Relation of Biochemical Mutations to Actinomycin Synthesis in *Streptomyces antibioticus*

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SUMMARY

Biochemical mutants of *Streptomyces antibioticus* were isolated and tested for their capacity to produce actinomycin. The yield of actinomycin produced in minimal medium plus the required amino acid, by strains requiring an amino acid not present in the molecule of actinomycin, was not significantly different from that obtained from the wild-type strain. On the other hand, all the strains which required an amino acid, which was also a precursor of the antibiotic, showed on minimal medium a drastic decrease in the production of actinomycin. The results have been interpreted by assuming a different utilization by the cell of exogenous and endogenous amino acid pools for antibiotic and protein synthesis.

INTRODUCTION

Biochemical mutations can determine an alteration of antibiotic yield when introduced into strains of fungi or *Streptomyces* which produce antibiotics. Bonner (1947) isolated fifty-five mutants of *Penicillium notatum* which were unable to synthesize penicillin or which produced less than 5–10 units/ml. Sermonti (1957, 1959) and MacDonald, Hutchinson & Gillett (1963) found that a large proportion of auxotrophic mutants of *P. chrysogenum* showed a decrease of penicillin production. Similar results were reported by Alikhanian, Mindlin, Goldat & Vladimirov (1959) for the production of oxytetracycline by auxotrophic strains of *Streptomyces rimosus*; they also showed that antibiotic production by some auxotrophs was increased when the specific growth factor was added in excess of the amount required for normal growth. Mach, Reich & Tatum (1963), in a study of the biosynthesis of the polypeptide tyrocidine by *Bacillus brevis*, obtained several strains which were incapable of producing the antibiotic, but they did not succeed in isolating strains which were auxotrophic for amino acids present in the polypeptide. The present paper describes the effect of auxotrophy on the production of actinomycin by biochemical mutants of *S. antibioticus*. It will be shown that the nutritional dependence on the presence of an amino acid component of the polypeptide antibiotic had a more drastic effect on actinomycin yield than a nutritional dependence for other amino acids not components of the antibiotic. A possible interpretation of the results is given on the basis of a different utilization by the organisms of the exogenous and endogenous amino acid pools for protein and antibiotic synthesis.

METHODS

Culture media. Bennett's agar (Waksman, 1961) was used for the maintenance of strains and for plating ultraviolet-irradiated spores. Medium no. 40 of Magni & von Borstel (1962), a minimal medium adjusted to pH 7, was used in replica plating for the isolation of biochemical mutants. The complete medium V6, used for vegetative growth, had the following composition (g./l.): yeast extract (A. Costantino, Favria, Turin, Italy), 5; beef extract (as above), 5; peptone (as above), 5; casein hydrolysate (as above), 3; glucose, 20; NaCl, 1.5; distilled water to 1000 ml.; adjusted to pH 6.6; sterilized at 115° for 30 min. The complete medium V6 and the minimal medium of Goss & Katz (1960), in which galactose was replaced by glucose, were used for the fermentations.

Table 1. *Biochemical mutants of Streptomyces antibioticus*

Code	Genotype*	Derivation
AB 2	(<i>isol</i> + <i>val</i>) ⁻	1692 by u.v. irradiation
AB 8	(<i>isol</i> + <i>val</i>) ⁻	
AB 107	(<i>isol</i> + <i>val</i>) ⁻	
AB 111	<i>met</i> ⁻ <i>thr</i> ⁻	
AB 1	<i>cys</i> ⁻	
AB 3	<i>arg</i> ⁻ <i>ur</i> ⁻	
AB 9	<i>arg</i> ⁻ <i>ur</i> ⁻	
AB 115	<i>arg</i> ⁻ <i>ur</i> ⁻	
AB 114	<i>arg</i> ⁻	
AB 4	<i>his</i> ⁻	
AB 101	<i>his</i> ⁻	
AB 102	<i>his</i> ⁻	
AB 103	<i>his</i> ⁻	
AB 113	<i>ad</i> ⁻	
AB 6	<i>phe</i> ⁻	
AB 106	<i>leu</i> ⁻	
AB 7	<i>leu</i> ⁻	
AB 10	<i>leu</i> ⁻	
AB 110	<i>met</i> ⁻	AB 2 by u.v. irradiation
AB 109	<i>tyr</i> ⁻	
AB 2/1	<i>isol</i> ⁻ / <i>val</i> ⁻ <i>met</i> ⁻	
AB 2/2	<i>isol</i> ⁻ / <i>val</i> ⁻ <i>met</i> ⁻	
AB 2/5	<i>isol</i> ⁻ / <i>val</i> ⁻ <i>ad</i> ⁻	
AB 2/3	<i>isol</i> ⁻ / <i>val</i> ⁻ <i>met</i> ⁻	
AB 2/4	<i>isol</i> ⁻ / <i>val</i> ⁻ <i>met</i> ⁻	
AB 2/6	<i>isol</i> ⁻ / <i>val</i> ⁻ <i>met</i> ⁻	spontaneous back mutation
AB 2/7	<i>isol</i> ⁻ / <i>val</i> ⁻ <i>met</i> ⁻	
AB 2/21	<i>met</i> ⁻	
AB 2/51	<i>ad</i> ⁻	
AB 2/52	<i>ad</i> ⁻	
AB 2/10	Prototroph	
AB 2/22	Prototroph	

