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Gene Recombination in *Streptomyces coelicolor*

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SUMMARY: Prototrophic recombinant strains were obtained by plating on minimal medium spores from mixed cultures grown on limiting agar, of two strains each with a different double nutritional requirement. Clones showing new associations of properties were isolated on partially supplemented media. Some showed one 'mutant' character derived from one of the starting strains and 'wild' characters derived from the other; others showed two 'mutant' characters, each derived from one of the starting strains.

Actinomycetes are generally regarded as asexual micro-organisms. However, different parasexual processes have been discovered in the last ten years in a few species of bacteria and asexual fungi (Lederberg & Tatum, 1946; Zinder & Lederberg, 1952; Pontecorvo, Roper & Forbes, 1953; Pontecorvo & Sermonti, 1954) leading to gene recombination otherwise than by sexual reproduction. The properties of the actinomycetes are intermediate between those of the bacteria and the fungi, so that the existence of a process of parasexual recombination was to be expected in this group of micro-organisms as well. A preliminary report of the present work appeared elsewhere (Sermonti & Spada-Sermonti, 1955).

METHODS

Organisms. A strain of *Streptomyces coelicolor* Reiner-Müller, supplied by the courtesy of Dr A. Tonolo of this laboratory, was used. The colonies consist of a colourless primary mycelium and a thick layer of whitish secondary or aerial mycelium. The medium becomes intensely coloured by a blue pigment formed by the organism (Tonolo, Casinovi & Marini-Bettolo, 1954). The authors were also supplied by Dr Tonolo with an achromogenic variant in which the primary mycelium is red and the aerial mycelium greyish pink.

Media. The media employed were as in the genetic research on *Penicillium chrysogenum* carried out in this laboratory (Sermonti, 1954): viz. a minimal medium (MM) (modified Czapek-Dox) and a complete medium (CM) containing yeast extract, casein hydrolysate, nucleic acid hydrolysate, corn steep and a mixture of vitamins. All the cultures were incubated at 30°.

Isolation of mutants. The spores were subjected to ultraviolet irradiation after surface plating on CM agar (Pontecorvo, 1953) to give a mortality ranging from 98 to 99.7 %. Mutants were selected from the surviving colonies by the total isolation method (Fries, 1948). At first, spores from every surviving colony were point-transferred to MM agar; later, spores collected from each single colony were streaked on to MM agar by means of a loop containing a drop

of sterile water. The latter procedure turned out to be more efficient and was adopted later for recognition of nutritional requirements of recombinant strains. The clones which showed no growth on MM were recovered and subjected to Beijerinck's auxanographic test. A list is given in Table 1 of 19 mutants obtained by this procedure. Nine different types of mutant with determined nutritional requirements were isolated, and two mutants whose requirements were not determined. One of these latter (*4 un pigm*) grows on MM supplemented with casein hydrolysate, and the other (*18 un nic pigm*) requires added peptones. In four of the experiments the rate of mutant occurrence was higher than 1 % of the colonies tested.

Table 1. *Isolation of biochemical mutants in Streptomyces coelicolor*

Ultraviolet irradiation of Petri dishes of CM agar each plated with about 10,000 spores. Survival rate between 2 and 0.3 %. Total isolation of surviving colonies.

Strain irradiated. Symbol*	Colonies isolated (no.)	Mutants among isolates	
		(no.)	Symbol*
1 +	725	1	3 <i>hist</i>
2 <i>pigm</i>	1565	1	4 <i>un pigm</i>
3 <i>hist</i>	2112	1	5 <i>me hist</i>
2 <i>pigm</i>	1179	4	6 <i>glu pigm</i>
			7 <i>as pigm</i>
			8 <i>nic pigm</i>
			9 <i>nic pigm</i>
7 <i>as pigm</i>	336	4	10 <i>cys as pigm</i>
			11 <i>me as pigm</i>
			12 <i>me as pigm</i>
			13 <i>arg as pigm</i>
6 <i>glu pigm</i>	346	2	14 <i>pr glu pigm</i>
			15 <i>arg glu pigm</i>
3 <i>hist</i>	180	2	16 <i>me hist</i>
			17 <i>ur hist</i>
8 <i>nic pigm</i>	566	1	18 <i>un nic pigm</i>

* The symbols consist of a code number and the symbols of the induced mutations in inverse order of induction with the following meanings: *arg* = arginine requirement; *as* = aspartic acid requirement; *cys* = cysteine requirement; *glu* = glutamic acid requirement; *hist* = histidine requirement; *me* = methionine requirement; *nic* = nicotinamide requirement; *pigm* = absence of blue pigment; *pr* = proline requirement; *un* = unknown requirement; *ur* = uracyl requirement; + represents the wild type.

