Recombination in *Actinomyces aureofaciens*

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(Received 27 October 1960)

SUMMARY

Biochemical mutants of *Actinomyces aureofaciens* were obtained after treatment with ultraviolet irradiation or ethyleneimine. A specificity of the mutational process in a series of *A. aureofaciens* strains was observed, revealing itself mainly in formation of arginine-requiring mutants. Prototroph formation was shown to result from crosses of *A. aureofaciens* biochemical mutants with either the same or different amino acid requirements. Colonial morphology and antibacterial activity of some prototrophs were studied.

INTRODUCTION

The possibility of obtaining hybrids or heterozygous diploids in various species of imperfect fungi was shown for the first time by Pontecorvo (1956) and others. This was extended to antibiotic-producing actinomycetes by Sermonti & Spada-Sermonti (1955, 1956) and by Sermonti (1957). Later the methods developed by these authors were used by Alikhanian & Mindlin (1957) for obtaining recombinants in *Actinomyces rimosus*. The aim of this investigation was to study the possibility of hybridization in *A. aureofaciens* so as to use it for the purposes of selection.

METHODS

Biochemical mutants were obtained after ultraviolet (u.v.) irradiation and ethyleneimine treatment of *Actinomyces aureofaciens*. At the very beginning of our studies we found that this organism differed greatly from other actinomycetes in the extreme specificity of the mutational process in a number of strains, such as 536, II, Bd, BMK, B-16. These strains of *A. aureofaciens* differed from each other in their origin, colonial morphology and antibiotic-producing properties. The biochemical mutants were isolated on the following media: corn steep liquor (N12) medium, containing (w/v) 0.5% corn steep liquor, 0.4% $(\text{NH}_4)_2\text{HPO}_4$, 0.2% KH_2PO_4 , 0.29% MgSO_4 , 0.1% CaCO_3 , 2% starch, 2.0% agar; and two modifications of this medium, one with the addition of yeast extract and another with the addition of yeast extract and casein hydrolysate. The data on selection of biochemical mutants in *A. aureofaciens* are presented in Table 1.

As is evident from Table 1 our data do not conform to the regularities of biochemical mutant formation described in the literature in that from all strains, independent of the nutrient medium used, the occurrence of arginine-requiring (*arg*) mutants only was in general observed (99 cases), and only in two cases other amino acid requiring mutants (histidine (*his*) and isoleucine + valine (*isl + val*)) were detected.

The occurrence of arginine-requiring mutants in almost all cases could not but direct our attention to study hybridization in *Actinomyces aureofaciens*. The prototrophs were obtained by the method described by Alikhanian & Mindlin (1957).

Table 1. *The frequency of isolation of biochemical mutants from different strains of Actinomyces aureofaciens treated with various mutagenic factors*

Strain	Mutagenic factor	Medium	No. of biochemical mutants	Nutritional requirements	No. of isolates examined
BMK	u.v. radiation	N12	21	Arginine	1300
		N12+yeast extract	7	Arginine	—
		N12+yeast extract and casein	5	Arginine	—
	Ethyleneimine	N12	0	—	1075
		N12+yeast extract	0	—	—
		N12+yeast extract and casein	0	—	—
Bd	u.v. radiation	N12	19	Arginine	1211
		N12+yeast extract and casein	4	Arginine	—
	Ethyleneimine	N12	0	—	1200
		N12+yeast extract	2	Arginine	—
		N12+yeast extract and casein	1	Arginine	—
	536	u.v. radiation	N12	5	Isoleucine + valine Arginine (4)
N12+yeast extract			10	Arginine	—
N12+yeast extract and casein			0		
Ethyleneimine		N12	4	Arginine	1273
		N12+yeast extract	7	Arginine	—
		N12+yeast extract and casein	1	Arginine	—
B-16	u.v. radiation	N12	6	Arginine	2006
		N12+yeast extract	3	Arginine	—
		N12+yeast extract and casein	4	Histidine (1) Arginine (3)	— —
	Ethyleneimine	N12	0	—	1740
		N12+yeast extract	0	—	—
		N12+yeast extract and casein	0	—	—
II	u.v. radiation	N12	1	Arginine	3071
		N12+yeast extract	0	—	—
		N12+yeast extract and casein	0	—	—
	Ethyleneimine	N12	0	—	1600
		N12+yeast extract	0	—	—
		N12+yeast extract and casein	0	—	—

