FOLIA MICROBIOLOGICA

Vol. 5, 1960

Antagonistic Properties and Mutual Relationships of Some Actinomycetes

By Z. ŘEHÁČEK, LIBUŠE DOLEŽILOVÁ and Z. VANĚK

Department of Microbiology, Institute of Biology, Czechoslovak Academy of Sciences, Prague

Received September 4, 1959

Fundamental research on the antagonistic properties of microorganisms has not hitherto contributed substantially to the elucidation of the possibilities of a planned search for antibiotic microorganisms with desired properties. Consequently the search for suitable antagonistic microorganisms is a complicated task and the finding of a new antibiotic is still more or less a matter of accident. It was for the above reasons that we attempted in this work to investigate the antibiotic properties and mutual relationships of freshly isolated antagonistic actinomycetes, which are the most frequent source of antibiotic substances. We proceeded from the fact that different antibiotics are formed under different conditions (Reynolds & Waksman, 1948; Kupferberg *et al.* 1950), from the discovery of formation of antibiotic mixtures (Vaněk, Doležilová & Řeháček, 1958) and from the assumption that one of the ways of imposing a certain order in the problem of obtaining antagonistic microorganisms and their antibiotics, consists in investigating ecological aspects of these microorganisms.

MATERIALS AND METHODS

Actinomycete strains were obtained from different soil samples by centrifuging (Řeháček, 1959a). In 93 freshly isolated antagonistic strains, the antibiotic spectra were determined by a previously described method (Řeháček, 1959b). Mutual relationships between the individual strains were investigated by the method of agar blocks simultaneously on a complex medium (C) (Krasil'nikov, 1950) and on a synthetic Czapek-Dox medium (CD). The strains were evaluated taxonomically according to Gauze *et al.* (1957). For the purpose of determining the solubility of antibiotics liberated into the agar nutrient media, paper chromatography was used, along with a system of ten solvents (Ševčík, Podojil & Vrtišková, 1957). The ionic character of the antibiotics was assayed by block tests using plates with an agar layer containing fine-grained cation exchanger (Ševčík & Podojil, 1957). In antibiotics of a polyene type, the absorption spectra were measured in the UV range, using ethanol solutions of the preparations obtained from the agar medium by extraction with acetone. Antibiotic substances were classified in five groups, as suggested by Waksman and Lechevalier (1953):

I. Antibiotics soluble in organic solvent and to a limited degree in water. Production strains form yellow, orange ranging to red pigments and display an antagonistic effect mostly against Gram-positive bacteria and fungi, and only slightly against Gram-negative bacteria. This group comprises actinomycins, rhodomycins, rhodomycetin, actinorhodin, lithmocidin, coelicolorin.

II. Antibiotics of a basic character, readily soluble in water, insoluble in most organic solvents, effective against Gram-positive and Gram-negative bacteria and partly effective against fungi. This group comprises streptothricins, streptomycins, neomycins etc.

III. Antibiotics with poor water solubility, readily soluble in organic solvents, effective against Gram-positive and Gram-negative bacteria and partly effective against fungi. This group includes neutral antibiotics such as chloramphenicol, chlortetracycline, oxytetracycline, flaveolin and basic antibiotics of the erythromycin-carbomycin group.

IV. Antibiotics with a pronounced anti-yeast and a less marked antifungal effect.

V. Antibiotics of a polyene type with characteristic absorption spectra in UV light, effective against fungi, ineffective against bacteria.

