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# Regulation of Biosynthesis of Pesticidal Metabolic Complexes in *Streptomyces griseus*

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ABSTRACT. The complex of pesticidal metabolites produced by *Streptomyces griseus* LKS-1 consists of a peptide antibiotic (A), nonactic acids (B), macrotetrolides (C), pyrrolizines (D), and of cycloheximide. The latter unwanted phytotoxic compound was eliminated by treatment with mutagens. Combined approaches, including both genetic and physiological manipulations, resulted in the following alterations in the biosynthetic capacity: (*1*) A more than 80-fold increase in the production of C under a substantial decrease in the yields of A, B and D, the ratio of the components of C being steered toward the required more active ones; (*2*) a more than 300-fold increase in the production of B under suppression of the formation of A and C; (*3*) a 10-fold increase in the yields of D under suppression of A and C; (*4*) a significant increase in the yields of A with eliminating B, C and D. The level of inorganic phosphate in fermentation media and the sensitivity of the organism to carbon catabolite repression were important factors participating in the regulation of the above biosynthetic processes.

The diverse biological activity of *Streptomyces griseus* LKS-1 (Blumauerová *et al.* 1989) results from combined action of four groups of metabolites (Table I) co-produced with cycloheximide. To increase the yield of the insecticidal complexes **B** (nonactic acids), **C** (macrotetrolides) and **D** (pyrrolizines) with simultaneously suppressing the unwanted formation of the phytotoxic components (peptide antibiotic **A** and cycloheximide), the production organism was subjected to both genetic and physiological manipulations, the results of which are reported in the present communication. On the basis of our previous findings on the biosynthesis of **B** and **C** in *S. globisporus* (Blumauerová *et al.* 1987, and *unpublished data*), physiological studies were done by using three complex fermentation media containing different levels of inorganic phosphate; the sensitivity of *S. griseus* to glucose repression was also examined in a preliminary way. Since mutagenesis was not the main goal of this work but one of the means of attaining the objective, only some more relevant data on mutagenic experiments are included here. For greater clarity the individual groups of pesticides are discussed separately.

**Table I.** Pesticidal metabolites co-produced with cycloheximide in *S. griseus* LKS-1

| Group | Effect | | | | | References |
|---|---|---|---|---|---|---|
| | Anti-bacterial | Anti-fungal | Insecticidal | Acaricidal | Herbicidal | |
| A[a] | + | – | – | – | +[b] | |
| B[c] | – | – | + | – | – | Jizba *et al.* 1992*a*, 1993*b* |
| C[d] | + | + | + | + | – | Beran *et al.* 1988 |
| D[e] | – | – | + | – | – | Jizba *et al.* 1992*b* |

[a]Peptides; isolated by column chromatography on Sephadex G-50; *M* about 20 kg/mol (detailed structure not determined). **A** is stable only when bound to a protective component of the fermentation broth whereas under storing in a purified state its activity decreases (Přikrylová, *unpublished results*).

[b]In dicotyledonous plants, higher concentrations of **A** inhibit seed germination, lower concentrations cause a negative geotropism in seedlings (Blumauerová *et al.* 1989).

[c]Nonactic acids; biosynthetic precursors of **C** (*see below*). Major components: **B1**, nonactic acid; **B2**, homononactic acid. 2-Epihomononactic acid (**B3**) was identified as one of minor components.

[d]Macrotetrolides. **C1**, nonactin; **C2**, monactin; **C3**, dinactin; **C4**, trinactin. **C1** consists of **B1** only. The other components differ mutually in the increasing content of **B2** (Keller-Schierlein *et al.* 1967). In the strain LKS-1, tetranactin (**C5**) consisting only of **B2** (Ando *et al.* 1971*b*) was not found.

[e]Pyrrolizines. Major components: 5,6,7,7*a*-tetrahydro-3*H*-pyrrolizin-3-one (**D1**) and 5,6,7,7*a*-tetrahydro-3*H*-pyrrolizin-7*a*-ol-3-one (**D2**).

## MATERIAL AND METHODS

*Microorganism and cultivation. Streptomyces griseus* strain LKS-1 (Blumauerová *et al.* 1989) and its mutant derivatives (obtained in the course of a three-step mutation and selection procedure; Fig. 1) were maintained on yeast extract-malt extract agar (Shirling and Gottlieb 1966). To detect mutants insensitive to glucose repression of the formation of aerial mycelium, the complex organic agar (reproductive medium 1 after Hopwood and Sermonti 1962) supplemented with 1, 2 or 5 % glucose was employed as a selective plating medium. Fermentation studies were performed under rotary shaking (2.7 Hz) at 28 °C in three types of media (Table II), using 60 mL per a 500-mL flask. Cultures grown for 4 d were seeded with a 1-d vegetative inoculum cultivated under the same conditions (if not otherwise stated). The level of inorganic phosphate ($P_i$) in the media was determined according to Weil-Malherbe and Green (1951). The effect of $P_i$ on the biosynthetic activity of cultures was investigated after additional supplementing with $K_2HPO_4$ (46 mg per flask, in 1 mL of sterile water solutions) at the beginning or in the course of cultivation. Also stock solutions of amino acids (120 g/L) and biotin (1.2 g/L) were sterilized separately (100 °C, 30 min) and added (1 mL per flask) to cultures at the appropriate time.

