Variations in Strains of *Streptomyces griseus* Isolated from a Degenerating Streptomycin-Producing Culture

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Abstract

Nomi, Ryosaku (Rutgers, The State University, New Brunswick, N.J.). Variations in strains of *Streptomyces griseus* isolated from a degenerating streptomycin-producing culture. Appl. Microbiol. **11**:84–89. 1963.—Streptomycin-resistant strains were isolated from a degenerated streptomycin-producing culture of *Streptomyces griseus*. From 250 resistant strains, 3 low, 2 intermediate, and 2 high potency strains were selected; these were compared in their morphological, cultural, physiological, and streptomycin-producing properties. Though no definite correlation between streptomycin production and the other properties could be obtained, the following correlations were considered as distinct differences among the low, intermediate, and high potency strains. (i) When streptomycin-producing ability degenerates, more submerged spore formation or fragmentation of mycelium into shorter filaments appears to occur. (ii) On agar medium, low and intermediate potency strains often show finely wrinkled growth; high potency strains do not show such characteristics. (iii) High potency strains excrete a distinct yellow soluble pigment on synthetic agar medium and on glucose-yeast extract agar, but low and intermediate potency strains show little or no ability to form this soluble pigment. (iv) In low and intermediate potency strains, inositol and arginine did not stimulate streptomycin production as they did in high potency strains. Streptamine showed some stimulating effect in the high potency strains and, in contrast, a depressive effect in intermediate potency strains, though streptidine showed a distinctly stimulating effect in all groups of strains employed.

When streptomycin-producing strains of *Streptomyces griseus* are transferred serially on agar slants or in liquid media, the ability of the cultures to produce streptomycin often decreases. Waksman, Reilly, and Johnstone (1946) suggested that streptomycin-enriched medium be utilized for obtaining active strains from degenerated cultures of this organism. They also pointed out that streptomycin-enriched media would allow the growth not only of active strains but also some inactive strains, although the tendency is to eliminate many of the inactive strains.

Szabó, Barabàs, and Vályi-Nagy (1961) compared three nonstreptomycin-producing mutants with the parent strain and found that the former had a shorter life cycle in submerged culture than the latter. Alikhanian and Teterjatnik (1962) reported that a nonstreptomycin-producing mutant could elaborate streptomycin from the fermented fluid of the high-producing parent strain.

To examine the natural variation or the degeneration of various properties of *S. griseus* associated with streptomycin production, the author isolated various potency strains of streptomycin-producing cultures grown on streptomycin-enriched plate cultures; from these streptomycin-resistant strains, seven were selected and compared as to their morphological, cultural, physiological, and streptomycin-producing characteristics. The seven strains were grouped as low potency, intermediate potency, and high potency groups.

Materials and Methods

The culture used in these experiments was *S. griseus* Z-38 (Dulaney, 1951), which originally formed white spores and produced about 2,000 units of streptomycin per ml under optimal conditions. This culture underwent considerable variation; it formed a pale-yellowish to greenish-gray aerial mycelium and usually produced 100 to 300 units of streptomycin per ml. This culture was inoculated in 40 ml of glucose-yeast extract medium, which contained 1% glucose, 1% yeast extract, 0.5% NaCl, 0.025% $MgSO_4 \cdot 7H_2O$, and 0.001% $FeSO_4 \cdot 7H_2O$ (LePage and Campbell, 1946), in a 250-ml Erlenmeyer flask and cultured at 28 C for 7 days on a reciprocating shaker. The broth was then filtrated through sterilized filter paper and diluted with sterile water; 0.15 ml of the diluted suspension, containing about 10 to 11 million spores, was spread on the surface of streptomycin-enriched glucose-yeast extract agar plate and cultured at 28 C. Three pairs of plates were used, two of each containing 3,000, 5,000, or 10,000 units of dihydrostreptomycin per ml; they gave about 200, 100, or 10 colonies per plate for each of the above streptomycin concentrations, respectively. A total of 250 colonies were picked from six plates and transferred to slant cultures. Among the isolated colony strains, 29 produced 800 to 1,000 units of streptomycin per ml and 6 produced only 4 to 100 units of streptomycin per ml in a glucose-Soytone-yeast extract medium, which consisted of 2.5% glucose, 2% Soytone (Difco), 1% yeast