* The following abbreviations have been used: *isol* = isoleucine; *val* = valine; *met* = methionine; *thr* = threonine; *cys* = cysteine; *arg* = arginine; *ur* = uracil; *his* = histidine; *ad* = adenine; *phe* = phenylalanine; *leu* = leucine; *tyr* = tyrosine.

Difco antibiotic medium 3 was used for the microbiological determination of actinomycin production.

Isolation of mutants. Biochemical mutations were induced by ultraviolet (u.v.)

irradiation. A spore suspension (0.5 ml. containing 5×10^7 spores in a Petri dish diam. 40 mm.) was irradiated with a low-pressure mercury lamp to a survival of 10^{-2} . Irradiated spores were plated on Bennett agar medium and incubated at 28°. After 4 days the colonies were replicated on minimal medium. Colonies which did not grow in minimal medium were isolated and their growth requirements characterized.

Production of actinomycin. Medium V6 (5 ml.) was added to an agar slope culture and the spore suspension used to inoculate 100 ml. of the same medium in a 500 ml. Erlenmeyer flask. The cultures were incubated at 28° on a reciprocating shaker for 24 hr. The mycelium was recovered by centrifugation, washed twice with the minimal fermentation medium and finally resuspended in 100 ml. of similar medium; 5 ml. of such a suspension was used as inoculum for each 100 ml. of fermentation media appropriately supplemented with the required nutrients. The fermentation flasks were incubated for 72 hr under the conditions given above for the growth of the inoculum. All experiments were done in triplicate.

Bioassay of actinomycin. Liquid cultures were centrifuged and the supernatant solution was used for antibiotic assay with *Bacillus subtilis* ATCC 6633 as a test organism. The antibiotic titre was determined by the filter paper disk method, with crystalline actinomycin D as standard.

Growth curves. Cultures in appropriately supplemented minimal medium of two auxotroph strains were prepared as described above and incubated for 48 hr on a reciprocating shaker. Samples (10 ml.) of the cultures were removed at various time intervals and were centrifuged in graduated tubes for 15 min. at 2500 rev./min. to determine the content of the mycelium, as % of the total volume of the liquid culture. Each experiment was done in triplicate.

Organism. All the isolates used were derived from *Streptomyces antibioticus* no. 1692 (culture collection of the Institute of Botany, University of Pavia). A list of isolated strains is given in Table 1.

RESULTS

The actinomycins are a family of chromopeptide antibiotics which differ only in the peptide portion of the molecule (Brockmann, 1960). *Streptomyces antibioticus* strain 1692 produces in a minimal medium actinomycins C₁, C₂ and C₃. The peptide portion of these actinomycins contains L-threonine, L-proline, sarcosine, L-N-methylvaline, D-valine and/or D-alloisoleucine (Fig. 1). Recent studies have shown that: L-proline is the precursor of the L-proline found in the actinomycins (Katz, Prockop & Udenfriend, 1962); L-valine is the precursor of both the D-valine and L-N-methylvaline residues of the actinomycin molecule (Katz & Weissbach, 1963); L-isoleucine is the precursor of the D-alloisoleucine (Albertini, Cassani & Ciferri, 1964); L-threonine is the precursor of the L-threonine and, to a lesser extent, of the D-alloisoleucine (Albertini *et al.* 1964); glycine or sarcosine are the precursors of sarcosine (Ciferri, Albertini & Cassani, 1964) in the molecule. Therefore, among the auxotrophs listed in Table 1, only those which require isoleucine and valine (*e.g.* AB2, AB8, AB107) and threonine (AB111) carry a deficiency for the synthesis of amino acids present in the proteins and in the antibiotic. On the other hand, the remaining strains are mutated in genes which control the synthesis of amino acids present only in the proteins.

All isolates listed in Table 1 were tested for actinomycin production in complete medium and in minimal medium plus supplement, i.e. amino acid(s) required by the strain, at 10 μ g. and 100 μ g./ml.

The results are collected in Tables 2-4.

In complete medium, the average production of actinomycin by auxotrophs which required amino acids not present in the polypeptide was of the order of

Fig. 1. Actinomycin C_2 . In actinomycin C_1 the D-alloisoleucine is replaced by D-valine; in actinomycin C_3 the D-valine is replaced by D-alloisoleucine.