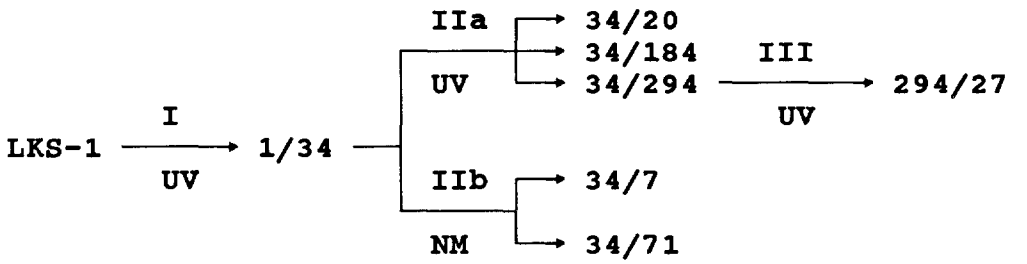


Fig. 1. Origin of mutant derivatives of *S. griseus* LKS-1. I, II, III – selection steps. Mutagen treatments (performed in parallel with natural selection): UV, ultraviolet light; NM, methylbis(2-chloroethyl)amine (nitrogen mustard). Step I was aimed predominantly at obtaining cycloheximide-blocked mutants and mutants resistant to glucose repression of morphogenesis (Table IV); for the latter purpose, plating media with 1, 2 or 5 % glucose was employed. In step II and III (using yeast extract – malt extract agar with 0.4 % glucose and, for checking the stability of the induced resistance, also the selective medium with 5 % glucose), mutants producing lower amounts of A (Table V) and increased levels of B, C or D were screened. The activity of the isolates was tested, at first, in fermentation medium M1 (Table VI) and later in M2 and M3 (Tables VII – IX) containing different levels of $P_i$ (Table II).

Table II. Composition of fermentation media M1–M30 (in g/L)[a,b]

| Component | M1[c] | M2 | M3 |
|---|---|---|---|
| Glucose | – | – | 25 |
| Starch (soluble)[d] | 40 | 40 | – |
| Soybean flour | 20 | – | 15 |
| Distiller's solubles | 10 | 7 | – |
| Molasses | 20 | – | – |
| Dried yeast[e] | – | 5 | – |
| NaCl | – | – | 3 |
| $CaCO_3$ | 3 | – | 3 |

[a]The initial values of $P_i$ (mmol/L) 0.4 (in M1), 2.6 (in M2) and 0.2 (in M3). In some studies on the production of B (Table VII) and C (Table VIII), the levels of $P_i$ were enhanced by additional supplementing with $K_2HPO_4$ to 4.8 (in M1), 7.0 (in M2) and 4.6 mmol/L (in M3).

[b]In all media. pH was adjusted (before sterilization) to get 6.9 – 7.3 after autoclaving; at the end of fermentation, pH was 7.3 – 8.0 in all media. Glucose was sterilized separately as a 50 % solution and added to M3 before inoculation.

[c]Used as control medium (Jizba *et al.* 1991).

[d]In some studies on the production of C (Table VIII), starch in M1 and M2 was replaced by 2.5 % glucose.

[e]Or yeast autolyzate.

*Bioassays of* A *and cycloheximide.* The presence of antibiotic A and of cycloheximide was tested by the conventional agar plate method using *Bacillus subtilis* and *Saccharomyces cerevisiae*, respectively*, in filtrates of the fermentation broth, in extracts of mycelium and on bioautographs of chromatograms. Extracts of the mycelium (*see below*) evaporated to dryness were dissolved in

*Due to low water solubility, C formed only small and unclear inhibition zones (cf. Ando *et al.* 1971*a*) that did not interfere with the clear, sharp-edged halos of A and cycloheximide.

methanol and diluted with a 1:1 mixture of methanol and phosphate buffer (pH 6.0). The same mixture was used to dilute methanolic solutions of the cycloheximide standard. Concentrations of cycloheximide found in extracts were recalculated with regard to the initial volume of cultures. In assays of **A**, the activity was evaluated only by measuring inhibition zones due to lack of any stable standard (Table I).