extract, 0.5% sodium chloride, 0.025% magnesium sulfate, and 0.001% ferrous sulfate (pH 7.0). From the isolated strains, isolates 107 and 210 were selected and used as high potency strains, isolates 51 and 206 as intermediate potency strains, and isolates 228, 10, and 47-2 as low potency strains (Table 1).

These strains were examined for their morphological, physiological, and streptomycin-producing properties.

Inoculum for surface cultures was suspension in sterile water of spores or mycelia which were harvested from slant cultures of glucose-yeast extract agar grown at 28 C for 14 days. Inoculum for the shaking culture was prepared as follows. Three loopfuls of spores or mycelia were inoculated in 40 ml of glucose-yeast extract medium in a 250-ml Erlenmeyer flask and cultured on a reciprocating shaker at 28 C for 2 days. Every streptomycin-producing medium was inoculated with 0.5 ml of this broth and cultured at 28 C under a reciprocating shaking condition of 98 or 110 strokes per min in a 10-cm distance.

In streptomycin production, glucose-yeast extract medium was used as basal medium which was supplemented with various substances.

RESULTS

Cultural and physiological characteristics. The nine strains isolated belonging to three groups and the parent strain were examined for their cultural and physiological characteristics. Although there were some fluctuations in some properties among the strains in each group, distinct differences were shown between the different groups. The characteristics of each group are summarized in Tables 2 and 3. From these tables, the following differences may be considered as distinct. (i) The low potency strains formed better aerial mycelium in synthetic media than did the strains of the other groups. Intermediate potency strains formed no aerial mycelium, except on starch agar. (ii) Low and intermediate potency strains often produced finely wrinkled growth; high-potency strains did not show such characteristics. (iii) High potency strains excreted a yellow soluble pigment on slant cultures of synthetic agar and on plate cultures of glucose-yeast extract agar, but low and intermediate potency strains showed little or no ability to form this soluble pigment.

(iv) Peptonization of protein, nitrate reduction, and dissolution of calcium malate were far weaker in intermediate potency strains, compared with the strong reaction of the strains belonging to the other groups.

(v) The three groups showed almost the same characteristics in starch hydrolysis and chromogenic property, although gelatin stab cultures of the high potency strains showed a little difference from the strains of the other groups in the excretion of a somewhat dark soluble pigment.

(vi) When streptomycin-producing ability degenerated, more submerged spore formation appeared to occur in submerged culture, or the submerged mycelium appeared to break up into shorter fragments.

(vii) No distinct differences were observed among three groups in the carbohydrate-utilization spectrum, although in the case of some carbohydrates there could be seen slight differences among the different strains. The low potency strains formed better aerial mycelium on several carbon sources than the strains of the other two groups.

TABLE 1. *Streptomycin production by various strains of Streptomyces griseus*

Strain no.	Group: potency	Streptomycin produced	
		Glucose-Soytone-yeast extract medium	Glucose-yeast extract medium
		µg/ml	*µg/ml*
228	Low	8–43	3–4
10	Low	8–58	2–9
47-2	Low	10–87	8–45
51	Intermediate	31–87	45–134
206	Intermediate	40–118	44–155
107	High	825–1,066	210–350
210	High	824–960	224–340
Z 38	Original culture	100–280	100–240

Microscopic morphology of aerial mycelium. The strains belonging to low and high potency groups showed cluster formation, but no spirals. These represent the typical morphology of *S. griseus*. However, in old cultures of the low potency strains, the terminal hyphae were sometimes considerably flexous and could easily be mistaken as spiral forms under low microscopic magnifications. The low potency strains sometimes formed coremium of various sizes. In the intermediate potency strains, aerial mycelium was not formed in almost all culture media used, except plate cultures of starch agar, on which these strains showed the typical cluster forms.