RESULTS

Taxonomy and antibiotic properties of investigated actinomycetes. According to the results of a taxonomic evaluation of strains (Table 1) the population investigated consisted mainly of actinomycetes of the albus (72%) and helvolus (23%) series. A minute number of strains belonged to the aureus (3%), griseus (1%) and ruber (1%) series. In the helvolus series there was no basic quantitative difference between actinomycetes antagonistic to the individual test microorganisms. There were 16 strains effective against Gram-

Table 1. Taxonomy of investigated actinomycetes and review of their antibiotic properties

Series	Group	Number of strains				
		Total	Effective against			
			Gram-posit. bacteria	Gram-negat. bacteria	Yeasts	Fungi
Ruber	*Act. oidiosporus* Kras 1941	1	1	1	1	1
Helvolus	*Act. globisporus* Kras 1941	6	3	3	5	4
	Act. globisporus var. *caucasicus* Gauze 1957	4	4	2	4	3
	Act. globisporus var. *flavofuscus* Gauze 1957	9	8	5	9	9
	Act. streptomycini Kras 1955	1	0	0	0	1
	Act. phaeochromogenes Conn 1917	1	1	0	0	0
Albus	*Act. candidus* Kras 1941	12	8	4	11	12
	Act. wedmarensis Millard et Burr 1926	8	5	1	8	8
	Act. coroniformis Kras 1941	14	9	1	14	14
	Act. albidoflavus Duché 1934	24	15	7	19	21
	Act. candidus var. *alboroseus* Gauze 1957	1	1	0	1	1
	Act. griseolus Waksman et Curtis 1916	6	6	2	5	5
	Act. mirabilis Ruschman 1952	2	2	0	2	2
Griseus	*Act. acrimycini* Gauze 1957	1	1	0	0	0
Aureus	*Act. graminearus* Berestnev 1897	2	0	0	2	0
	Act. olivaceus Waksman et Henrici 1919	1	1	1	1	0

Table 2. Antibiotic formation by some taxonomically coherent actinomycetes

Series	Group	Number of strains (%) producing antibiotic of group				
		I	II	III	IV	V
Albus	*Act. candidus*	0	38	0	15	46
	Act. wedmarensis	0	14	0	14	70
	Act. coroniformis	17	0	22	7	64
	Act. albidoflavus	4	30	8	4	50
	Act. griseolus	0	25	0	38	38
Helvolus	*Act. globisporus*	0	33	17	0	50
	Act. globisporus var. *caucasicus*	50	25	25	0	0
	Act. globisporus var. *flavofuscus*	11	56	0	11	22

positive bacteria, 10 strains against Gram-negative bacteria, 18 strains against yeasts and 17 strains against fungi. In actinomycetes of the albus series greater overall differences were observed, especially in the proportion of strains effective against yeasts or fungi and of those with antibacterial properties. The growth of Gram-positive bacteria was inhibited by 46 strains of this series, the growth of Gram-negative bacteria by 15 strains only, while yeasts were inhibited by 60 strains and fungi by 63 strains.

Results of the investigation of the character of antibiotics formed by strains of the albus and helvolus series are apparent from Table 2 and Fig. 1. Numerous strains which were otherwise taxonomically identical formed antibiotics of different characters. The quantitative proportion of strains producing the individual types of antibiotics was different not only in the two taxonomic series but also within some of their groups. In the albus series strains forming antifungal

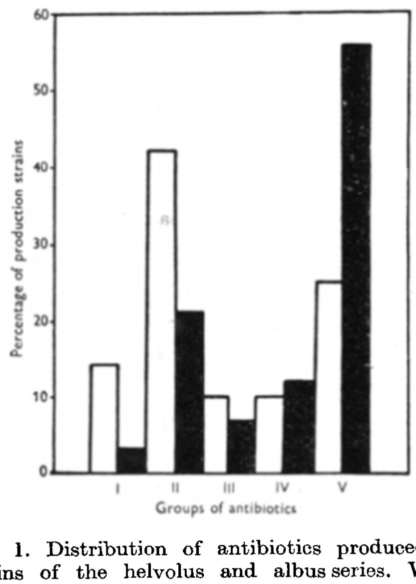

Fig. 1. Distribution of antibiotics produced by strains of the helvolus and albus series. White columns — helvolus series; black columns — albus series.