*Extraction procedures and chemical analyses.* Two combined parallel cultures were centrifuged. The supernatant (100 mL) was concentrated under vacuo to $^1/_5$ volume, supplemented with 1 g of diatomaceous earth (Celite no. 545)* and evaporated to full dryness. The residue was extracted with ethyl acetate (3 × 30 mL) and the extracts were combined, filtered and evaporated. The mycelium was washed with distilled water (50 mL) and extracted with methanol (2 × 50 mL) and with methanol–chloroform (1:1; 1 × 50 mL); the combined extracts were supplemented with 1 g of Celite no. 545 and evaporated. The dry residue was extracted with chloroform–methanol (95:5; 2 × 30 mL) and combined extracts were filtered. The sediment on filter was extracted with the same chloroform–methanol mixture (2 × 20 mL) and combined extracts were filtered. The two filtrates were combined and evaporated. To determine the content of **B**, **C**, **D** and cycloheximide, dry residues resulting from the extraction of both mycelium and supernatant were processed successively as follows.

*1.* The content of **D** was analyzed by TLC on Silufol (*Kavalier*, Czech Republic) in chloroform–methanol–27 % ammonium hydroxide (90:8:0.02) or, under double development, in ethyl acetate–methanol–water–27 % ammonium hydroxide (90:8:0.02:0.22). The components of **D** were detected by spraying with Ehrlich's reagent and their amounts were estimated visually by comparing the intensity of the red-purple coloration of the spots**.

*2.* Assays of **C** and of cycloheximide. The extracts remaining after the analyses of **D** were supplemented with 0.1 mol/L $Na_2CO_3$ (0.5 mL) and with water to the final volume of 40 mL. The mixture was extracted with chloroform (2 × 20 mL); the extracts were combined, evaporated to dryness and further processed as follows.

*a.* The dry residues were dissolved in 10 mL dichloromethane and 1 mL of the solution was used for spectrophotometric assays (at 380 nm) of the total content of **C** by the method of Suzuki *et al.* (1971), based on the formation of a complex of **C** with sodium picrate. A mixture of **C1** (nonactin) : **C2** (monactin) : **C3** (dinactin) : **C4** (trinactin) (1:1:1:1) was used as reference sample.

*b.* The solutions remaining from step (*a*) were evaporated and the dry residues were dissolved in 1 mL of methanol–chloroform (3:2) and analyzed by TLC on Kieselgel 60 (0.25 mm, 200 × 200 mm) in ethyl acetate–chloroform–dichloromethane (7:3:1) for the components of **C** (Jizba *et al.* 1991). After double development, the spots were visualized by spraying with concentrated sulfuric acid and by heating to 150 °C for 10 min. The ratios of the components were evaluated quantitatively by densitometry at 590 nm on a Shimadzu CS-930 spectrophotometer.

*c.* The solutions remaining from step (*b*) were evaporated; the residues were dissolved in methanol and used for TLC of cycloheximide on Silufol in heptane–chloroform–methanol (4:5:2). The spot of cycloheximide ($R_F$ 0.40) was visualized by bioautography (see *Bioassays*).

*3.* Assay of **B**. The aqueous phase remaining after extraction of **C** and cycloheximide (procedure 2) was acidified with 0.2 mol/L HCl to pH 6.0 and extracted with ethyl acetate (3 × 20 mL). The combined extracts were evaporated and used for TLC of B on Silufol in ethyl acetate–methanol–formic acid (90:1:0.05). Spots of B were visualized by spraying with anisaldehyde–ethanol–sulfuric acid (6:100:0.8) and by heating (130 °C, 3 min). After the color of the spots was stabilized (at laboratory temperature in daylight for 2 h), the concentration of the components of **B** was determined by densitometry (at 530 nm) on the Shimadzu spectrophotometer against the respective standards.

*Standards.* Cycloheximide (Actidione; *The Upjohn Company*, USA) and **C1** (Nonactin; *Sigma*, USA) were commercial preparations. The other compounds (components of **B**, **C** and **D**) were isolated from *S. globisporus* and *S. griseus* and the identity of purified samples was confirmed by NMR and mass spectrometry (Beran *et al.* 1988; Jizba *et al.* 1991, 1992*a*, 1993*a*).

*Mutagenesis and screening of mutants.* A three-stage improvement procedure (Fig. 1) was used in two independent lines including both treatments either with UV light or with methylbis(2-chloroethyl)amine (nitrogen mustard; NM) and natural selection. Conventional UV mutagenesis resulting in 0.01–1 % survival in different strains was employed. NM was applied as a 10 mmol/L solution in

*After supplementing with Celite, the efficiency of extraction increased by 20 % (probably due to the increased surface of the extracted samples).