Table 2. Antibiotic production by *Streptomyces antibioticus* auxotrophs requiring as nutrients nitrogen bases or amino acids not present in the peptide chain of actinomycin

Strain		Antibiotic production (μ g./ml.)		
Code	Requirement	Complete medium V 6	Minimal medium + 10 μ g./ml. of required growth factor	Minimal medium + 100 μ g./ml. of required growth factor
AB 1	cys*	135	35	38
AB 3	arg + ur	180	55	58
AB 9	arg + ur	119	37	39
AB 115	arg + ur	120	35	33
AB 114	arg	110	31	33
AB 4	his	208	26	31
AB 101	his	122	28	29
AB 102	his	108	36	38
AB 103	his	98	27	28
AB 113	ad	118	35	30
AB 6	phe	52	6.5	8
AB 106	leu	88	18	20
AB 7	leu	74	16	17
AB 10	leu	92	19	22
AB 110	met	31	6.5	7
AB 109	tyr	80	15	18
	Average	108.4	26.6	28
	Wild type			
	Average	106	25	—

* Abbreviations see Table 1.

108 $\mu\text{g./ml.}$, not significantly different from 106 $\mu\text{g./ml.}$, the average production of the parental prototroph strain. In complete medium, the mutants which required amino acids which are potential precursors of actinomycin showed a significant

Table 3. *Antibiotic production by Streptomyces antibioticus auxotrophs requiring amino acids which are present in protein and in the peptide chain of actinomycin*

Strain		Antibiotic production ($\mu\text{g./ml.}$)		
Code	Requirement	Complete medium V 6	Minimal medium + 10 $\mu\text{g./ml.}$ of required amino acid	Minimal medium + 100 $\mu\text{g./ml.}$ of required amino acid
AB 2	isol + val*	83	0.9	2.7
AB 8	isol + val	70	0.8	2.1
AB 107	isol + val	79	1.0	3.8
AB 111	met + thr	68	0.7	3.2
AB 2/1	isol + val + met	88	1.0	3.3
AB 2/2	isol + val + met	12.6	1.0	1.4
AB 2/5	isol + val + ad	78	0.9	1.2
AB 2/3	isol + val + met	30	0.8	1.8
AB 2/4	isol + val + met	20	0.9	2.1
AB 2/6	isol + val + met	45	1.1	2.1
AB 2/7	isol + val + met	40	0.8	1.2
Average		55.7	0.9	2.2
Wild-type average		106	25	—

* Abbreviations see Table 1.

Table 4. *Antibiotic production by strains of Streptomyces antibioticus back-mutated to isoleucine and valine independence**

Code	Requirement	Derivation	Antibiotic production ($\mu\text{g./ml.}$)		
			Complete medium V 6	Minimal medium + 10 $\mu\text{g./ml.}$ of required amino acid	Minimal medium + 100 $\mu\text{g./ml.}$ of required amino acid
AB 2/21	met	AB 2/2 <i>isol⁻/val⁻ met⁻†</i>	198	66	—
AB 2/51	ad	AB 2/5 <i>isol⁻/val⁻ ad⁻</i>	172	23	—
AB 2/52	ad	AB 2/5 <i>isol⁻/val⁻ ad⁻</i>	175	20.5	—
AB 2/22	—	AB 2/2 <i>isol⁻/val⁻ met⁻</i>	157.5	—	21.5
AB 2/10	—	AB 2 <i>isol⁻/val⁻</i>	100	—	12.5

* The strain AB 2/22 is back-mutated also to methionine independence.

† Abbreviations see Table 1.

but not very marked decrease in production of actinomycin (average 56 $\mu\text{g./ml.}$). In minimal medium plus supplement of the amino acid required for growth, however, all the isolates showed a decrease in the synthesis of actinomycin. The yield of actinomycin was decreased on an average to 25 % in the parental prototroph strain and in the auxotroph strains which required for growth amino acids not present in

the antibiotic. The actinomycin yield was not significantly increased in this group of amino acid requiring mutants when the required amino acid supplement was increased from 10 to 100 $\mu\text{g./ml.}$ (Table 2).

On the other hand, all the isolates which required for growth an amino acid which was a precursor of actinomycin (Table 3) showed in minimal medium (supplemented with the amino acid required for growth) a marked decrease in the production of actinomycin (about 2% of the yield in complete medium). The increase in the specifically required amino acid(s) from 10 to 100 $\mu\text{g./ml.}$ resulted in a threefold increase of actinomycin yield; this was still very far from that of the other isolates listed in Table 2.