RESULTS

It was first necessary to demonstrate the possibility of recombination in *Actinomyces aureofaciens* in order that crosses might be made between all arginine-requiring mutants to explain their genetic nature. For this purpose we used the isoleucine + valine-requiring mutant, the histidine-requiring mutant, and a number of arginine-requiring mutants. The data on the investigation of combinations in the three groups are presented in Table 2.

Table 2. *Biochemical mutant combinations and the frequency of prototroph formation from these combinations*

Combination group	Biochemical combination	Prototroph frequency (%)	Frequency biochemical mutant reversions
I	<i>arg-1</i> × (<i>isl</i> + <i>val</i>)	0.13	0
	<i>arg-2</i> × (<i>isl</i> + <i>val</i>)	0.06	0
	<i>arg-3</i> × (<i>isl</i> + <i>val</i>)	0.0009	0
	<i>arg-4</i> × (<i>isl</i> + <i>val</i>)	3.3	0
	<i>arg-5</i> × (<i>isl</i> + <i>val</i>)	0.0004	0
	<i>arg-6</i> × (<i>isl</i> + <i>val</i>)	0.13	0
	<i>arg-7</i> × (<i>isl</i> + <i>val</i>)	0.01	0
	<i>arg-8</i> × (<i>isl</i> + <i>val</i>)	0.03	0
	<i>arg-9</i> × (<i>isl</i> + <i>val</i>)	0.006	0
	<i>arg-10</i> × (<i>isl</i> + <i>val</i>)	0.0	0
	<i>arg-11</i> × (<i>isl</i> + <i>val</i>)	0.004	0
	<i>arg-12</i> × (<i>isl</i> + <i>val</i>)	0.02	0
	<i>arg-13</i> × (<i>isl</i> + <i>val</i>)	0.0025	0
	<i>arg-14</i> × (<i>isl</i> + <i>val</i>)	0.0003	0
II	<i>arg-2</i> × <i>his</i>	0.0015	0
	<i>arg-7</i> × <i>his</i>	0.0007	0
III	<i>his</i> × (<i>isl</i> + <i>val</i>)	0.9	0

Biochemical mutants *arg-1* to *arg-5* were derived from strain Bd; *arg-6* to *arg-14* from strain BMK; (*isl* + *val*) from strain 536; *his* from strain B-16.

It should be pointed out that these crosses gave rise to a comparatively high frequency of prototrophs, reaching 3.3% in some combinations. This was higher than the frequencies observed in crosses of *Actinomyces rimosus*.

The prototrophs, obtained from the various combinations, independent of the starting strains, belonged morphologically to the classical wild-type strain of *A. aureofaciens* as for example strain 536 type. Such prototrophs formed flat colonies of mouse-grey or dark-grey coloration with aerial mycelium and buff-yellow substrate mycelium. Some prototrophs were characterized by a different rate of sporulation but this cannot serve as a distinguishing feature. Prototrophs from combination *arg-7* × *his* may be regarded as an exception, since they were characterized by dingy-beige coloured spores and low rate of sporulation.

In contrast, the biochemical mutants from which the prototrophs were derived differed greatly in colonial morphology from the wild-type strains. Thus, all arginine-requiring mutants were characterized by flat colonies, not highly folded or having radial lines, asporogenic, with no aerial mycelium or specific pigment (Plate 1). The histidine-requiring mutant formed light-grey, flat, compact colonies, elevated above

the agar surface and characterized by a low rate of sporulation and limited growth. The mutant requiring isoleucine and valine differed from the wild-type strains only by the production of colonies with poor sporulation and a broad asporogenic periphery.