**The color developed by Ehrlich's reagent was not suitable for densitometry.

0.2 mol/L phosphate buffer (pH 7.8) for 30 min, at $10^{-3}$–$10^{-2}$ % survival. The biosynthetic activity of 200–400 isolates from each mutagenized population was evaluated by a four-step analytical procedure at the next improvement stage (Fig. 1). For the 1st step, a simplified method was developed that permitted simultaneous assays of large series of isolates to be done (each grown in one flask only). Fermentation broth (15 mL) was centrifuged (supernatant was used for bioassays of **A**), mycelial sediment was washed with water (5 mL) and extracted with methanol (2 × 5 mL) and with methanol–chloroform (4:1; 1 × 5 mL). The combined extracts were evaporated and subjected to preliminary TLC of **B**, **C** and **D**, and to bioassays of cycloheximide. Samples exhibiting the highest content of **C** on chromatograms were reanalyzed by the picrate method (*see above*)*. In the strains showing higher yields of **B** and **D**, TLC was repeated using extracts of evaporated supernatants of the whole broth. In steps 2, 3 and 4 (including only the isolates preselected in the 1st step), two whole parallel cultures of each strain were elaborated as described in *Extraction procedures and chemical analyses*. The isolate exhibiting the best properties with regard to the objective of the work was used as a parent strain for mutagen treatment at the next improvement stage.

## RESULTS

*Occurrence of pesticide in shaken cultures.* Antibiotic **A** was found in the supernatants of the fermentation broth only while the components of **C** were present predominantly in the mycelium and other pesticides occurred in both parts of the culture in different proportions (Table III).

Table III. Occurrence of pesticides in cultures of *S. griseus*

| Group[a] | Content (%)[b] in fraction | |
|---|---|---|
| | Supernatant | Mycelium |
| A | 100[c] | 0 |
| B | 80[d] | 20[d] |
| C | 2 | 98 |
| D | 90[e] | 10[f] |
| Cycloheximide | 10 | 90 |

[a] See Table I.

[b] The percentage ratios were similar in all strains tested. With the exception of B (*see below*) they were unaffected by the composition of the medium.

[c] Due to its high polarity, A was present only in native supernatants and not in extracts.

[d] In M1 and M2. Cultures grown in M3 showed the ratio of 5:95 for supernatant *vs.* mycelium.

[e] In the initial strain LKS-1, the ratio of D1 : D2 in the supernatant fraction was 1 : 2; by mutagen treatment (Fig. 1; Table VI) it was changed to 2 : 1 (in some isolates from populations tested in selection steps II and III) or 1 : 1 (the highest C-producer 34/20 isolated in step IIa).

[f] Predominantly D2 (in all strains tested).

*Elimination of cycloheximide.* In the initial strain of *S. griseus* LKS-1 grown in the control medium M1, the total yield of cycloheximide (obtained from both supernatant and mycelium extracts) was 43 mg/L. The replacement of M1 with M2 or M3 resulted in a 30–50 % decrease in the yields. The desired full elimination of cycloheximide from the produced pesticidal complex was achieved only by UV-irradiation followed by selection of mutants resistant to glucose repression of morphogenesis (step I in Fig. 1; Table IV). The resulting stable cycloheximide-blocked mutant 1/34 used as parent strain for subsequent mutagen treatments (steps IIa and IIb in Fig. 1) exhibited also a moderate decrease in the formation of **A** (Table V) and apparent increases in the production of **B** (Table V) and apparent increase in the production of **B** (Table VII), **C** (Table VIII) and **D**.

*Values of C obtained by this simplified method were about 20 % lower than those resulting from the complete extraction but they were sufficient to recognize higher C producers among the other isolates.

**Table IV.** Summarized results of strain selection[a] in *S. griseus* aimed at cycloheximide-blocked mutants and mutants insensitive to glucose repression of morphogenesis

| Aim | Population | Total number of individuals tested | Stable mutants[b] | |
|---|---|---|---|---|
| | | | Number | % |
| Induction of resistance to glucose repression[c] | untreated | 5 990 | 0 | 0 |
| | treated with UV light | 1 874 | 5[d] | 0.2 |
| Induction of block in cycloheximide biosynthesis[e] | untreated | 200 | 0 | 0 |
| | treated with UV light | 400[f] | 1[g] | 0.2 |

[a]Step I in Fig. 1.

[b]Not reverting either after many repeated subcultures on nonselective media or after subsequent mutagen treatment.

[c]The initial strain LKS-1 formed sporulating aerial mycelium on media with 1 % and 2 % glucose. After increasing the concentration of glucose to 5 %, the formation of aerial mass was suppressed completely both in this strain and in its glucose-sensitive progenies.

[d]In contrast with the sensitive bald colonies, it formed well-developed aerial mycelium also on 5 % glucose medium.

[e]Strain LKS-1 grown in M1 produced 43 mg/L of cycloheximide. In M1 and M3 the production decreased to 23 and 15 mg/L, respectively. The activity of isolates from untreated and UV-irradiated populations (tested in M2) varied in ranges of 20–140 and 0–440 %, respectively, as compared with that of strain LKS-1 (taken as 100 %).