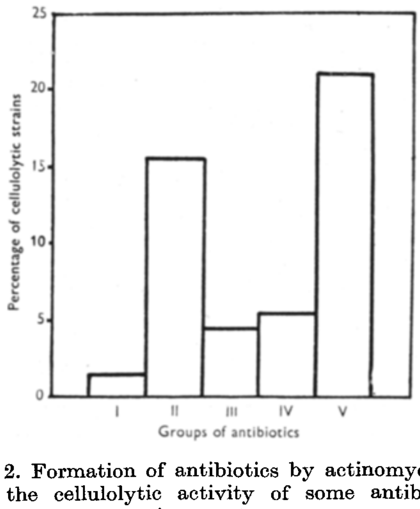

Fig. 2. Formation of antibiotics by actinomycetes and the cellulolytic activity of some antibiotic actinomycetes.

Table 3. Mutual relationships between actinomycete strains which are taxonomically coherent but produce different antibiotics. Group *Act. candidus*

Strain No.	Groups of antibiotic formed	Number of antagonistic strains forming antibiotics of group I	II	III	IV	V
1288	II	0	2	0	2	5
+1097	II	0	3	0	0	4
1431	II	0	0	0	2	3
1492	II	0	4	0	2	4
+1315	II	0	3	0	1	6
1318	IV	0	4	0	1	2
+1618	IV	0	4	0	0	4
1640	V	0	2	0	0	1
1253	V	0	5	0	1	2
1581	V	0	3	0	1	1
1003	V	0	2	0	1	4
1006	V	0	4	0	1	3
1023	V	0	4	0	0	4

Table 4. Mutual relationships between actinomycete strains which are taxonomically coherent but produce different antibiotics. Group *Act. coroniformis*

Strain No.	Group of antibiotic formed	Number of antagonistic strains forming antibiotics of group I	II	III	IV	V
1239	I	0	0	0	0	1
1008	III	0	0	0	0	4
1434	III	0	0	0	0	3
1566	III	0	0	0	0	1
1228	IV	0	0	0	0	1
1002	V	0	0	0	0	3
1096	V	0	0	0	0	1
+1156	V	1	0	2	1	9
1208	V	0	0	0	0	1
1236	V	0	0	0	0	1
1251	V	0	0	0	0	1
1437	V	0	0	0	0	1
1446	V	0	0	0	0	3
1919	V	0	0	0	0	1

antibiotics of a polyene type (Group V) predominated, in the helvolus series basic antibiotics of the type of streptothricin, streptomycin and neomycin (Group II) were most frequent. It follows from Fig. 2 that strains with polyene antibiotics most frequently possessed cellulolytic properties; the cellulolytic capacity of strains producing basic antibiotics of

Table 5. Mutual relationships between actinomycete strains which are taxonomically coherent but produce different antibiotics. Group *Act. albidoflavus*

Strain No.	Group of antibiotic formed	Number of antagonistic strains forming antibiotics of group I	II	III	IV	V
1173	I	0	6	1	1	6
1016	II	0	4	0	0	4
1245	II	1	5	0	0	1
1418	II	1	8	1	0	6
1575	II	1	6	0	0	2
1619	II	0	1	0	0	8
1620	II	1	5	1	1	6
1623	II	0	5	0	1	8
1624	II	0	5	2	0	8
1311	II	1	6	1	1	9
+1014	III	1	4	2	0	2
1354	III	1	8	0	0	6
1538	IV	1	5	2	0	3
+1022	V	1	9	0	0	5
1073	V	0	3	1	0	4
+1179	V	0	3	1	1	5
1215	V	0	6	0	0	2
1387	V	0	5	0	0	3
1482	V	0	4	1	0	3
1501	V	1	7	0	0	1
1511	V	0	5	1	0	4
1553	V	0	6	1	1	5
1572	V	0	4	1	1	6
+1579	V	1	5	0	0	2

+ = suppresses its own culture.