Segregation patterns of prototrophs

The prototrophic recombinants gave rise to various types of segregants with differing frequencies. In order to study in detail the various segregation patterns we used prototrophs from eleven combinations, eight combinations of group I (*arg-1* to

Table 3. *Distribution of prototrophs from combinations according to the segregation types*

Combination group	Biochemical mutant combination	Prototrophs studied	Segregation type
I	<i>arg-1</i> × (<i>isl</i> + <i>val</i>)	No. 1	I
		No. 2	I
		No. 3	I
		No. 4	II
		No. 5	VI
	<i>arg-2</i> × (<i>isl</i> + <i>val</i>)	No. 1	I
		No. 2	I
		No. 3	I
		No. 4	IV
		No. 5	VI
		No. 6	VI
		No. 7	VI
	<i>arg-3</i> × (<i>isl</i> + <i>val</i>)	No. 1	I
		No. 2	II
		No. 3	III
	<i>arg-4</i> × (<i>isl</i> + <i>val</i>)	No. 1	I
		No. 2	I
	<i>arg-5</i> × (<i>isl</i> + <i>val</i>)	No. 1	I
		No. 2	I
		No. 3	II
	<i>arg-6</i> × (<i>isl</i> + <i>val</i>)	No. 1	I
		No. 2	I
		No. 3	I
		No. 4	IV
		No. 5	IV
	<i>arg-7</i> × (<i>isl</i> + <i>val</i>)	No. 1	I
		No. 2	I
		No. 3	V
	<i>arg-8</i> × (<i>isl</i> + <i>val</i>)	No. 1	IV
II	<i>arg-2</i> × <i>his</i>	No. 1	I
		No. 2	II
		No. 3	VI
		No. 4	VI
		No. 5	VI
	<i>arg-7</i> × <i>his</i>	No. 1	I
		No. 2	IV
III	<i>his</i> × (<i>isl</i> + <i>val</i>)	No. 1	III
		No. 2	VI
		No. 3	VI
		No. 4	VI
		No. 5	VI

arg-8 × (*isl* + *val*) and all combinations of group II (*arg-2* × *his*; *arg-7* × *his*) and group III (*his* × (*isl* + *val*)). As a result of this study the segregation patterns which are summarized in Table 3 were observed.

In all, the behaviour of 41 prototrophs from eleven combinations were studied. As far as their segregation pattern is concerned, six types were observed which were produced to a varying extent from each combination.

Segregation of type I. When plated out, the prototrophs of this type resembled the starting prototrophs, but segregated one of the starting biochemical mutants, in this instance the arginine-requiring mutant, but only in very small amounts (0.02–0.7 %).

The arginine-requiring segregant in its turn segregated in the first and sometimes in the second generation a few prototrophic colonies (0.5 %), morphologically similar to the arginine-requiring mutant. Further segregation of such colonies was not observed. It should be noted that the arginine-requiring mutants never segregated prototrophic or other auxotrophic forms. Neither were reverse spontaneous mutations observed.

Segregation of type II. A considerably smaller number of the prototrophs segregated the other parental form, that is the form similar to the isoleucine + valine-requiring mutant. The amount of such segregants was 1.5 to 0.14 %.

The isoleucine + valine-requiring segregant in further generations segregated no new forms.

Segregation of type III. A single prototroph (one prototroph among 41 prototrophs studied) which segregated both parental biochemical mutants (1.4 to 0.1 %).

Segregation of type IV. In this type of segregation, the prototroph segregated morphologically changed prototrophs. The number of such prototrophs was not high (0.15 %). In most cases, asporogenic forms similar to the arginine-requiring mutant were observed. Sometimes solid colonies with black substrate mycelium and various degrees of sporulation were encountered.