[f]395 sensitive and 5 resistant (designated 1/34) were found among the five glucose-resistant isolates. It showed also a decrease in the production of **A** (Table V) and significant increases in the production of **B** (Table VII), **C** (Table VIII) and **D**.

[g]The cycloheximide-blocked mutant (designated 1/34) was among the five glucose-resistant isolates. It showed also a decrease in the production of **A** (Table V) and significant increases in the production of **B** (Table VII), **C** (Table VIII) and **D**.

**Table V.** Decreased production of **A** achieved in the initial strain *S. griseus* LKS-1 and in its improved mutant derivatives (resulting from selection step I–III)[a] in fermentation media M1–M3[b,c]

| Strain | M1 | M2 | M3 |
|---|---|---|---|
| LKS-1 | 27 | 26 | 22 |
| 1/34 | 25 | 23 | 22 |
| 34/249 | 24 | 21 | 16 |
| 249/27 | 24 | 16 | 14 |

[a]See Fig. 1. None of the isolates from both untreated and NM-treated populations (steps I–III and IIb, respectively) was blocked completely in the biosynthesis of A. In populations mutagenized with UV (steps I, IIa and III), the A-blocked mutants occurred at frequencies lower than 1 %, but they always gave either considerably decreased or no yields of B, C and D. For the production of the latter metabolites in strain LKS-1 and its lower A-producing derivatives see Tables VII–IX. In contrast with both the block in cycloheximide and the resistance to glucose repression (Table IV), the decreased ability to produce A was a rather unstable marker and thus an occasional natural selection was necessary to keep the levels of **A** down.

[b]Due to lack of pure standard (Table I), the data summarized here represent only sizes of the inhibition zones (in mm) found in bioassays using *B. subtilis*. Each value is a mean of 20–40 independent experiments.

[c]For the content of $P_i$ in the media see Table II. After enhancing the $P_i$ concentration in M1 from 0.4 to 4.8 mmol/L the production of **A** increased moderately in all strains tested. In M2 and M3 no effect of $P_i$ supplements on the levels of **A** was found.

[a]Steps I – III in Fig. 1.

[b]Either of all the three groups simultaneously or only one of them. For data on the production of cycloheximide (eliminated in step I) and of A (decreasing gradually in all the three steps) see Tables IV and V, respectively.

[c]Series of 100 – 200 isolates from each untreated population and of 200 – 400 isolates from each UV-irradiated (step I, IIa and III) or NM-treated (step IIb) population were tested. In preliminary analyses of the large series, a simplified assay method was employed using mycelium extracts only (see *Mutagenesis and screening of mutants* in *Material and Methods*). In later tests of selected mutants, extracts of both mycelium and supernatants of fermentation broth were examined with regard to different shares of metabolites in them (Table III) to obtain complete data on the yields; U – untreated, T – treated.

**Table VI.** Summarized results of strain selection[a] in *S. griseus* aimed at increasing yields of B, C and D[b]

| Metabolic group in question | Population[c] | Total range of production activity %[d] | Yield of more active mutants[e,f] %[g] |
|---|---|---|---|
| B | U | 40 – 200 | < 1 |
| | T | 0[h] – 700 | 1 – 5[i] |
| C | U | 40 – 200 | < 10 |
| | T | 0[h] – 600 | 10 – 20[i] |
| D[j] | U | tr[k] – 200 | < 1 |
| | T | 0[h] – 500 | < 1 |

[d]The activity of individual isolates (tested in M1) was expressed as the percentage of that of the respective parent strain. Similar activity ranges were obtained in both UV- and NM-treated population; however, the NM-induced mutations were less stable.

[e]Reaching at least 150 % of the respective parent activity.

[f]In mutants giving higher yields of any of the three groups of metabolites (in M1), no strict correlation with the levels of the other complexes was found.

[g]The percentage of the total number of isolates in individual steps.

[h]Full blocks in the production of any of the three groups of metabolites were always associated with a drastic reduction or complete elimination of formation of the other complexes.

[i]The range of frequencies found in different selection steps, the highest values being obtained in step IIa and IIb.

[j]The levels of D could be estimated only approximately (see *Assay of* D in *Material and Methods*).

[k]Traces.

**Table VII.** Yields of components of B[a] (mg/L) in *S. griseus* LKS-1 and its improved mutant derivatives grown in fermentation media M1 – M3[b-d,g]

| Strain | Medium | B1 | B2 | B3 | B[e] |
|---|---|---|---|---|---|
| LKS-1 | M1 | 0.01 | 0.09 | tr | 0.1 |
| | M2 | tr | tr | tr | tr |
| | M3 | 0.5 | 0.4 | tr | 0.9 |
| 1/34 | M1 | 0.2 | 0.4 | tr | 0.6 |
| | M2 | 0.09 | 0.05 | tr | 0.14 |
| | M3 | 2.0 | 2.5 | tr | 4.5 |
| 34/249 | M1 | 15.5 | 24.6 | 1.3 | 41.4 |
| | M2 | 0.7 | 0.9 | tr | 1.6 |
| | M3 | 6.9 | 7.4 | 4.6 | 18.9 |
| 249/27[f] | M1 | 24.9 | 32.4 | 4.9 | 62.3 |
| | M2 | 1.7 | 2.2 | tr | 3.9 |
| | M3 | 11.3 | 12.9 | 7.9 | 32.7 |

[a]See Table I.

