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(From the Department of Antibiotics, Institute of Experimental Medicine of the Hungarian Academy of Sciences, Debrecen, Hungary)

Group-wise growth of Streptomyces in a medium containing streptomycin

By

T. VÁLYI-NAGY and G. SZABÓ

Wit 3 Figures in the text

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In our previous investigations (SZABÓ and VÁLYI-NAGY 1956) we examined the development of resistance of *Streptomyces griseus* against streptomycin. In the course of these experiments we often noticed on Petri-dishes containing streptomycin that the *Str. griseus* colonies were not uniformly distributed over the agar surface, but were grouped around certain centres, single colonies rarely appearing between the groups (Fig. 1). This phenomenon is caused by a substance (or substances) produced by germinating *Str. griseus* spores. In this report we summarize our results about the properties of the substance and the experiments standardizing this phenomenon.

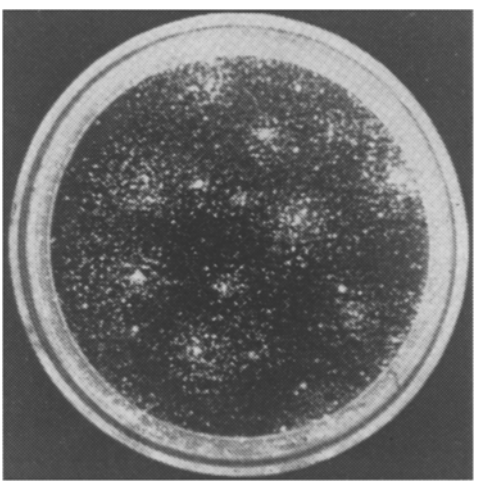

Fig. 1.
Group-wise growth. *Streptomyces* colonies grouping around certain centres on agar media containing streptomycin

Materials and methods

Throughout the present experiments two strains of *Str. griseus* used in the industry were applied, other strains were isolated from natural sources. The solid media used for cultures had the following composition: potato extract prepared by boiling with tap-water, 900 ml.; liquid yeast extract 100 ml.; $Na_2HPO_4 \cdot 12\,H_2O$ 10 g.; KH_2PO_4 1 g.; agar—agar 20 g.; adjusted to p_H 7.5; sterilized at a pressure of 1.5 atm. for 20 minutes in autoclave. After sterilization the p_H value was readjusted to p_H 7.5.

Streptomycin sulphate containing 600 (later 800) μ/mg. was used. Spore suspensions for inoculations were always freshly prepared by flooding 5—10 days old *Str. griseus* cultures grown on agar slants, with a 0.9% NaCl solution. If needed the

number of spores in suspension was counted in a haematocytometer. Previous tests indicated that between 5—10 days, the age of the spores does not influence resistance to streptomycin.

Tenfold dilutions of spore suspensions were made and volumes of 0.5—1.0 ml. of these dilutions were mixed with agar medium of 45° C. 10 or 20 ml. portions were used to pour out plates in Petri-dishes. Experiments were carried out at least in triplicate. Results were read in 2—3—4 days after inoculation and incubation at 27° C.

In experiments to produce the phenomenon mentioned (Fig. 1), the medium was melted and cooled to 45° C. before the appropriate spore suspensions and streptomycin were added to give a final concentration of 600 μ/ml. After the Petri-dishes were poured and solidified, agar blocks were cut out with a ten mm. diameter cork-borer from the center of the Petri-dishes. Into the holes we put spore suspensions mixed with agar medium at equal volumes. After some incubation time we got the following pictures (Fig. 2). This way we were always able to produce essentially the same phenomenon we had noticed before with a special *Str. griseus* strain. For certain reasons the method was modified later (see below).

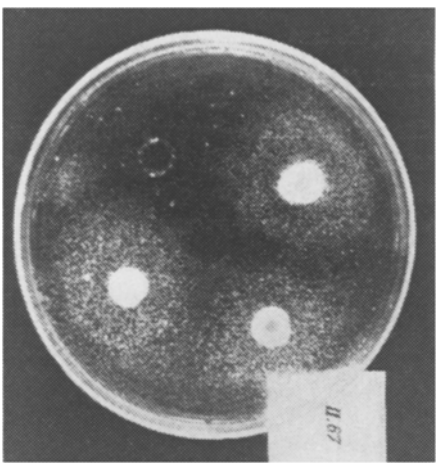

Fig. 2. Experiment II/67. The picture shows the group-wise growth phenomenon in a standardized form. Spores of a *Str. griseus* strain were mixed in agar medium containing streptomycin at inhibiting concentration. Into three holes was put any of our *Str. griseus* strain

Results

First of all we examined whether all *Streptomyces* strains could produce the phenomenon when placed in the central holes (see Fig. 2), that is, whether they were able to induce spores to grow out to visible colonies at inhibiting streptomycin concentrations. Out of 37 morphologically different *Streptomyces*, isolated from natural sources, only 3 strains gave positive results. Their classification and characterization requires further work.

