Folia Microbiol. 19, 133–138 (1974)

The Study of Variability and Strain Selection in *Streptomyces atroolivaceus*

I. UV Light and Nitrous Acid as Effective Agents in the Improvement of Mithramycin Production

M. BLUMAUEROVÁ, D. A. S. CALLIERI*, K. STAJNER and Z. VANĚK

Department of Biogenesis of Natural Substances, Institute of Microbiology, Czechoslovak Academy of Sciences, 142 20 Prague 4

Received July 2, 1973

ABSTRACT. A seven-step selection procedure (repeated UV irradiation, single-step application of nitrous acid, and natural selection, following each mutagenic treatment) made it possible to increase production of mithramycin by *Streptomyces atroolivaceus* from 40–50 μg/ml to 680–830 μg/ml, *i.e.* roughly by 15 to 19-fold. The UV radiation was more effective when applying lower doses, yielding about 5% survival, 2.3% survival was obtained after the treatment with nitrous acid. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine applied in a buffer pH 9.0) at doses yielding more than 99% killing was less effective than the two former mutagens.

When studying possibilities of biological transformation of antibiotics by streptomycetes (Vaněk, 1973) we concentrated mainly on transglucosidations (Hovorková *et al.*, 1974*a*, 1974*b*; Matějů *et al.*, in press) and transglycosidations (Vaněk *et al.*, 1973). Species producing glycosides of phenolic type containing minor sugars appear particularly useful for this kind of study. However, the successful progress of these studies depends to a considerable extent on selection of suitable mutant strains. Variants producing increased quantities of a given glycoside must first be isolated from low-producing wild strains. In addition, blocked mutants, unable to form the aglycone of the antibiotic molecule, but yet capable of biosynthesis of nucleotides of corresponding sugars, are required. In the present experiments *Streptomyces atroolivaceus* (Gauze *et al.*, 1967), a producer of the glycosidic antibiotic mithramycin (aureolic acid) with antitumor activity (Grundy *et al.*, 1953; Rao *et al.*, 1962; Bakhåeva *et al.*, 1968) was applied. This communication summarizes results of a multistage selection of high-producing variants from a standard strain, related with the wild type, including both the application of mutagens and screening without mutagenic treatment. Isolation of different types of mutants blocked in biosynthesis of mithramycin will be published later.

* *Present address*: Department of Industrial Microbiology, Institute of Microbiology, University of Tucumán, Argentina.

MATERIALS AND METHODS

Working procedure and microorganism. The scheme of the used working procedure is illustrated in Fig. 1. In each selection step about 200 isolates were evaluated in four experiments performed under submerged conditions (according to the scheme recommended by Alikhanyian *et al.*, 1957); among variants exceeding the activity

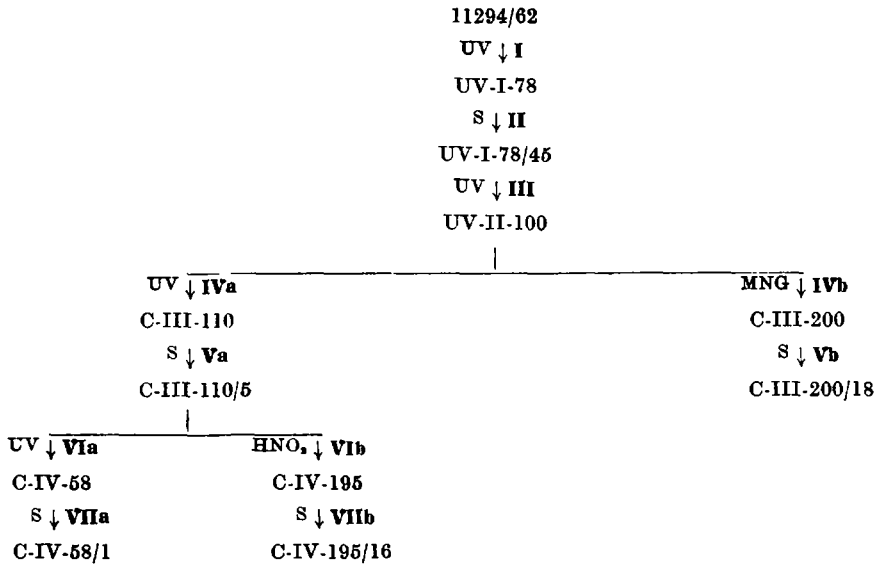

FIG. 1. Scheme of selection of improved strains in *Streptomyces atroolivaceus* producing mithramycin. UV, ultraviolet light; S, natural selection; MNG, N-methyl-N'-nitro-N-nitrosoguanidine, HNO_2, nitrous acid; bold-face numerals, number of selection step.

of the parent strain by more than 20%, the most stable strain was selected for further work. The first selection step was performed with a standard strain *Streptomyces atroolivaceus* 11294/62 (derived from the wild type; Gauze *et al.*, 1967) obtained from the Institute of New Antibiotics in Moscow.