Comparison of streptomycin production. Since it was open to question that, in addition to streptomycin, other antibiotics might be produced by the low and intermediate potency strains, streptomycin-resistant *Escherichia coli* IMRU 103 and a cation-exchange resin, Amberlite IRC 50 (Doery, Mason, and Weiss, 1950) were used. The antibiotics thus produced by these strains could not inhibit streptomycin-resistant *E. coli*, and were adsorbed completely to Amberlite IRC 50 and then eluted by hydrochloric acid. The question appeared to be unnecessary.

In the production of streptomycin, arginine and inositol were found to exert a stimulating effect (Saunders, 1950; Severina, Gorskaya, and Gracheva, 1959; Egorov, 1959; Galanina and Agatov, 1959; Majumdar and Kutzner, 1962*a*). Majumdar and Kutzner (1962*a*) reported that these compounds stimulated streptomycin production of the same culture used by me as the original culture. I examined the effect of these two compounds on all strains employed (experiment 1, Table 4). In the high potency group, both arginine and inositol showed good stimulating effect, which agreed with the results presented by Majumdar and Kutzner (1962*a*). However, in the intermediate

potency group, arginine seemed to have a depressive effect, and inositol showed almost no effect. In the low potency group, streptomycin production was too low to draw any conclusion concerning the effect of supplemented substances, although, in no. 47–2, arginine seemed to have some stimulating effect. It is clear that there are distinct differences in the utilization of arginine and inositol between three groups of strains derived from the same parent culture.

Oxygen tension in the medium is said to play an im-

TABLE 2. *Cultural and physiological characteristics of Streptomyces griseus isolates**

Determination	Low potency group	Intermediate potency group	High potency group	Original culture
Glucose-nitrate agar	(A) W to sGrG; (R) pY to dOY; (P) n to sdY on late stage	(A) n; (V) dY to B, wrinkled; (P) sY in early stage to OY in late stage	(A) s, W; (R) Y to YO; (P) distinctly vivid Y → O	Same with high potency group, but excretion of (P) delays
Glucose-asparagine agar	(A) W to sYGr; (R) C to dpY to Y; (P) n	(A) n; (V) C to dpY; (P) n	(A) s, W; (R) Y; (P) Y	(A) n; (V) C to sdY; (P) n
Glucose-yeast extract agar	(A) pYGr, wrinkled; (R) DYB to DB; (P) almost n to sDY or sDB	(A) n; (V) dY → B, wrinkled; (P) almost n to sDY or sDB	(A) pYGr; (R) YB to B; (P) sdB	(A) pYGr; (R) B; (P) almost n to sDY
Soybean meal agar	(A) pYGr to pGr, wrinkled; (R) Y → B	(A) n; (V) pYB to lB, wrinkled; (R) same with (V)	(A) GGr to lGr or GrW; (R) Y → B	(A) GGr to YGr; (R) Y → B
Glucose-nutrient agar	(A) s, W, wrinkled; (R) OY; (P) n	(A) n; (V) dpY to O, wrinkled; (P) O	(A) W; (R) Y; (P) sY	(A) s, W; (R) Y; (P) Y
Glucose-yeast extract agar plate	(A) GrW to Gr to GGr, wrinkled; (R) dY to BD; (P) n	(A) n; (V) C to B, wrinkled; (P) n	(A) pYGr; (R) Y to DB; (P) Y	
Gelatin stab	Liquefied; (P) OY; more surface growth than other groups	Slower liquefaction than other groups; (P) OY	Liquefied; (P) DY to lDB	Same with high potency group
Litmus milk	Fairly rapid peptonization becoming alkaline; no coagulation	Slow growth, slow peptonization, and slow alkalifying; no coagulation	Rapid peptonization becoming alkaline; no coagulation	Same with high potency group
Starch	Hydrolyzed	Hydrolyzed	Hydrolyzed	
Calcium malate agar plate	(A) GrW to pGGr to GGr; (R) Y → DB; (P) Y; Ca-malate is dissolved	(A) n to s, W; (V) YB to B to partially YG; (P) n to sYB; Ca-malate is dissolved very slowly and rather in old stage of growth	(A) pGGr to GGr. (R) dY → DB; (P) distinct Y; Ca-malate is dissolved	Same with high potency group
Chromogenicity	Not chromogenic	Not chromogenic	Not chromogenic	Not chromogenic
Nitrate reduction	Nitrite produced	Nitrite weakly produced†	Nitrite produced	Nitrite produced
Microscopic observation of broth at end of fermentation	Numerous spores and relatively few fragmented mycelia which are short and single to short and simply branched	Many fragmented mycelia, which are short and single to fairly longer and more branched than low potency group; almost no spores	Many long and highly branched mycelia; relatively few spores	Many fragmented mycelia, which are short to relatively long and often branched; many spores