[b]The yields of B in M2 and M3 were always in inverse proportion to those of C (cf. Table VIII).

[c]After additional enhancement of the level of $P_i$ in M3 (0.2 mmol/L; Table II) to 4.6 mmol/L at the beginning of cultivation, the total yields of B increased twice (without changing the ratio of the B components) in all strains tested; later supplements were ineffective in this respect. In M1 and M3 supplemented with $P_i$ the yields of B decreased.

[d]In all strains, the low yields of B in M2 were enhanced by about five times (without significantly affecting the ratio of B1 – B3 and the levels of C) by seeding the respective cultures with washed cells of a 1-d inoculum grown in M3. The transfer of inoculum from M1 to M3 was ineffective in this respect.

[e]Trace amounts of B3 are not included in the sums.

[f]Total yields of B in strain 249/27 increased by 622-fold (in M1) and by 327-fold (in M3) as compared to the initial level in strain LKS-1 grown in M1.

[g]tr – traces (undeterminable quantitatively by densitometry).

*Decrease in the production of* **A**. In spite of the fact that no strict correlation between the yields of **A** and those of **B**, **C** and **D** was found in the majority of **A**-producing strains tested, all mutants blocked completely in the biosynthesis of A exhibited also a drastic reduction in the production of the latter metabolites; consequently, the selection (Fig. 1) was aimed at obtaining only lower **A**-producing mutants (Table V) under conditions favorable for the biosynthesis of **B**, **C** or **D** (*see below*). In mutant

249/27 resulting from step III, the production of **A** in M2 and M3 decreased by more than 50 % as compared with that of the initial strain LKS-1 grown in M1 (Table V).

[a]For the ratio of C components see Table IX.

[b]The table includes several examples of both more and less active strains (with regard to the production of C in the control medium M1) selected in different steps (Fig. 1) to demonstrate that the significant increase in the yields of C in M2 (in contrast with M3) was remarkable especially in the lower C-producers. As mentioned in Table VII, the levels of C produced in M2 and M3 were correlated inversely with the accumulation of **B**.

[c]The percentage values are calculated for each strain separately with regard to its activity in M1.

[d]After additional enhancement of the levels of $P_i$ (Table II) from 0.4 (in M1), 2.6 (in M2) and 0.2 mmol/L (in M3) to 4.8, 7.0 and 4.6 mmol/L, respectively, at the beginning of cultivation, the total yields of C increased by about 20 % in all strains. Later supplements were almost ineffective in this respect.

**Table VIII.** Effect of fermentation media M1 – M3 on total yields of C (mg/L, %)[a] in *S. griseus* LKS-1 and some of its mutant derivatives from selection steps I – III[b-e]

| Strain | M1 | M2 | | M3 | |
|---|---|---|---|---|---|
| | mg/L | mg/L | % | mg/L | % |
| LKS-1 | 16 | 117 | 731 | 8 | 50 |
| 1/34 | 60 | 231 | 385 | 17 | 28 |
| 34/7 | 76 | 476 | 626 | 10 | 13 |
| 34/71 | 82 | 237 | 289 | 8 | 9 |
| 34/184 | 9 | 110 | 1 222 | 11 | 122 |
| 34/249 | 350 | 626 | 178 | 27 | 7 |
| 249/27[f] | 980 | 1 423[g] | 145 | 96 | 9 |

[e]In spite of the fact that the mutant 1/34 and its derivatives (step II and III; Fig. 1) were resistant to glucose repression of morphogenesis in agar cultures, the biosynthesis of C (in contrast with that of B and D) in shaken cultures of these strains remained sensitive to the glucose catabolic effect. The replacement of starch in M1 and M2 with glucose (Table II) resulted in a 30 – 50 % decrease in the yields of C in both strain LKS-1 and all mutants tested.

[f]The activity of the superior mutant 249/27 increased by 6120 % (in M1) and 1200 % (in M2 and M3) strain LKS-1 in the respective media.

[g]The total increase from the initial 16 mg/L (found in the strain LKS-1 in M1) represents 8 890 %.