Table 4. *Quantitative distribution of prototrophs according to the segregation types*

Segregation type	No. of prototrophs	Segregation type	No. of prototrophs
I	18	IV	5
II	4	V	1
III	2	VI	11

Segregation of type V. In the first generation prototrophs belonging to this type segregated only morphologically-changed prototrophs, while in the second generation segregation of arginine-requiring variants was also observed.

Segregation of type VI. Prototrophs of type VI proved to be stable and segregated no new forms in a number of generations. The quantitative distribution of all 41 prototrophs according to these six types of segregation is presented in Table 4. As is evident from Table 4, the prototrophs belonging to type I, as well as stable prototrophs were the most common.

It should be pointed out that prototrophs segregating according to different types were detected from the same combination. Thus, five prototrophs obtained from the combination *arg-7* × (*isl* + *val*) segregated according to three segregation types. Three of them belonged to type I, one to type II, and one to type VI.

Antibacterial activity of prototrophs

110 prototrophic colonies were studied with respect to the amount of chlortetracycline produced. The antibacterial activity was estimated on submerged cultures by the colorimetric assay method after acid hydrolysis of the culture fluid. Biochemical mutants and the wild-type strains, from which they were derived, served as the controls. The activity of the biochemical mutants was never greater than 100 $\mu\text{g./ml.}$, the activity of the arginine-requiring mutants in most cases being equal to zero, while the activity of the wild-type strains was 850–1000 $\mu\text{g./ml.}$ The activity of all prototrophs was many times higher than that of the biochemical mutants and reached the activity of the starting strains. Sometimes the activity of all the prototrophs from one combination was higher than that of the starting strains by 5–20 %. Examples of the antibacterial activity of prototrophs from three combinations ($\text{arg-1} \times (\text{isl} + \text{val})$; $\text{arg-4} \times (\text{isl} + \text{val})$; $\text{arg-3} \times (\text{isl} + \text{val})$) are presented in Table 5 and the corresponding diagram (Fig. 1).

The activity of prototrophs from combinations $\text{arg-2} \times (\text{isl} + \text{val})$; $\text{arg-7} \times (\text{isl} + \text{val})$; $\text{arg-8} \times (\text{isl} + \text{val})$; $\text{arg-5} \times (\text{isl} + \text{val})$, $\text{arg-7} \times \text{his}$; $\text{his} \times (\text{isl} + \text{val})$ was no higher than that of any of the wild-type strains similar to the activity of prototrophs from combination $\text{arg-4} \times (\text{isl} + \text{val})$. Some prototrophs from combinations $\text{arg-6} \times (\text{isl} + \text{val})$ and $\text{arg-2} \times (\text{isl} + \text{val})$ were superior with respect to their activity as compared with the wild-type strains, resembling those prototrophs from combination $\text{arg-1} \times (\text{isl} + \text{val})$. Combination $\text{arg-3} \times (\text{isl} + \text{val})$ was the only one among the eleven combinations studied, which produced prototrophs always superior in their chlortetracycline activity to the wild-type strains.

Table 5. *Chlortetracycline activity of the wild-type strains, biochemical mutants and prototrophs from combinations $\text{arg-4} \times (\text{isl} + \text{val})$, $\text{arg-1} \times (\text{isl} + \text{val})$ and $\text{arg-3} \times (\text{isl} + \text{val})$*