Media and cultivation. Cultures on Petri dishes and agar slants were incubated for 7—10 days at 28°C on a sporulation medium according to Herold *et al.* (1956) which was found to be most useful of five media tested*. Submerged cultivation was performed on a reciprocal shaker (96 strokes/min, amplitude 10 cm) in 500 ml flasks containing 80 ml of the fermentation medium of composition (%, w/v): glucose, 2.5 (inoculum) or 5.0 (second vegetative generation); soybean meal, 1.5; NaCl, 0.3; $CaCO_3$, 0.3**. The basic medium containing all compounds with the exception of

* In addition to the medium according to Herold *et al.* (1956), containing sucrose, dextrin, urea, peptone, beef extract and mineral salts, also glucose-asparagine agar, Czapek-Dox medium with sucrose and a mineral medium No. 1 with starch (Gauze *et al.*, 1967), and reproductive medium 1 with glucose, peptone, yeast extract and caseine hydrolysate (Hopwood and Sermonti, 1962) were tested.

** A modification of the medium according to Gauze *et al.* (1967) containing glucose instead of starch. By this modification it was possible to increase the production of mithramycin by the standard strain by about 10%. On the contrary, sucrose had an unfavourable effect on the production, as well as supplementation of the medium with certain complex sources of nutrients, *e.g.* corn steep, molasses, yeast extract, peptone or caseine hydrolysate (Blumauerová, unpublished data).

glucose was adjusted to pH 6.1—6.2 with 20% HCl; pH after sterilization was 6.7 to 6.8. A 50% solution of glucose was sterilized separately and added to the flasks just before the inoculation. Flasks of the second vegetative generation were inoculated with 5% of 30 h old inoculum and incubated at 28°C for 96 h.

TABLE I. Spontaneous and induced variability in the production of mithramycin discovered in the course of selection of *Streptomyces atroolivaceus*.

Selection step	Parent strain	Mutagen	Spread in productivity*		Frequency of superior producers*** %	Frequency of inactive mutants %
			Total range	The most frequent class**		
I	11294/62	none	0—140	0— 20	16.6	15.5
		UV	0—420	0— 20	20.6	29.8
II	UV-I-78	none	0—160	80—120	4.3	1.8
III	UV-I-78/45	UV	0—200	60—120	16.7	0.5
IVa	UV-II-100	UV	0—260	80—120	26.2	1.8
IVb		MNG	0—380	0—140	17.2	2.0
Va	C-III-110	none	0—160	100—120	29.4	2.3
Vb	C-III-200	none	0—140	80—100	2.3	1.1
VIa	C-III-110/5	UV	0—160	80—140	22.7	4.9
VIb		HNO_2	10—160	60—120	3.0	0
VIIa	C-IV-58	none	40—180	100—140	38.8	0
VIIb	C-IV-195	none	20—200	140—160	76.4	0

* Percentage of the respective parent activity;
** Including more than 50% of the total number of isolates tested;
*** Frequency of the variants reaching more than 120% of the respective parent activity in the whole population tested.

Mutagenesis. When irradiating the cells with UV light two parallel exposures yielding about 5% and less than 1% survival, *i.e.* 50 and 70 sec (selection steps I and III) or 70 and 90 sec (steps IVa and VIa) exposures, respectively, were employed; these doses corresponded to 3,000, 4,200 and 5,400 erg mm^{-2} sec^{-1}. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNG) (Koch-Light) was applied in a 0.05M Tris-maleate buffer (pH 9.0) at a concentration of 3 mg/ml. The treatment lasted for 30, 60 and 120 min (Delić *et al.*, 1970), all three doses being lethal for 99% cells in the population. Detailed data concerning experimental conditions of the UV and MNG mutagenesis have already been published (Blumauerová *et al.*, 1973*a*). In the experiments with nitrous acid, 0.05M $NaNO_2$ in a 0.2M acetate buffer (pH 4.0) was applied for 20 min (Tessman *et al.*, 1964), yielding 2.3% surviving cells.

Assay of antibiotic activity. Production of mithramycin was assayed by means of the plate diffusion technique using *Bacillus subtilis* as a test microorganism. The standard preparation of mithramycin was obtained from the All-Union Research Institute of Antibiotics in Moscow.