Table 6. The effect of the medium on antagonistic phenomena in 93 actinomycete strains

Strains tested: Number	Strains tested: Group of antibiotic formed	Antagonism: one-stage + C	one-stage + CD	one-stage — C	one-stage — CD	two-stage C	two-stage CD
6	I	4	1	4	1	6	0
26	II	6	20	21	5	12	14
7	III	7	0	3	4	6	1
12	IV	8	4	5	7	7	5
42	V	30	10	11	31	27	15

C = number of cases in which Krasil'nikov's medium was more conducive to antagonism; CD = number of cases in which synthetic Czapek - Dox medium was found more suitable; + = strains were antagonistic; — = strains were inhibited.

Table 7. The effect of nutrient medium and cultivation time on autoinhibition in some antagonistic actinomycetes

Actinomycete	Synthetic medium (CD)							Complex medium (C)						
	Length of cultivation (days)													
	2	3	4	5	6	7	8	2	3	4	5	6	7	8
	Diameter inhibition zones (mm.)													
Act. candidus 1315	0	0	0	14	0	0	(12)	0	0	n	(10)	0	0	0
1097	n	10	n	0	0	0	0	n	n	10	0	0	0	0
1618	11	10	n	0	0	12	(11)	0	0	10	0	0	0	0
Act. coroniformis 1156	0	12	11	0	n	11	11	11	14	12	12	12	12	10
Act. albidoflavus 1014	0	10	0	0	0	0	0	11	10	10	11	n	0	0
1022	0	0	n	n	n	n	0	n	10	10	n	0	0	0
1179	n	n	10	10	0	0	n	12	11	11	10	10	10	n
1579	0	0	0	n	0	0	n	12	10	11	10	0	0	0

( ) = indistinct inhibition zones; n = indication

Group II was less frequent but was still more so than among strains forming antibiotics of Groups I, III and IV.

Mutual relationships between antibiotic actinomycetes. As far as the mutual relationships of the investigated actinomycetes are concerned microbial strains producing basic antibiotics of the type of streptothricin, streptomycin and neomycin (Group II) appeared to be the most effective antagonists and at the same time the most sensitive organisms. This is evidenced by the finding of a high number of cases of two-step, i.e. bilateral antagonism. The most resistant actinomycetes were those producing polyene antibiotics. Most antagonists of these strains were producers of antibiotics of Group II or V, only rarely of Group I, III or IV. Some strains, as shown in Tables 3, 4, 5, were suppressed by taxonomically coherent strains producing antibiotics of an identical type and eight strains were found to inhibit their own younger cultures. These phenomena were especially marked in strains forming antibiotics of a polyene type.

One of the factors affecting the mutual relationships between the investigated actinomycetes was the nutrient medium. According to the results shown in Table 6 the effect of the medium was especially pronounced in strains producing antibiotics of Group II and in strains producing polyene antibiotics (Group V) .Strains with the Group II antibiotics displayed their antiactinomycete effect much more markedly on a synthetic medium, while they were more frequently inhibited by strains grown on a complex medium. These characteristics were reversed with actinomycetes producing polyene antibiotics. The two-step (bilateral) antagonism was more pronounced on a complex medium.

Autoinhibition. An inhibitory effect on younger cultures of the same strain was shown in eight strains (15%). According to the data shown in Table 7, autoinhibition was not found to be a constant phenomenon. It could be affected, for example, by the nutrient medium and by the length of cultivation of cultures on blocks of nutrient medium. In strains of *Act. coroniformis* and *Act. albidoflavus* the complex medium was more conducive to autoinhibition. In strains of *Act. candidus*, autoinhibition was more expressed on a synthetic medium. In most cases two-, three- or four-day cultures were found to be optimal for inhibition of their own younger cultures. With increasing age the autoinhibitory capacity of strains usually decreased or disappeared completely. Strains with demonstrated autoinhibition were inhibited primarily by producers of polyene antibiotics and of antibiotics of Group II. Strains forming antibiotics of Group I, III and IV (Table 8) were more rarely antagonistic toward the above actinomycetes. Strains inhibiting their own cultures were frequently inhibited by taxonomically related strains which formed antibiotics of the same group as their own.

