (R⁺) parent, tested by a simplified procedure (see Materials and Methods, last paragraph).

^b Selective transfer medium: minimal medium plus methionine, histidine, arginine, uracil, and streptomycin.

^c See footnotes to Table 1.

^d The nontested markers are given in parentheses.

FIG. 5. Percentage of heteroclones carrying various donor markers, after different preincubation times. The points are calculated from the data of Table 3. Heteroclones are selected for the presence of +*phe-2* (+p), and tested, after different incubation times, for the occurrence of other donor markers: *ura-1* (u), *acr-3* (ac), and +*met-2* (+m). The percentage of heteroclones carrying unselected donor markers increases with time. The order of first appearance as well as the order in final frequency of the unselected markers correspond to their location with respect to the selected +p marker.

recombinants, simply because they come in a later stage.

As a matter of fact, practically all the minute tufts crowding the cultures preincubated for a long period are genetically heterogeneous. Only the very rare ones appearing after the shortest preincubations sometimes turn out to be homogeneous.

At any rate, these homogeneous minute tufts represent a very small fraction of the total number of tufts that eventually appear on the mixed culture. We can therefore conclude that recombination takes place in *S. coelicolor* essentially through a transitory merozygotic phase, lasting for several nuclear generations (5, 11). No other important mechanism of recombination is operating under the adopted experimental conditions, such as the formation of nonreproducible zygotes, or any kind of infective heredity.

The development of heteroclones on media on which no heterokaryotic growth is allowed clearly indicates that the heterokaryotic growth is not prerequisite to karyogamy. The occurrence of a very short heterokaryotic stage developed before transfer cannot, however, be excluded. On the minimal transfer media without streptomycin, heteroclones appear after short preincubations, side by side with the heterokaryotic growth, thus supporting the idea that zygote formation is independent of heterokaryon formation. In mixed cultures between sterile strains (R⁻ × R⁻), only heterokaryons can be detected. The mentioned observations suggest a process of conjugation quite similar to that described in *Escherichia coli* K-12 (14), in which chromosome transfer occurs between adjacent cells without actual plasmogamy, or heterokaryotic phase.

A closer parallel with the genetic system of *E. coli* K-12 (for review, see 8) appears from the pattern of genetic constitution of different minute tufts selected on various transfer media. The

FIG. 6. Heterokaryotic growth appearing as large tufts on the zones of overlapping between colonies with complementary phenotype. Mixed culture as in Fig. 3. Grown for 26.5 hr on complete medium, 48 hr on minimal medium. Oblique light; $\times 80$.

genome of *S. coelicolor* A3(2) appears as a continuous structure, the two originally defined linkage groups (3, 5) being joined in such a way that *acr-3* on group I is connected with *ura-1* on group II (Fig. 1). This is in accordance with the conclusion reached by Hopwood (4) through quite a different procedure. The evidence in favor of a continuous genome presented in this paper is not based on any linkage data, but on the mere fact that one, and only one, connection of the two linkage groups is such that, whatever the contri-

bution of one parent (the streptomycin-sensitive one) to the zygote, in no case are two of its markers present in a heteroclone if all the markers between them are not also present. The evidence of a continuity across the *str-1* gene, possibly "closing" the map, is still unavailable, as the $+str-1$ allele is forcibly absent among the markers of the streptomycin-sensitive parent. The data presented in Table 2 also show clearly that the two ends of the genome segment contributed by the streptomycin-sensitive parent are not constant, a situation assumed to occur on the male side in the $F^+ \times F^-$ conjugal crosses of *E. coli* K-12.

A further similarity between *S. coelicolor* conjugation and that of *E. coli* K-12 is in the progressive completion of the zygotes with time (13). The longer the presence of the mixed culture of *S. coelicolor* on the complete medium, the larger is the contribution of the donor (R^+) parent to the zygotes. This observation implies that the zygote formation is progressive and also that the passage of the conjugating hyphae from a complete medium onto a streptomycin-supplemented minimal medium involves stopping or slowing down in the genome transfer process. The present results do not give any evidence of an "oriented transfer" of the donor genome during conjugation (14). The ordered and progressive appearance of the R^+ markers in the heteroclones, observed in the experiment reported in Table 3 and in Fig. 6 (see also Table 2, second selective medium), is due to the particular selective conditions. The selected donor marker ($+phe-1$) is adjacent to the donor marker selected against ($+str-1$) so that growth is allowed only of heteroclones bearing the $+phe-1$ markers and adjacent donor markers distal to it (in respect to the counter-selected $+str-1$ allele). The constant origin of the donor contribution is therefore only apparent. The type of selective conditions adopted can, however, provide a technical tool for ordering genes on a linear array, besides the already adopted procedures (5).

Although the picture of the conjugation process in *S. coelicolor* appears very similar to that of *E. coli* K-12 $F^+ \times F^-$ conjugal crosses, the evidence presented in this paper is of a very different kind, and in some ways is complementary to that provided by the work on *E. coli* K-12 (8). Whereas most of the evidence in the latter species comes from the selective analysis of the pooled progeny of many transient zygotes, the single composition of which is but indirectly deduced, the picture obtained in *S. coelicolor* is based mainly on the examination of isolated heterozygous clones (heteroclones).

The variable "ends" of the transferred donor chromosome in the $F^+ \times F^-$ crosses of *E. coli*

K-12 were assessed after the isolation from one F^+ clone of several Hfr subclones having different patterns of chromosome transfer (7). In *S. coelicolor* A3(2) no Hfr subclones were isolated, but the examination of the genetic composition of various heterozygotes derived from the same $R^+ \times R^-$ cross gives a similar indication. The influence of the period of effective contact between parental cells on the completion of the zygotes was deduced in *E. coli* K-12 from the progressive appearance of new donor markers among the $Hfr \times F^-$ progeny (13), but the progressively more complete heterozygotes had never been analyzed separately, a situation that has now been approached in *S. coelicolor*.

The use of the minute tufts for genetic analysis presents some disadvantages as compared with the use of heteroclones derived from plating of mixed cultures (6). This is partly due to the greater incompleteness of the heteroclones obtained as minute tufts, which can however be overcome by using nutritionally unsupplemented transfer media, and possibly also omitting the addition of streptomycin to the latter media. A more serious difficulty lies in the possibility that a minute tuft may sometimes represent the result of more than one multiplying heterozygote, particularly in the crowded plates. More accurate analyses will be necessary to rule out this possibility, which is, however, irrelevant for the main conclusions drawn in this note.

Finally, we wish to point out that, from a merely practical point of view, the time for the isolation of the heteroclones, which was 10 days (5), has now been reduced to 3 days. Moreover, the isolation of the heteroclones by the cellophane technique no longer requires the adoption of two closely linked markers in repulsion, and can therefore be achieved with any pair of auxotrophic strains, one resistant and the other sensitive to a drug like streptomycin (resistance to which is achieved by recessive mutations). This might be of importance for workers wishing to investigate the genetics of new species of *Streptomyces*.

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