RESULTS

Production of 'syntrophic tufts'

The first attempts to set up a parasexual process were along the lines followed successfully in the experiments with *Penicillium chrysogenum* (Sermonti, 1954): isolation of tufts of exuberant growth between colonies with complementary requirements growing on limiting medium. The first strains to be employed were *3 hist* and *4 un pigm*. A suspension, containing about 2000 spores/ml., of each of the two strains was spread at the rate of 0.1 ml. (200 + 200 spores) per plate on the surface of a series of Petri plates containing

MM mixtures of MM and CM in various proportions (MM : CM = 1 : 20, 1 : 4 and 1 : 1) or CM agar. After 4 days of incubation tufts of exuberant growth ('syntrophic tufts') appeared in the areas of contact between colonies of the two types on all the dishes except those containing MM alone. These resembled those already observed in *P. chrysogenum* (Sermonti, 1954). They were very obvious on the seventh day, and in sharp contrast with the adjacent colonies in height and richness of spore formation (see Pl. 1, figs. 1, 2). Only a very slight growth, probably due to strain *4 un pigm*, was observed on MM. The tufts were especially noticeable on the 1 : 1 MM : CM media and on CM. The strains used showed slightly less growth on this latter medium, with spores forming either very late or not at all. Increase of the quantity of MM in the medium led to greatly decreased growth. Transfer of tufts to MM gave rise in some cases to very stunted colonies with an irregular edge, very delayed spore formation limited to the centre of the colony, and late and scanty production of the blue pigment. No growth was observed after plating on MM several tens of thousands of spores collected from one of these colonies; on CM, colonies of the two component types reappeared, forming exuberant tufts in their contact zones. The same results were obtained on plating several tens of thousands of hyphal fragments in a suspension obtained by grinding finely in distilled water the spore-free margin of a colony. The experiments were discontinued, since strain *4 un pigm* showed traces of growth on plating on MM.

Syntrophic tuft production was next tried with strains *5 me hist* and *14 pr glu pigm* on CM, following the same procedures. In this case the occurrence of the phenomenon was sporadic and concentrated in particular areas of the dish. Sixteen tufts were transferred to MM agar, and produced irregular colonies like those already described. Two of these after a few days produced sectors of greater height and richer spore formation, which overgrew the original colony (Pl. 2, fig. 3). A spore suspension (about 1000 spores/ml.) from one of these sectors was well broken up so that it consisted almost entirely of single spores and was then plated on CM and on MM agar at the rate of 0.1 ml./dish. Thirty colonies grew on three CM agar dishes, as against 267 colonies on four MM agar dishes. All the colonies produced the blue pigment. Ninety-six colonies were transferred from the CM agar to fresh MM agar, and turned out to be prototrophic and to produce the blue pigment. One of the colonies which had been grown on CM agar was used to obtain a spore suspension, which was again plated on fresh CM agar. A total of 17,700 colonies was observed on fifty Petri plates, and all of them turned out to be blue pigment-producing. Sixty-two of the more stunted colonies were transferred to MM agar and turned out to be prototrophic and pigment-producing.

Several preliminary experiments were carried out to determine conditions for regular reproducibility of the formation of syntrophic tufts. Strains *3 hist* and *8 nic pigm* did not produce syntrophic tufts on CM, but the phenomenon appeared regularly with these strains on CM enriched with yeast autolysate at a concentration of 1-2 % (w/v). The two strains produced no spores on this medium. The colonies formed were prominent and gelatinous, with tufts looking like opaque white spindles.

Mixed cultures on agar slopes of limiting medium

A search was made for a procedure to obtain syntrophic growth, like that observed in the zone of contact between two colonies in the experiments just described, but homogeneously over the whole of an agar surface. This was achieved by spreading a thick mixed suspension of spores of two different kinds on the surface of agar slopes of limiting agar. The limiting agar was obtained as already described, by mixing CM and MM in various proportions. Two strains, each with two requirements, were used: *5 me hist* and *14 pr glu pigm*. Separately streaked on CM agar slopes, these strains grew well and formed spores. They also grew well and formed spores freely when grown on MM supplemented by the substances on which they were dependent, in the following concentrations: 0.68 mM-DL-methionine, 1.30 mM-L-histidine HCl, 1.30 mM-L-proline, 11.8 mM-glutamic acid. Growth was poor and spores were not formed on limiting media composed of a mixture of MM and CM in the proportion 1 : 2.3, or with lower proportions of CM (see Pl. 2, fig. 4). Strain *5 me hist* produced no pigment under these conditions.