* Abbreviations: (A) aerial mycelium; (V) vegetative mycelium; (R) reverse side of colony; (P) soluble pigment; B = brown or brownish; C = colorless; D = dark; G = green or greenish; Gr = gray or grayish; O = orange or orangish; W = white; Y = yellow or yellowish; d = dull; l = light; n = none; p = pale; s = slight.

† In test tube, growth occurred in bottom. Shaking culture was simultaneously used.

portant role in streptomycin production. Experiment 1 (Table 4) was carried out under shaking conditions of 98 strokes per min. When 110 strokes per min were applied to the cultures in the intermediate and high potency groups, a distinct improvement in the total production of streptomycin occurred in the former; however, arginine and inositol did not show any stimulating effect as compared with that observed in the high potency group (experiment 2, Table 4).

Majumdar and Kutzner (1962*b*), using radioisotopes, reported that inositol or its metabolic products may be involved in streptidine biosynthesis. On this assumption, it was supposed that the intermediate potency group might have some metabolic block in the biosynthetic pathway from inositol to streptidine. If it were true, then streptidine should have a stimulatory effect on streptomycin production. Streptidine sulfate was prepared from dihydrostreptomycin sulfate by the method of Hunter, Herbert, and Hockenhull (1954), and used to supplement the basal medium (Table 5). Streptidine had a distinct stimulatory effect on all groups employed, although the total production by the low potency group was still low. The quantity of streptidine employed (0.56 g/liter) corresponded to about one-half of the inositol employed above. The streptidine experiment was repeated, using concentrations of 0.5 and 1 g/liter. A concentration of streptidine equal to that of inositol was not suitable, but rather a lower concentration of streptidine was needed to stimulate streptomycin production, not only in the intermediate-potency group but also in the high potency group (experiment 2, Table 5).

The fact that streptomycin production by the intermediate potency group was stimulated by streptidine, but not by inositol and arginine, seems to indicate that the intermediate potency group has a metabolic block in the biosynthetic pathway from inositol to streptidine. Streptamine and strepturea may be considered as probable intermediates on this pathway, although Hunter (1956) concluded that streptamine did not appear to be on a direct pathway from glucose to streptomycin, and that supplemented streptamine was incorporated to a greater extent into *N*-methyl-L-glucosamine than into streptidine. Streptamine sulfate and strepturea were prepared from streptidine sulfate by the method of Peck et al. (1946) and were added to the basal medium. In the intermediate potency group, the effect of streptamine was not stimulative but rather depressive; the combination of streptamine with arginine did not improve the production of streptomycin (Table 6). In the high potency group, streptamine showed stimulatory effect but not a distinct one. However, in the latter the combination of arginine with streptamine showed less production than streptamine