**Table IX.** Effect of fermentation media M1 – M3 on the composition of C[a] in *S. griseus* LKS-1 and its improved mutant derivatives[b-d]

| Strain | Medium | Ratio of components, % | | | |
|---|---|---|---|---|---|
| | | C1 | C2 | C3 | C4 |
| LKS-1 | M1 | 27 | 40 | 33 | 0 |
| | M2 | 15 | 48 | 37 | tr |
| | M3 | 23 | 42 | 35 | 0 |
| 1/34 | M1 | 19 | 37 | 44 | 0 |
| | M2 | 12 | 40 | 38 | 10 |
| | M3 | 16 | 52 | 32 | 0 |
| 249/27 | M1 | 13 | 42 | 44 | tr |
| | M2[e] | 5 | 27 | 50 | 18 |
| | M3 | 11 | 47 | 33 | 9 |

[a]See Table I.

[b]For total yields of C see Table VIII.

[c]After supplementing the media with exogenous $P_i$ the total yields of C increased by about 20 % (Table VIII), but the ratios of the C components were no more affected significantly.

[d]tr – traces.

[e]In M2, strain 249/27 produced also traces of the most active component C5. The yield of the least active C1 was 62 % lower than that formed by this mutant in M1, and 82 % lower than that in the initial strain LKS-1 grown in M1.

*Increase in the production of* **B**. The total yield of **B** in strain LKS-1 grown in M1 was only 0.1 mg/L. This low level, as well as the ratio of **B1**, **B2** and **B3** and even the ratio of the yields of **B** and **C** were improved not only by mutagenesis but also by changing media: M1 was suitable for obtaining both **B** and **C**, M2 was more favorable for formation of **C**, while in M3 **B** accumulated predominantly under simultaneous suppression of the production of **C** (cf. Tables VI and VII). As a result of the combined genetic and physiological approach, the total yield of B in the mutant strain 249/27 (Fig. 1, step III) grown in M1 and M3 increased by more than 620 and 320 times, respectively, as compared with the initial level in strain LKS-1 grown in M1, and the ratio of the components of **B** was changed (in favor of **B1** and **B3**). Additional improvement in the production of **B** was achieved in M3 by 23-fold enhancement of the level of $P_i$, and in M2 by using cells precultivated in M3 (Table VI).

*Increase in the production of* **C**. In addition to mutagen treatments, the composition of media played an important role also in the regulation of the biosynthesis

of C. After replating M1 with M2 (unsuitable for the accumulation of B; Table VI), the yields of C in various strains increased 1.5–12 times; a seven-fold enhancement of the C production was achieved even in the mutagen-untreated strain LKS-1 in this way. In the superior mutant 249/27 the total yield of C in M2 (1423 mg/L) was more than 88 higher than the initial C level (16 mg/L) found in strain LKS-1 in M1, while cultures of the same mutant grown in M1 exhibited only a 12-fold increase in this respect (Table VII). Moreover, in all strains grown in M2 the biosynthesis of the four components of C (Table I) was directed toward the most active ones; the share of the least active C1 in the whole C complex decreased gradually along with the increasing biosynthetic potency of the selected strains; in mutant 249/27 the yield of C1 was reduced by more than 80 % (as compared to that in strain LKS-1 grown in M1) in favor of C3 and C4 and, in addition, also traces of the most active C5 were formed *de novo* under these conditions (Table IX). Additional enhancements of the levels of $P_i$ in M1, M2 or M3 (by 12, 2.6 or 23 times, respectively) increased the production of C in all strains by about 20 % (Table VIII) without significantly affecting the ratios of the C components.

*Increase in the production of* D. The most suitable medium (as compared with M1) for the biosynthesis of D (under suppressing of the formation of C; Table VIII) was M3 (favorable also for the production of B; Table VII), while in M2 the yields of D decreased by about 10 times in all strains tested. After mutagen treatment (Table VI), the total yields of this complex in the improved mutants 1/34 and 34/20 (Fig. 1) grown in M3 increased by about five and ten times, respectively, as compared with the initial low level in strain LKS-1. The ratio of components D1 and D2 (Table I) was changed in this way as well (Table III). An additional increase (by 3–5 times) in the total yields of D (without affecting the D1 : D2 ratio) was achieved in strain LKS-1 and other lower D-producers by growing in M3 supplemented with proline, arginine or biotin at the beginning of cultivation (later supplements as well as adding methionine were inefficient). In M2 the low yields of D (similarly to those of B; Table VII) were improved by seeding washed cells of 1-d inoculum precultivated in M3. On the other hand, the biosynthesis of D in M1, M2 and M3 was reduced considerably (in contrast with the production of C; Table VIII) by additional increase of the levels of $P_i$.