On streaking together spores of the two strains in approximately equal quantities (about 200,000 of each strain per tube) on agar slopes of limiting medium, exuberant and precocious growth was produced (Pl. 2, fig. 4). By the

Table 2. *Selection on minimal agar of prototrophic colonies derived from mixed culture of strains 5 me hist and 14 pr glu pigm, and controls*

Spores derived from	Spores plated		Prototrophic colonies	
	Total (no.)	Per plate (no.)	Total (no.)	Per plate (no.)
Strain <i>5 me hist</i>	24.7×10^6	1.30×10^6	0	0
Strain <i>14 pr glu pigm</i>	12.0×10^6	0.75×10^6	0	0
Separate { <i>5 me hist</i> cultures* { <i>14 pr glu pigm</i>	33.6×10^6 †	1.98×10^6	0	0
Mixed { <i>5 me hist</i> culture‡ { <i>14 pr glu pigm</i>	46.8×10^6 †	1.17×10^6	10,832	271

* Spores obtained from separate cultures on CM and mixed together at the moment of plating on MM agar. 22 % strain *14*.

† Corrected for viable counts.

‡ Spores obtained from 4-day old mixed culture on MM + CM (1:1) agar slopes: 17 % strain *14*.

third day, spore formation was heavy and the blue pigment had been densely produced. No trace of growth was observed on MM agar slopes. A suspension of spores from a mixed (MM:CM = 1:1) 4-day culture, in distilled water, was filtered through a Permax gamma filter to eliminate fragments of mycelium, and washed by centrifugation in distilled water; it was then plated at the density of about 1.4×10^6 spores/plate on forty MM agar plates. Part of the suspension was plated on four CM agar plates at 100 spores/plate; 342 colonies grew on CM, of which 61 (17 %) showed the red colour of strain *14* and the rest produced the blue pigment. Colonies began to appear on the MM agar on the third day

after plating. On the fifth day they were small but prominent and had formed spores (Pl. 2, fig. 5), and the blue pigment was beginning to diffuse into the medium. The average number of colonies/plate was calculated to be 271 ± 6 , equal to 0.023 % of the viable spores plated. As a control, strains 5 and 14 separately and a mixed suspension of spores of the two strains obtained from separate culture, were plated on MM agar (Table 2). No growth on MM was observed after 2 weeks, either from the spores of the two separate strains or from the mixed suspension. The conclusion was drawn that the colonies which grew on MM from spores of the mixed culture were to be regarded as prototrophic recombinants.

Isolation of prototrophic recombinants was carried out on MM from another nine mixed culture agar slopes of strains 5 and 14. Eight tubes gave positive results, and the frequency of prototrophic recombinants from one of them was over 40 % of the viable spores plated. Prototrophic recombinants were also obtained from a mixed culture on limiting medium of strains 8 *nic pigm* and 5 *me hist* at a rate of 0.029 % of the viable spores plated.

Segregants from prototrophic recombinants

Attempts were made, on the hypothesis that the recombinants obtained were heterozygous diploids (Roper, 1952), to obtain from the prototrophic recombinants described in the last section strains in which one or more of the markers of the parent strains was present. Spores of some of the prototrophic recombinants were plated on CM agar and hundreds of the colonies formed were observed for colour or tested, but without success in any case, with the exception of one colony which was found to have numerous red sectors, and from which three non-pigment-producing clones were isolated.

The spores were subjected to ultraviolet irradiation in order possibly to increase the frequency of any segregation process; the surviving clones were tested for prototrophism once in the first generation and once in the second. No segregants were found. The attempts were discontinued at this stage and the study of auxotrophic recombinants as described in the next section begun. The experiments described above, however, indicate the stability of the prototrophic recombinants tested.