TABLE 3. *Utilization of carbohydrates**

Carbohydrate	Strain no. 228	10	47-2	51	206	107	210	Z 38
Arabinose	−	+	+	−−±	−−±	−−±	−−±	−−±
d(+)-Xylose	+	+	++	±	±−+	+	+	+
d(−)-Xylose	−	−	−	−	−	−	−	−
Rhamnose	−	−	−	−	−	−	−	−
Glucose	++	++	++	+	+	+	+	+
Galactose	+	++	++	+	+	+	+	+
Fructose	++	++	++	±−+	±−+	+	+	+
Mannose	++	++	++	+	+	+	+	+
Mannitol	+	++	++	+	+	+	+	+
Sorbitol	−	−	−−±	−−±	−−±	−	−	−
Cellobiose	+	++	++	+	+	+	+	+
Maltose	++	+	++	+	+	+	+	+
Sucrose	−	−	−	−	−	−	−	−
Lactose	++	++	++	+	+	+	+	+
Salicin	+	+	+	+	+	+	+	+
Raffinose	−	−	−	−	−	−	−	−
Inulin	−	−	−	−	−	−	−	−
Dextrin	++	++	++	+	+	+	+	+
Myo-inositol	−	−	−	−	−	−	−	−

* Symbols: − = no growth; ± = no aerial mycelium and growth restricted; + = good growth but no aerial mycelium to moderate aerial mycelium; ++ = good growth and good aerial mycelium.

TABLE 4. *Effect of arginine and inositol on streptomycin production*

Expt no.	Strain no.	Supplement* Control	Arginine†	Inositol‡	Arginine + inositol
1 (98 rev/min)	228	3	3	3	4
	10	6	6	8	7
	47-2	9	14	9	11
	51	45	35	44	38
	206	44	37	42	44
	107	260	344	332	448
	210	332	356	356	496
2 (110 rev/min)	51	120	102	102	105
	206	105	108	117	112
	107	210	256	384	410
	210	248	290	396	452
	Z 38	216	272	330	396

* Results expressed as μg/ml of streptomycin produced.
† At 0.2 g per liter.
‡ At 0.5 g per liter.

TABLE 5. *Effect of streptidine sulfate on streptomycin production**

Strain no.	Expt 1 Control	Expt 1 Streptidine (0.56 g/liter)	Expt 2 Control	Expt 2 Streptidine (0.5 g/liter)	Expt 2 Streptidine (1.0 g/liter)
228	4	6			
10	3	5			
47-2	5	26			
51	134	204	114	396	114
206	155	280	86	180	102
107	300	644	264	564	300
210	320	670	320	644	50
Z 38			240	546	272

* Results expressed as μg/ml of streptomycin produced.

alone. This is also true in the case of the intermediate potency groups. On the other hand, strepturea showed no definite effect in either group. These results do not seem to support the possibility of coupling of streptamine with arginine, or formation of strepturea as the possible intermediate on the biosynthetic pathway from inositol to streptidine.

Although no positive information concerning the biosynthetic pathway of streptomycin in the above experiment was obtained, the three groups of strains seem to possess some distinct physiological properties from the point of streptomycin production; the low and intermediate potency groups seem to be mutants derived from the high potency group. There remains the question as to the reason which allowed the low potency strains to grow on highly enriched streptomycin medium when they were isolated. The basal medium was enriched with 100 μg of streptomycin per ml and inoculated with the low and high potency groups. The growth of each strain did not seem to be macroscopically affected by streptomycin enrichment. The low potency group seemed to inactivate a part of the added streptomycin, and the high potency group did not (Table 7). This phenomenon does not entirely explain the streptomycin tolerance of the low potency group, but is considered to be one of the reasons which allowed the strains to grow in highly enriched streptomycin medium.

TABLE 6. *Effect of streptamine sulfate and strepturea on streptomycin production**

Strain no.	Control	Streptamine (0.38 g/liter)	Streptamine (0.77 g/liter)	Streptamine (0.38 g/liter) + arginine (0.2 g/liter)	Streptamine (0.77 g/liter) + arginine (0.4 g/liter)	Strepturea (0.37 g/liter)	Strepturea (0.73 g/liter)
51	114	102	66	94	56	232	180
206	86	45	17	27	10	72	82
107	264	300	272	232	175	239	239
210	320	360	350	330	248	363	353
Z 38	240	290	272	240	210	192	185

* Results expressed as μg/ml of streptomycin produced.