RESULTS AND DISCUSSION

The original cultures of the standard strain of *Streptomyces atroolivaceus* 11294/62 used for the first selection step produced about 40—50 μg of mithramycin per ml; however, the productivity of the strain decreased to less than 10 μg/ml on subcultures on agar slants (apparently as a result of a high spontaneous variability of the standard strain). A relatively broad range of productivity was detected (0 to 140% of the original activity) in the progeny obtained after spraying conidia of the parent culture; most isolates belonged to the group reaching at most a 20% production (Table I). Blocked mutants without the antibiotic activity representing about 15% of the tested population were also classified in this group. High-producing variants exceeding the parent activity by more than 20% occurred with a similar frequency as the non-producing strains (*i.e.* 16.6% of the total number of isolates); however, it is apparent that on repeated subcultures of the parent culture these variants are gradually excluded from the population by a spontaneous selective pressure, the predominance of the more viable low-producing and non-producing forms leading to a rapid decrease of the total productivity*.

After the UV irradiation of the standard strain the production of mithramycin varied within 0—420% of the parent activity (Table I). However, also in this case low-producing and non-producing isolates predominated. Whereas the yield of high-producing variants increased by only 4% after the mutagenic treatment, frequency of blocked non-producing mutants was roughly two-fold.

A considerable morphological variability could be observed in untreated and irradiated populations of the standard strain. Most morphological mutants were low-producing or completely non-producing; on the other hand, colonies of variants with a middle or higher activity usually did not substantially differ from the parent strain. Thus, a visual detection of high-producing strains on Petri dishes was impossible. However, a correlation between the antibiotic activity and intensity of pigmentation could be observed under submerged conditions.

Several conclusions concerning the effectivity of the used selection procedure may be made on the basis of the results of further selection (*cf.* Table 1): (1) Beginning with the second selection step the productivity is increased, most isolates varying within 60—120% of the parent activity. At the same time, it was possible to decrease frequency of the non-producing mutants to about 1—2% of the total; in the last two steps the non-producing mutants were not detected at all. A considerable decrease or a complete loss of the original morphological variability were observed at the same time. (2) UV radiation alternating with natural selection is a very suitable means for improving strains of *Streptomyces atroolivaceus*. Lower exposures yielding roughly 5% surviving cells are usually most effective. Evaluation of the UV radi-

* A decrease of activity caused by a genetic non-homogeneity of the cultures was also observed in other antibiotics producing streptomycetes, during their subculturing, storage or after lyophilization (Stark *et al.*, 1971).

ation as a highly effective mutagen for increasing the productivity is in agreement with results described *e.g.* in *Streptomyces griseus*, *Streptomyces noursei* (Thoma, 1971), *Streptomyces rimosus* (Mindlin and Alikhanyian, 1958) or *Streptomyces aureofaciens* (Goldat, 1961; Blumauerová, 1973*b*). (3) After the MNG treatment (selection step IVb) a broad productivity range was obtained (both with respect to total variability and within the most frequent group), however, the yield of high-producing variants was lower than among isolates obtained after a parallel UV irradiation (step IVa). Most highly active variants selected after the application of MNG were unstable and their productivity further greatly decreased. Also the natural selection

TABLE II. Improved productivity of some strains of *Streptomyces atroolivaceus* resulting from successive selection.

Selection step	Strain	Average activity μg/ml	Ratio to the activity of 11294/62 %
—	11294/62	42	100
I	UV-I-78	88	215
VIa	C-IV-58	666	1,600
VIIa	C-IV-58/1	683	1,640
VIb	C-IV-195	713	1,710
VIIb	C-IV-195/16	830	1,990

of the resulting most stable isolate C-III-200 (step Vb) was relatively ineffective and resulted in an almost 13-times lower yield of (+)-variants than a parallel spread of the strain C-III-110 (step Va). Therefore, the use of MNG was avoided in further selections.

It is apparent that conditions chosen for the application of MNG, useful for the isolation of auxotrophic mutants in streptomycetes (Delić *et al.*, 1970; Blumauerová *et al.*, 1973*a*) are not optimal for the induction of mutations *in loci* controlling the excessive metabolism. A similar conclusion could be reached also when studying mutagenesis in *Streptomyces aureofaciens* (Blumauerová *et al.*, 1973*b*). (4) The application of nitrous acid (step VIb) was seemingly far much less effective than the UV irradiation (step VIa), however, relatively high yield of high-producing variants could be obtained after the spread of the resulting isolate C-IV-195 in step VIIb (76.4% of the total number of isolates). This result shows that the expression of mutations induced with nitrous acid is delayed, thus indicating that the natural selection should necessarily be included in the improvement procedure after each use of this mutagen.

Final results of the selection studies are summarized in Table II. By the used seven-step procedure it was possible to increase the production of mithramycin by 15 to 19-fold. As shown by later chromatographic analysis (Stajner *et al.*, in press) no other biologically active compound was responsible for this increased antibiotic titre. It can be assumed that a further selection could undoubtedly result in a further increase of the production of the antibiotic.