Auxotrophic recombinants obtained directly from mixed cultures

After this lack of success in obtaining segregants from prototrophic recombinants, a search was made for auxotrophic recombinants directly from the mixed cultures on limiting medium. Spores from mixed cultures of strains 5 and 14 were plated on MM agar supplemented with pairs of the nutrients required by the strains used, in all possible combinations except those which would permit the growth of either parental strain (Table 3); the nutrients were used in the concentrations already given. Colonies which developed on these media were transferred by streaking (see above) to MM agar. Some of them did not grow. The MM agar under each streak of these clones was divided into two parts (see above) and transferred to two minimal media, one supplemented with one of the nutrients present in the original partially supplemented

medium, and the other with the other. The clones which grew on one only of these media were regarded as dependent on the substance added to the medium on which they did grow. Two clones which grew on neither of the two media were transferred to CM and tested by the auxanographic technique. Both showed requirements for both methionine and proline. Many other strains with a single requirement were tested by the auxanographic technique.

Table 3. *Study of new recombinant types from the spores of a mixed culture of 5 me hist and 14 pr glu pigm*

Mixed culture medium (MM:CM)	Age of the mixed culture (days)	Medium on which spores plated (MM supplemented by)*	Rate of occurrence of recombinants (%)	Colonies tested† (no.)	Recombinant types obtained	
					(no.)	(type)‡
1:2:3	7	Proline and histidine	3.1	36	3	<i>pr</i>
					4	<i>hist</i>
					29	+
1:2:3	7	Glutamic acid and methionine	1.4	482	1	<i>me</i>
					481	+
1:1	16	Histidine and glutamic acid	19	168	9	<i>hist</i>
					7	<i>pigm</i>
					136	+
1:1	16	Methionine and proline	4.6	300	34	<i>pr</i>
					2	<i>pr me</i>
					264	+

* The substances were added in the optimal concentration as indicated in the text.

† The test of colonies was carried out by streaking the spores of the colonies from supplemented MM on to MM, and isolating streaks which did not grow.

‡ The symbol of the mutant characters stands for the phenotype (see Table 1, note *).

Table 3 gives the detail of the experiments. The strains isolated included three kinds of recombinants: (a) those with all the 'wild' characters of the original strains; (b) those with some of the 'wild' characters of one strain and one of the 'mutant' characters of the other strain; (c) those with 'mutant' characters from both strains.

As a check on the possible presence of recombinants with more than two requirements, a mixed culture 51 days old (MM : CM = 2.3 : 1) was plated on CM. From each of 270 colonies point-transferences were made to MM supplemented by both the requirements of one of the parents, and to MM supplemented by both the requirements of the other; 47 (17 %) of the colonies grew on both media. All the others grew on either one or the other. Thus no strain with more than two deficiencies was detected in this preliminary test.

DISCUSSION

This work is still in a preliminary stage, and no genetic conclusions can be drawn. The precautions taken and the controls carried out are sufficient to exclude the attribution of the appearance of new types to any processes independent of the interaction of the different strains, such as back-mutation

or adaptation. The direct evidence for the interaction between two strains with complementary nutritional requirements is striking enough: see Pl. 1, figs. 1 and 2, for one example of this and Pl. 2, fig. 5, for another. Selection of prototrophic clones from mixed cultures has been amply demonstrated. The stability of some of these clones for two or three subcultures has been checked. It is thus legitimate to conclude that the appearance of new stable prototrophic clones is the result of the interaction of two auxotrophic clones. The negative result obtained on plating together spores of strains 5 and 14 on MM indicates that the two strains require a period of common growth before they will give rise to recombinants. The clearest evidence for the occurrence of recombination is the finding of 51 strains carrying one 'mutant' character from one of the two strains and 'wild' characters from the other, and especially the finding of two strains with one 'mutant' character from one of the two strains and one 'mutant' character from the other.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Syntrophic tufts on CM agar between colonies of strains *3 hist* (dark colonies) and *4 un pigm* (light colonies). About natural size.

Fig. 2. Enlarged detail of fig. 1.

PLATE 2

Fig. 3. Prototrophic sectors which have overgrown a colony derived from a syntrophic tuft transferred to MM agar (strains *5 me hist* and *14 pr glu pigm*). Magnification about $\times 7$.

Fig. 4. Growth on limiting agar slopes (MM:CM=1:1) of strains *5 (me hist)* and *14 (pr glu pigm)* separately and in mixed culture *5+14*. Note exuberant growth of the mixed culture, and dark pigment diffused into the agar.

Fig. 5. Growth on MM agar of prototrophic recombinants after plating 1.4×10^6 spores from a limiting agar (MM:CM=1:1) mixed culture of strains *5 me hist* and *14 pr glu pigm*.

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(Facing p. 616)