TABLE 7. *Effect of streptomycin-enriched media on streptomycin production*

Strain no.	Days				
	1	2	3	4	5
No inoculation	85/0*	86/0	88/0	90/0	92/0
228	67/2	62/5	59/4	60/4	53/3
10	84/2	75/2	67/2	58/2	53/2
47-2	92/8	92/8	80/8	74/8	75/8
107	170/75	320/190	330/240	360/210	300/216
210	180/90	320/204	330/224	330/204	310/186
Z 38	175/87	310/224	310/210	276/198	256/186

* First figure represents streptomycin produced in media enriched with 100 μg/ml of streptomycin; second figure represents streptomycin produced in media without enrichment. A part of streptomycin enriched in glucose-yeast extract medium appeared to be inactivated by some ingredient in the medium, because streptomycin content of the enriched and noninoculated medium was a little less than the supplemented one. Results expressed in μg/ml.

DISCUSSION

When three groups of strains isolated from a potent streptomycin-producing culture of *S. griseus* were compared in their morphological, cultural, physiological, and streptomycin-producing properties, several differences could be observed. Each of these is not considered to be so significant as to justify any change in species name. The results of this experiment must be taken into consideration to show the variability inherent in *S. griseus*.

The three groups of strains produced different amounts of streptomycin. Unfortunately, there was no decisive correlation from a taxonomic point of view between the examined properties and streptomycin production. However, it is important to consider the fact that the yellow soluble pigment produced by high potency strains is not produced or becomes considerably weaker in the low and intermediate potency strains. The chemical characteristics of this compound are still unknown, so that speculation of the relationship between the yellow pigment and streptomycin production is difficult. It remains to be determined whether the results obtained with the culture of *S. griseus* Z 38 and the corresponding subcultures concerning yellow pigment are also applicable to other streptomycin-producing cultures.

In the intermediate potency strains, peptonization of protein, nitrate reduction, and dissolution of calcium malate were weaker than in the high potency strains. However, as the low potency strains had almost the same abilities as the high potency strains in those characteristics, speculation concerning the relationship of those characteristics to streptomycin production is not possible.

The microscopic morphology of submerged cultures seems to have some significance. The submerged spore formation, or the fragmentation of the mycelium into shorter elements, seems to be inversely proportional to streptomycin production. Similar results have been reported by Williams and McCoy (1953), Schatz and Waksman (1945), and Carvajal (1947). It will be very interesting to determine what physiological significance spore formation or mycelium fragmentation has.

The disappearance of aerial mycelia has been said to be accompanied by depressed streptomycin production (Waksman et al., 1946; Williams and McCoy, 1953; Schatz and Waksman, 1945). Also, in this experiment, the intermediate potency strains, which did not form aerial mycelia, showed depressed streptomycin production. On the other hand, it was clear from this experiment that the depression of streptomycin production does not always appear together with deterioration of aerial mycelia. The low potency strains sometimes formed better aerial mycelia

than the high potency strains. However, the development of aerial mycelia can be an important criterion for the maintenance of streptomycin-producing ability, because the high potency descendant strains always show almost the same aerial mycelia formation as the original high potency strain after serial transfers.

Inositol, arginine, streptidine, streptamine, and strepturea were examined as to their effect on streptomycin production. The results showed distinct physiological differences between three groups of strains used. It is important that the degeneration of streptomycin-producing ability accompanied the degeneration of ability to utilize inositol and arginine for streptomycin production; the degenerated strains still could elaborate streptomycin. This seems to indicate that there is a biosynthetic pathway from glucose to streptomycin other than through inositol. Thus, inositol appears to participate in the biosynthetic pathway of streptomycin from glucose through some metabolic steps which probably exclude formation of streptamine or strepturea in free form.

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