Fig. 2

Fig. 3

Fig. 2. Growth curve and actinomycin production by the *Streptomyces antibioticus* mutant AB3 $\text{arg}^- \text{ur}^-$. Δ — Δ , $\bigcirc$ — $\bigcirc$, mycelium (% by volume of the total cultural liquid) in minimal medium supplemented with 100 and 10 $\mu\text{g./ml.}$ of the required growth factors, respectively. Δ — Δ , $\bigcirc$ — $\bigcirc$, antibiotic production in minimal medium supplemented with 100 and 10 $\mu\text{g./ml.}$ of the required growth factors, respectively.

Fig. 3. Growth curve and actinomycin production by *Streptomyces antibioticus* mutant AB2/2 $\text{isol}^- \text{val}^- \text{met}^-$. Δ — Δ , $\bigcirc$ — $\bigcirc$, mycelium (% by volume of the total cultural liquid) in minimal medium supplied with 100 and 10 $\mu\text{g./ml.}$ of the required growth factors, respectively. Δ — Δ , $\bigcirc$ — $\bigcirc$, antibiotic production in minimal medium supplied with the required growth factors 100 and 10 $\mu\text{g./ml.}$, respectively.

The fact that this behaviour was determined by the specific amino acid growth requirement is shown by the data of Table 4. Isolates carrying mutations for two kinds of amino acids, i.e. one required for actinomycin and protein synthesis and the other required for protein synthesis only, produced in minimal medium extremely low yields of antibiotic (Table 3). When these strains reverted to ability to synthesize the amino acid present in the antibiotic, the production of actinomycin became equal to that of the strains of the first group (Table 4).

A simple explanation for the above results could be that a very limited utilization of isoleucine, valine and threonine by all mutants requiring isoleucine, valine and threonine might result in scanty growth of the micro-organisms and therefore in a low production of antibiotic. This possibility was ruled out by the experiments reported in Figs. 2 and 3 which show that, despite very different yields of actinomycin in minimal media appropriately supplemented, two strains of different genotype showed equal growth rates which should indicate equal protein synthesis.

The decrease in yield of actinomycin by the auxotrophs which required for growth a specific amino acid also present in the polypeptide chain should therefore be ascribed to a different phenomenon.

In the case of *Bacillus anthracis*, Gladstone (1939) observed that omission from the growth media of isoleucine, valine or leucine inhibited the growth of the micro-organism. Such inhibition could be overcome by adding a mixture of all three amino acids. A similar 'toxic' effect brought about by threonine was removed by adding valine. It could be postulated, then, that such amino acids could exert a specific inhibition of the synthesis of actinomycin by *Streptomyces antibioticus*. However, the results obtained in the case of the prototroph *S. antibioticus* (Katz, 1960) showed that even very high levels of valine or isoleucine stimulate, rather than inhibit, growth and antibiotic production. Experiments performed on our mutant strains that carry a deficiency for valine and isoleucine revealed that the addition of a tenfold excess of leucine did not stimulate production of actinomycin. Nor did the addition of an excess of valine to fermentation media of the threonine-requiring strains have a beneficial effect.

DISCUSSION

MacDonald *et al.* (1963) suggested that 'the diminution of penicillin yield in *Penicillium chrysogenum* was most likely due to pleiotropic effects of the auxotrophic locus'. If this is an effect that occurs at random among mutants to auxotrophy, it is unlikely to explain our data because only mutations affecting the synthetic pathways of amino acids present in the polypeptide chain of actinomycin decrease the yield of actinomycin.

Our data are, at least in part, at variance with the data of Alikhanian *et al.* (1959), who observed in *Streptomyces rimosus* that the yield of antibiotic from some auxotrophs could be increased by the addition of an amino acid, for which the auxotroph was dependent, in amounts higher than those required only for growth. In our case, the production of actinomycin by the mutants of the first group (Table 2) seems to be unaffected by the concentration of the amino acid supplied to the culture.

The results obtained in minimal media may be explained as follows. The cell may recognize and utilize to different extents the amino acids absorbed from the environment and those synthesized by the cell itself, as reported, for instance, by Sercarz & Gorini (1964) for the control of repressor formation by endogenous *vs.* exogenous arginine. Such different pools, or compartments, may be utilized rather efficiently and to approximately the same extent for protein synthesis. On the other hand, for the synthesis of the antibiotic polypeptide chain, the utilization of the external pool may be very inefficient since the production of actinomycin is decreased to 1/50 of the normal at amino acid concentrations which support normal protein synthesis. Even a tenfold increase in the amino acid supplied stimulates only a threefold increase in the production of the antibiotic. Assuming that such a hypothesis is correct, one would then have to explain why in complete medium the production of antibiotic by the auxotrophs of Table 3 approaches a value near to that of the other mutants and of the prototroph. But it cannot be excluded *a priori* that the capacity of the cell to recognize two amino acid pools does not affect the utilization of small peptides or other possible precursors of the antibiotic present in the complete medium.

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