Table 8. Relationships between strains exhibiting autoinhibition and taxonomically identical strains

Strains with autoinhibition: Classification	Group of antibiotic produced	Number of antagonists producing antibiotics of group: I	II	III	IV	V
Act. candidus 1097	V	0	2	0	1	4
1315	II	0	3	0	0	4
1618	II	0	4	0	2	4
Act. coroniformis						
1156	V	1	0	2	1	9
Act. albidoflavus						
1014	III	1	4	2	0	2
1022	V	1	9	0	0	5
1179	V	0	3	1	1	5
1579	V	1	5	0	0	2

DISCUSSION

The discovery of the formation of several antibiotics by a single actinomycete strain, whether alone or in a combination, suggests that the antagonist possesses several types of specific metabolic chains, the end-products of which are antibiotics. It was confirmed in this work that in order to demonstrate the synthesis of antibiotic substances, careful selection of nutrient media is of paramount importance, as different antibiotics are produced under different conditions. A careful choice of testing methods is also essential since the response of a microorganism toward the presence of an antibiotic substance is influenced by the concentration of this substance and by the environmental conditions in the broadest sense of the word. Hence it follows that the relationships between antibiotic biosynthesis and general metabolism of a production strain are affected by the environmental conditions of the natural occurrence of the microorganism. If ecology is understood as the interaction between the microorganism and its environment it must be assumed that the formation of antibiotics is influenced by ecology.

On the basis of the results obtained it is assumed that different antagonistic actinomycetes are capable of forming under corresponding conditions different sets of antibiotics, characterized not only by the qualitative incidence of the antibiotics but also by a different degree of fixation of the formation of these substances in microbial metabolism. In the quantitatively distinct types of antibiotics, produced by strains of some taxonomic series or groups, a new method of application of antibiotic production by actinomycetes can be envisaged, with reference to the classification or systematics of these microorganisms. The high number of strains producing polyene antibiotics which were inhibited by taxonomically coherent strains also forming polyene antibiotics, in addition to the previously demonstrated frequent occurrence of polyene antibiotics in mixtures with other antibiotics (Vaněk, Doležilová & Řeháček, 1958) indicates, in our opinion, that the biosynthesis of some polyene antibiotics is most likely not very closely associated with the metabolic pattern of a microorganism. The antifungal properties of strains with polyene antibiotics, their resistance toward numerous actinomycetes and the frequently occurring cellulolytic capacity are, in our opinion, the most important causes of the abundant incidence of these actinomycetes, grouped mostly in the albus series, under natural environmental conditions. The ecological significance of polyene-type antibiotics is stressed by the previously demonstrated formation and presence of a polyene antibiotic in nonsterile soil samples (Řeháček, 1958).

The sensitivity of some antagonistic strains toward low concentrations of their own antibiotics is regarded as a consequence of labile fixation of antibiotic formation in microbial metabolism and as the probable basis of autoinhibition. The dependence of these phenomena on the composition of the nutrient medium and on the length of cultivation supports our view on the possibilities of microorganisms possessing different antibiotic characteristics under different environmental conditions.

SUMMARY

In a group of 93 freshly isolated antagonistic actinomycetes the following characteristics were investigated: taxonomy, antibiotic properties, the character of produced antibiotics and mutual relationships between individual strains. Numerous taxonomically identical strains of the albus and helvolus series produced different types of antibiotics. The quantitative representation of producers of the individual types of antibiotics was different not only in the two taxonomic series but also within some of their groups. As far as their mutual relationships are concerned the most effective antagonists and at the same time the most sensitive microorganisms were those producing antibiotics of the type of streptothricin, streptomycin and neomycin; strains with polyene antibiotics were very resistant. Mutual relationships were affected in varying degrees by the nutrient medium. Eight strains displayed autoinhibition, which was influenced by the nutrient medium and the length of cultivation. Strains exhibiting autoinhibition were inhibited by taxonomically coherent strains producing antibiotics of the same group as their own.