All of our *Str. griseus* strains sensitive or resistant to streptomycin at proper spore number, could bring about the phenomenon. We also examined whether all kinds of *Str. griseus* spores were able to grow in media containing 600 μ/ml. streptomycin and a germinating spore suspension in the center of the plate. At 600 μ/ml. streptomycin concentration our common *Str. griseus* strains did not reveal increased resistance in the neighbourhood of the central, growing culture. If, however, we determine the inhibiting streptomycin concentration for the *Str. griseus* strains (Table 1) and perform an experiment at a streptomycin concentration which is just above the inhibiting concentration (in Table 1 e.g.

50 μ/ml.) we are always able to reproduce the phenomenon: spores are growing around a *Str. griseus* culture at streptomycin-concentrations otherwise inhibiting.

Does this colony destroy the streptomycin in its neighbourhood? We poured special flat-bottomed Petri-dishes with agar media containing streptomycin. One series of them was inoculated with *Str. griseus* spores at the center as we always did in experiments when we produced the phenomenon mentioned. The other series was likewise incubated and served as a control. After 2—3 days we cut out agar-blocks of equal size placed the blocks on media[1] inoculatd with *Bac. subtilis* and determined their streptomycin concentration in the same way as if streptomycin solutions were concerned. As it is to be seen in Table 2, the *Str. griseus* colony did not excrete substance(s) which adsorb, inactivate or destroy the streptomycin in its neighbourhood incorporated in the media.

Table 1. *Colony count of Streptomyces griseus inoculated with the same spore number at increasing streptomycin concentrations*

Streptomycin concentration μ/ml.	Colony count/Petri-dish	Mean of colony count
0	47	44.5
	42	
20	23	22.5
	22	
50	∅	∅
	∅	
100	∅	∅
	∅	

The *Str. griseus* colony in the medium alters the p_H, but this also could not be the cause of the above mentioned increased streptomycin toleration, because buffers of different p_H were able to evoke such a phenomenon only if they consisted of ions inhibiting streptomycin (Table 3a, b). The substance could not be cristalloid and diffusible for the cultivation of *Str. griseus* spores in cellophane sacs gave negative results.

The *Str. griseus* colony produced the substance (s) only in media containing streptomycin. In liquid culture we were not able to demonstrate the presence of the substance, while the *Str. griseus* colony under the influence of streptomycin readily secretes the substance into agar-media. We could demonstrate this in the following way. We poured agar media or pure agar in distilled water. At the center we cut out blocks and in the place of them was put into Petri-dishes spore suspension in agar-medium containing streptomycin. From the neighbourhood of these *Str. griseus* colonies we cut out agar-blocks from time to time and placed them on so-called indicator plates, Petri-dishes. Indicator plates were produced of *Str. griseus* spores and streptomycin in agar-media in a way that a growing *Str. griseus* colony at their center was able to evoke increased streptomycin toleration of spores around the growing culture and gave a

[1] This medium had the following composition: Broth with 0.5% pepton and 1.8% agar-agar, adjusted to p_H 8.0.

picture shown by Fig. 2. In positive cases the *Str. griseus* colonies appeared around the blocks placed on these indicator plates earlier and in much greater number than in other places. With the help of this method

Table 2. *Change of the streptomycin concentration in agar blocks mixed previously into the agar medium in the neighbourhood of a growing Streptomyces griseus colony*

Diameter of inhibition zone in mm. produced by agar blocks from Petri-dishes without a growing *Streptomyces griseus* colony		Diameter of inhibition zone in mm. produced by agar blocks from Petri-dishes with growing *Streptomyces griseus* colony	
Number of Petri-dish		Number of Petri-dish	
1	2	1	2
22.5	22.1	22.0	22.0
22.1	21.8	21.5	22.5
22.2	22.2	21.5	23.0
22.5	22.1	22.0	22.2
22.8	22.0	22.2	22.0
22.5	22.1	22.0	22.0
21.5	22.1	22.1	22.0
23.0	22.4	22.0	23.0
22.0	22.5	22.0	22.2
22.1	22.2	22.0	23.3
22.1	22.4		22.5
			22.0
Mean: 22.32	**Mean: 22.18**	**Mean: 21.94**	**Mean: 22.42**

Table 3. *Capability of buffers to produce group-wise growth*

a) Universal buffer[1]		b) M/15 phosphate buffer	
pH	Measure of group-wise growth	pH	Measure of group-wise growth
1.5	++++	5.0	∅
2.0	++++	5.5	∅
2.5	++++	6.0	∅
3.5	+++	6.5	∅
4.5	+++	7.0	∅
5.5	++	7.5	∅
6.5	++	8.0	∅
7.5	+	Washed agar-agar	∅
8.5	+		
9.5	+		
10.0	∅		
10.5	∅		
11.0	∅		

[1] Universal buffer consisted of: 0.05 m oxalic acid, 0.2 m boric acid, 0.05 m succinic acid, 0.05 m natrium sulphate, 0.05 m borax, 0.05 m natrium carbonate.

we could demonstrate that the substance was produced by the *Str. griseus* colony in from 2 to 24 hours after inoculation. Before or after this time no effect was observed. (See the scheme of such an experiment in Fig. 3.)