## DISCUSSION

The diverse biosynthetic activity of *S. griseus* LKS-1 (Table I) represents a rather difficult model for any investigation; in addition, laborious analytical procedures are required for the study. In spite of the fact that the strain produces several groups of pesticides, its practical usefulness would be limited not only because of the low levels of the respective metabolites but also due to qualitative aspects. In contrast with the industrially important strain *S. aureus* S-3466 (Misato *et al.* 1977) producing only the most active C3, C4 and C5 (Oishi *et al.* 1970), the C complex of strain LKS-1 contained, under standard growth conditions in M1, mainly the less active C1 and C2 but no C4 and C5 (Table IX). Moreover, the coproduction of cycloheximide (Table I), the agricultural use of which is not recommended due to its high phytotoxicity (Misato *et al.* 1977), and the chemical separation of which from the mixtures with C would be difficult, was undesirable. If rough preparations (dried fermentation broth) of strain LKS-1 were used directly as agricultural insecticides, the presence of the phytotoxic antibiotic A would be problematic. The results of the present work (Table IV–IX) show, however, that the biosynthetic activity of the strain can be improved considerably and directed in several desired ways by both genetic and physiological methods. A series of cycloheximide-blocked mutants producing lower amounts of A and giving higher yields of C (in M2), B and D (in M3) or all of them (in M1) was obtained. At reduced levels of B and D, the total yield of C increased from 16 to 1423 mg/L, *i.e.* by more than 80 times (Table VIII) and its composition was enriched by C4 and C5, the ratio of C1 and C2 being changed from 27 : 40 to 5 : 27 (Table IX). At reduced levels of C, the yields of B (Table VII) and D increased by more than 2400-fold and by 10-fold, respectively, under simultaneous changes in the ratios of their components. Additional modifications of M2 resulting in a considerable increase in the yields of C4 and C5 have been reported elsewhere (Jizba and Skibová 1994).

With regard to the role of the B components as biosynthetic precursors of C (Keller-Schierlein *et al.* 1967), the observed inverse correlation between the levels of the two groups of metabolites (cf. Tables VII and VIII) is understandable. However, our present results, as well as those of previous experiments with other types of complex fermentation media (*unpublished data*), suggest that the final B :C ratio depends on the initial level of $P_i$ in the medium. In contrast with the biosynthesis of many other complex antibiotics that is inhibited at higher $P_i$ concentrations (for *a review see* Martin and

Demain 1980), the insertion of the **B** components (especially of **B2**; cf. Tables I and IX) into the **C** skeleton, catalyzed by the **C**-synthase (Nefelova *et al.* 1989), appears to be a high energy-requiring $P_i$-dependent process. In M3 containing $^1/_{12}$ the concentration of $P_i$ in M2 (Table II), the suppressed formation of **C** resulted in the accumulation of free **B** (cf. Tables VII and VIII) whereas additional provision of $K_2HPO_4$ increased the yields of **C** by about 20 % in all media. As demonstrated by transferring the inocula from M3 into M2 and *vice versa* (Table VII), lower $P_i$ concentrations were required for the start but not for the next subsequent course of **B** biosynthesis. Previous studies showed that both **B** and **C** appeared in the cultures already at early stages of incubation (Hejduková 1989). In M2 seeded with an inoculum grown in the same medium, the rates of **B** and **C** biosynthesis seem to be balanced whereas in M2 seeded with an inoculum from M3 a disturbance of the steady state takes places in favor of **B**. In agreement with previous findings (Blumauerová *et al.* 1987), a feedback inhibition of the **C**-synthase by superfluous free **B** might be considered as another regulation factor. M3 was the most suitable medium for separating the production of **B** from **C**; however, its poor composition was probably not sufficient for the increased biosynthetic potential of the superior strain 249/27 and other improved mutants. A new technology resulting in a remarkable increase in the yields of **B** (especially of the minor component **B3**) without changing the initial components of M3 has been described in another report (Jizba *et al.* 1994).

In contrast with **C**, the biosynthesis of **D** was inhibited by $P_i$. The stimulatory effect of supplementing with proline and arginine might be explained, with regard to the structure of **D** (Jizba *et al.* 1993), by a possible role of the two amino acids as biosynthetic precursors.

Previous studies of another producer of **B**, **C** and **D**, *S. globisporus* strain 0234, indicated the important role of carbon sources in catabolic regulation of the biosynthetic activity and morphology development of shaken cultures (Blumauerová *et al.* 1987). Results of the present work suggest the existence of a common regulatory mechanism controlling both the morphology of agar cultures and their activity under submerged conditions. Provided that the phenotype expression of the UV-induced mutant 1/34 (insensitive to the glucose effect of agar medium and altered positively in all other investigated markers; cf. Tables IV, VII–IX) resulted from a single mutation affecting the very beginning of culture development, this strain would represent a "living proof" of such a mechanism. On the other hand, the unfavorable effect of glucose in the fermentation media on the yields of **C** in both the initial strain LKS-1 and its mutant derivatives resistant to glucose repression of morphogenesis (Table VIII) indicates the existence of at least two different mechanisms participating in the sensitivity of *S. griseus* to the glucose effects.

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