Strain	Activity ($\mu\text{g./ml.}$)	Strain	Activity ($\mu\text{g./ml.}$)
Bd	990	<i>arg-1</i>	0
536	850	<i>arg-3</i>	0
<i>arg-4</i>	0	(<i>isl</i> + <i>val</i>)	50
Prototrophs from combination $\text{arg-4} \times (\text{isl} + \text{val})$			
No. 1	351	No. 6	316
No. 2	814	No. 7	582
No. 3	732	No. 8	632
No. 4	707	No. 9	420
No. 5	802	No. 10	772
Prototrophs from combination $\text{arg-1} \times (\text{isl} + \text{val})$			
No. 4	678	No. 9	1086
No. 5	256	No. 10	1143
No. 6	884	No. 11	1062
No. 7	241	No. 15	326
No. 8	466	No. 17	391
Prototrophs from combination $\text{arg-3} \times (\text{isl} + \text{val})$			
No. 1	1199	No. 9	1288
No. 2	1038	No. 11	1223
No. 3	1152	No. 14	1080
No. 5	1288	No. 16	1109
No. 7	1203	No. 17	996

It is interesting to note that in the combinations $arg-4 \times (isl + val)$, $arg-1 \times (isl + val)$ and $arg-3 \times (isl + val)$, all of which produced prototrophs differing in antibacterial activity, one and the same isoleucine + valine requiring mutant, derived from strain 536, was used. As the other crossing component in these combinations, one of the arginine-requiring mutants, $arg-4$, $arg-3$ or $arg-1$ derived from strain Bd, was used. These mutants differed neither in colonial morphology, nor in antibacterial activity. In addition to the arginine deficiency, each of these mutants is likely to possess specific physiological properties which determine the difference of the

Fig. 1. Antibiotic (chlortetracycline) activity of the wild-type strains, biochemical mutants and prototrophs. The activity levels are presented in absolute values. The interrupted line indicates the highest activity level of the wild-type strains.

prototrophs from the above combinations with respect to their antibacterial activities.

One prototroph which proved to be the most active, that is prototroph no. 11 from combination $arg-3 \times (isl + val)$, was selected for studying the range of induced variation in comparison with the wild-type strains. Antibacterial activity of the prototrophic-segregants was also studied. Usually the activity of non-sporulating prototrophs similar in their morphological properties to the arginine-requiring

mutants was zero, while the activity of segregants, such as segregant no. 3 from combination *arg-7* × (*isl* + *val*) reached 300 µg./ml. After these experiments, demonstrating recombinant formation in *Actinomyces aureofaciens*, combinations within the arginine-requiring mutants were studied.

In these combinations 56 arginine-requiring mutants of the 99 mutants isolated were used. All these strains were very similar in colonial morphology. They were characterized by asporogenic flat colonies, not highly folded nor having radial lines, and with no aerial mycelium or specific pigment.

Table 6. *Combinations of arginine-requiring mutants from which prototrophs were obtained*

No reversions of arginine-requiring strains were obtained

Combination	Biochemical mutant combination*	No. of prototrophs obtained
No. 24	<i>arg-19</i> × <i>arg-20</i>	8
No. 25	<i>arg-21</i> × <i>arg-9</i>	2
No. 52	<i>arg-24</i> × <i>arg-23</i>	1
No. 1108	<i>arg-15</i> × <i>arg-1</i>	1
No. 1073	<i>arg-18</i> × <i>arg-3</i>	1
No. 1107	<i>arg-15</i> × <i>arg-16</i>	1
No. 1176	<i>arg-11</i> × <i>arg-17</i>	1
No. 958	<i>arg-11</i> × <i>arg-1</i>	2
No. 1167	<i>arg-7</i> × <i>arg-22</i>	1
No. 1155	<i>arg-23</i> × <i>arg-22</i>	2
No. 306	<i>arg-25</i> × <i>arg-23</i>	1

* Biochemical mutants *arg-7*; *arg-9*; *arg-11*; *arg-15*; *arg-18*; *arg-19*; *arg-20*; *arg-21*; *arg-23*; *arg-24* were derived from strain BMK; *arg-1*; *arg-3*; *arg-16*; *arg-17* and *arg-25* from strain Bd; *arg-22* from strain B-16.