The discussion concerns the possibilities of microorganisms possessing different antibiotic properties under different environmental conditions and a new method of the possible application of the antibiotic properties of actinomycetes for their classification or systematics.

The authors are indebted to Mrs. L. Srbová, Mrs. V. Klicperová, Miss L. Pokorná and Mrs. B. Kratochvílová for technical assistance.

References

Gauze, G. F., Preobrazhenskaya, T. P., Kudrina, J. S., Blinov, N. O., Ryabova, I. D., Sveshnikova, M. A.: *Problems of classification of antagonistic actinomycetes.* Medgiz, Moscow 1957 (Гаузе, Г. Ф., Преображенская, Т. П. Кудрина, И. С., Блинов, Н. О., Рябова, И. Д. и Свешникова, М. А.: Медгиз, Москва 1957).

Krasil'nikov, N. A.: *Actinomycete antagonists and antibiotic properties.* Medgiz, Moscow 1950 (Красильников, Н. А.: Медгиз, Москва 1950)

Kupferberg, A. B., Styles, H., Singher, H. O., Waksman, S. A.: *The production of streptocin by different strains of Streptomyces griseus.* J. Bact. 59 : 523, 1950.

Reynolds, D. M., Waksman, S. A.: *Grisein, an antibiotic produced by certain strains of Streptomyces griseus.* J. Bact. 55 : 739, 1948.

Řeháček, Z.: *Růst a produkční schopnost antifungální aktinomycety 943 v půdních vzorcích.* Čs. mikrobiol. 3 : 54, 1958.

Řeháček, Z.: *Isolation of actinomycetes and estimation of their content in soil.* Mikrobiologiya 28 : 236, 1959a.

Řeháček, Z.: *Stanovení prvního protimikrobního spektra aktinomycet.* Rostl. výroba 5 : 373, 1959b.

Ševčík, V., Podojil, M.: *Orientierende Bestimmung des Ionencharakters der Antibiotika mit Hilfe von Plattentesten.* Naturwissenschaften 44 : 93, 1957.

Ševčík, V., Podojil, M., Vrtišková, A.: *Použití papírové chromatografie při výzkumu nových antibiotik.* Čs. mikrobiol. 2 : 175, 1957.

Vaněk, Z., Doležilová, L., Řeháček, Z.: *Formation of a mixture of antibiotic substances including antibiotics of a polyene character by strains of actinomycetes freshly isolated from soil samples.* J. gen. Microbiol. 18 : 649, 1958.

Waksman, S. A., Lechevalier, H.: *Guide to the classification and identification of the actinomycetes and their antibiotics.* Baillière, Tindall and Cox, Baltimore 1953.

АНТАГОНИСТИЧЕСКИЕ СВОЙСТВА И ВЗАИМООТНОШЕНИЯ НЕКОТОРЫХ АКТИНОМИЦЕТОВ

З. Ржегачек, Л. Долежилова, З. Ванек

В группе 93 свежевыделенных антагонистических актиномицетов исследовались кроме таксономии штаммов их антибиотические свойства, характер продуцируемых ими антибиотиков и взаимоотношения между отдельными. штаммами. Многочисленные таксономически сходные штаммы серии albus и helvolus образовали антибиотики различного характера. Количественная группировка продуцентов отдельных типов антибиотиков оказалась различной не только в обеих таксономических сериях, но и в некоторых их подгруппах. Что касается их взаимноотношений, наиболее энергичными антагонистами и в то же время наиболее чувствительными микробами оказались штаммы, продуцирующие антибиотики типа стрептотрицина, стрептомицина и неомицина. Штаммы с полиеновыми антибиотиками оказались весьма устойчивыми. Питательная среда различным образом влияла на эти взаимоотнощения. Для 8 штаммов было доказано наличие угнетения собственных культур и его зависимость от питательной среды и продолжительности культивации. Штаммы, проявлявшие автоингибицию, угнетались таксономически связанными с ними (когерентными) штаммами, которые образовали антибиотики, сходные в групповом отношении с их собственными антибиотиками.