Following the principles of this method we made many modifications. All of them indicated the importance of the solid agar environment around the colony. If a liquid phase was separated by an agar layer from the *Str. griseus* colony in media containing streptomycin we could demonstrate the substance in the liquid phase and thus we were enabled to

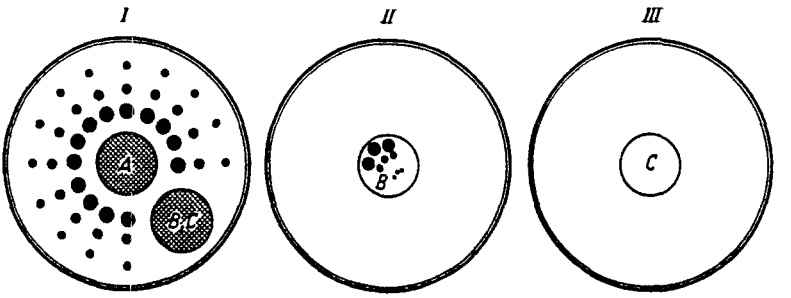

Fig. 3. Diagrammatic illustration of an experiment. The Petri-dish I. is called "indicator plate", the method of preparing being described on p. 283—284. On this, the growing colony of *Str. griseus* is put into the center (A). After 48—72 hours around "A" colonies emerge on the indicator plate (represented by black spots). After at least 2, last 24 hours, block "B" is cut out and is put on a simple agar-plate, II. In this block, contemporaneously with plate I, colonies of approximately equal size appear. Blocks cut out from indicator plate III—that is not provided with a *Str. griseus* colony (A)—put in place of "B" at least 24 hours after starting the experiment, do not display any growth

separate the active substance from the mycelia. Otherwise it was not possible to separate the substance responsible for the group-wise growth from the germinating spores. The attempts of boiling it 10 minutes in a water bath, killing the spores with uv. irradiation or filtration through asbestos or glass filters, did not result in separation. The above mentioned agar-layer around the *Str. griseus* colony is indispensable to separate the active substance from the *Streptomyces* culture.

In order to investigate the nature of the substance we mixed certain enzymes to the system. The tested enzymes were: cristalline ribonuclease (commercial), desoxyribonuclease (commercial), trypsin (G. Grübler et Co., or E. Merck), pancreatin, amylase (commercial). The first two had 250 μg./ml., the others 500 μg./ml. end concentrations. These enzymes did not influence the group-wise growth around the *Streptomyces* culture.

We have examined some enzyme-poisons for an influence on the production of the substance. After determining the concentration which did not inhibit by itself the growth of the spores we employed cyanide,

fluoride and azide of 1—5, 10 respectively 0.01 μg/ml concentrations. No effect was observed, much more colonies grew around the central colony than on other parts of the plate.

Discussion

From the experiments described we conclude that the ability of *Str. griseus* to grow in the presence of streptomycin is, in part, connected with the interaction of microcolonies arising from germinating spores. This phenomenon is probably due to the action of metabolites of colonies in a primary stage of growth or to autolytic substances of individuals killed by the toxic effects of streptomycin. The agent responsible for the inreased streptomycin toleration is probably a high molecular thermosensitive substance (or substances) of specific effect. It does not seem probable that any of the substance known from the literature (GREEN and WAKSMAN, 1948; GREEN, IVERSEN and WAKSMAN, 1948; BERKMAN, HENRY and HOUSEWRIGTH, 1947; DONOVICK et al., 1948; LIGHTBOWN, 1950; BERGMAN et al., 1954) as agents inhibiting the action of streptomycin in a non specific way should be responsible for the phenomenon described in the present paper. Several tests made with substances mentioned in the literature were negative.

The phenomenon reminds of the well known satellite growth, and this was also observed by DEAN and HINSHELWOOD (1952) with *Bacterium lactis aerogenes*. But while they demonstrated the phenomenon only occasionaly as a fact of interest, we could reproduce and define the desired circumstances. The strainspecific substance did not alter the streptomycin concentration but made the *Str. griseus* spores themselves more tolerant against increasing streptomycin concentrations. According to our observations this property is not stable, and is not inheritable. It is as yet not possible to decide whether this phenomenon plays any role in developing resistance under natural circumstances.

The production, concentration, purification and investigation of the substance will be undertaken.

Summary

We have described the observation that *Streptomyces griseus* colonies grow group-wise on agar media containing streptomycin. We have found that this phenomenon is due to a substance (s) produced by germinating *Str. griseus* spores in media containing streptomycin, and this substance made the neighbouring spores more tolerant to increasing streptomycin concentrations. The substance is produced specifically by *Str. griseus* strains. The substance has probably a great molecular size,

is thermolabile, not a nucleic acid and the applied enzymes did not inactivate it. Some investigated enzyme-poisons did not influence either its production or its effect on *Str. griseus* spores. We succeeded in carrying over the substance into liquid phase and separate it from the producing culture and this enables us to further purification and investigation of the substance.

Literature

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