The strains are very stable and segregate neither prototrophic, nor auxotrophic forms over a number of generations. An attempt was made to differentiate the mutants with respect to their requirements for intermediary products of arginine biosynthesis, that is for either glutamic acid, ornithine or citrulline; or according to an alternative way of synthesis, for urea or guanidine. It was found that all the arginine-requiring mutants isolated were similar in that the addition of any of the above intermediates to the medium did not restore the synthesis of arginine and permit growth. 1540 combinations of arginine-requiring mutants were studied. Prototrophs were obtained in eleven cases (Table 6). The frequency of prototrophs in these combinations is as low as 1 in 10⁷.

The prototrophs obtained differed in colonial morphology from one another and from strain 536 typical of *Actinomyces aureofaciens*. Most of them formed well sporulating colonies of white to light-grey coloration, with sporophores in the form of spirals with 0.5–1.0 µ coils, which is characteristic of *A. aureofaciens*. Some prototrophs formed asporogenic colonies, morphologically similar to those produced by arginine-requiring mutants (Plate 1).

The study of these prototrophs with respect to their segregation pattern showed that the majority of them were stable and segregated no new forms in a number of generations. Only one prototroph, that is no. 1073, segregated a small amount (0.3 %) of non-sporulating prototrophs. Therefore we had no data, with respect to

direct segregation of the starting biochemical mutants, to indicate the deviation of these prototrophs by recombination. However, certain changes were found in the antibiotic-producing properties of these prototrophs as compared with the arginine requiring mutants (Table 7).

Table 7. *Antibacterial activity of the wild-type strains, arginine-requiring mutants, and prototrophs from combinations of these mutants*

Strain	Chlortetra- cycline activity ($\mu\text{g./ml.}$)	Strain	Chlortetra- cycline activity ($\mu\text{g./ml.}$)
Starting strains			
BMK	950	B-16	1600
Bd	990		
Arginine-requiring mutants			
<i>arg-24</i>	0	<i>arg-7</i>	20
<i>arg-23</i>	80	<i>arg-22</i>	0
<i>arg-15</i>	0	Prototrophs from	
<i>arg-1</i>	80	<i>arg-23</i> $\times$ <i>arg-24</i>	0
<i>arg-18</i>	0	<i>arg-1</i> $\times$ <i>arg-15</i>	0
<i>arg-3</i>	0	<i>arg-18</i> $\times$ <i>arg-3</i>	0
<i>arg-11</i>	20	<i>arg-11</i> $\times$ <i>arg-17</i>	0
<i>arg-17</i>	16	<i>arg-7</i> $\times$ <i>arg-22</i>	0

DISCUSSION

As is evident from the data presented in Table 6, 16 arginine-requiring mutants out of 56, on crossing in definite combinations, produced the parental form, that is gave rise to prototrophs. It is possible to assume that they belong to different alleles, though probably not to different genes. All prototrophs from combinations of arginine-requiring mutants independent of the activity of the wild-type strains were unable to synthesize chlortetracycline. This was not observed in the other crossings of *Actinomyces aureofaciens* involving mutants having dissimilar biochemical requirements and many crossings of *A. rimosus*. When *A. aureofaciens* mutants with different nutritional requirements were crossed, even when the antibacterial activity of these mutants was either zero or as low as 50 $\mu\text{g./ml.}$, the prototrophs obtained were similar in their activity to the wild-type strains. On the basis of these data it is possible to assume that the arginine-producing locus is related to the chlortetracycline-producing locus. This assumption is confirmed by the specificity of biochemical mutant formation in *A. aureofaciens*, as well as by the fact that active hybrids are formed only in combinations, where one of the crossing components is a biochemical mutant requiring histidine or isoleucine + valine. It should be noted that the data presented are preliminary, and further studies on arginine-requiring mutants are needed.

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EXPLANATION OF PLATE

Colonies of arginine-requiring mutants and of prototrophs obtained from combinations of arginine-requiring mutants. Grown on medium N12 for 8 days.

Arginine-requiring mutants

Prototrophs obtained from combinations of arginine-requiring mutants