The improved strains differed from the standard type not only by their higher productivity but also by some other phenotypic characteristics, *e.g.* a changed pig-

mentation of submersed cultures (from light grey-brown to dark yellow-green-brown), increased sporulation ability and increased growth rate.

This work was supported by the International Atomic Energy Agency under research contract No. 845/ /RB. We should like to thank Academician G. F. Gauze (Institute of New Antibiotics, Academy of Medical Sciences of USSR, Moscow) for providing the initial strain of *Streptomyces astroolivaceus*, and Prof. A. D. Kuzovkov (All-Union Research Institute of Antibiotics, USSR Ministry of Medical Industry, Moscow) for the mithramycin standard. We also thank Mrs. V. Hummlová for her capable technical assistance.

REFERENCES

ALIKHANYIAN S. I., GOLDAT S. YU., KLEPIKOVA F. S., MINDLIN S. Z.: Utilization of ethyleneimine in selection of strains producing penicillin. (In Russian) *Antibiotiki* **1**, 33 (1957).

BAKHAEVA G. P., BERLIN YU. A., BOLDYREVA E. F., CHUPRUNOVA O. A., KOLOSOV M. N., SOIFER V. S., VASILYEVA T. E., YARTSEVA I. V.: The structure of aureolic acid (mithramycin). *Tetrahedron Letters* 3595 (1968).

BLUMAUEROVÁ M., ISMAIL A. A., HOŠŤÁLEK Z., CALLIERI D. A. S., CUDLÍN J., VANĚK Z.: Regulation of biosynthesis of secondary metabolites. XV. Isolation and characterization of auxotrophic mutants in *Streptomyces aureofaciens*. *Folia Microbiol.* **19**, 474 (1973*a*).

BLUMAUEROVÁ M., HOŠŤÁLEK Z., VANĚK Z.: Mutagenesis by UV-irradiation and N-methyl-N′-nitro-N-nitrosoguanidine in *Streptomyces aureofaciens*. Proc. Internat. Conf. "*The Bases of the Biological Effects of Ultraviolet Radiation*", Brno 1972. *Stud. Biophys.* **36/37**, 311 (1973*b*).

DELIĆ V., HOPWOOD D. A., FRIEND E. J.: Mutagenesis by N-methyl-N′-nitro-N-nitrosoguanidine (NTG) in *Streptomyces coelicolor*. *Mutation Res.* **9**, 167 (1970).

GAUZE G. F., MAKSIMOVA T. S., UKHOLINA R. S., BRAZHNIKOVA M. G., KRUGLIAK E. B.: *Act. atroolivaceus*, a new mithramycin producing organism. (In Russian) *Antibiotiki* **12**, 1059 (1967).

GOLDAT S. YU.: Selection of *Actinomyces aureofaciens* (a producer of chlortetracycline) by means of mutagen factors. (In Russian) *Trudy Inst. Mikrobiol. Akad. Nauk SSSR* **10**, 159 (1961).

GRUNDY W. E., GOLDSTEIN A. W., RICKHER C. J., HANES M. E., WARREN H. B., SYLVESTER J. C.: Aureolic acid, a new antibiotic. I. Microbiologic studies. *Antibiotics & Chemother.* **3**, 1215 (1953).

HEROLD M., BĚLÍK E., DOSKOČIL J.: Biosynthesis of chlortetracycline without maintenance of aseptic conditions. *Giorn. Microbiol.* **2**, 302 (1956).

HOPWOOD D. A., SERMONTI G.: The genetics of *Streptomyces coelicolor*. *Adv. Genet.* **11**, 273 (1962).

HOVORKOVÁ N., CUDLÍN J., MATĚJŮ J., BLUMAUEROVÁ M., VANĚK Z.: Microbial glucosidation of alizarin- and anthraflavin. *Coll. Czech. Chem. Commun.* **39**, 662 (1974a).

HOVORKOVÁ N., CUDLÍN J., MATĚJŮ J., BLUMAUEROVÁ M., VANĚK Z.: Microbial glucosidation of monohydroxyanthraquinones. *Coll. Czech. Chem. Commun.* **39**, in press (1974b).

MINDLIN S. Z., ALIKHANYIAN S. I.: A study on UV-induced variation of *Actinomyces rimosus* (oxytetracycline producer) and its selection. (In Russian) *Antibiotiki* **3**, 18 (1958).

RAO K. V., CULLEN W. P., SOBIN B. A.: A new antibiotic with antitumor properties. *Antibiotics & Chemother.* **12**. 182 (1962).

STARK W. M., KNOX N. G., WILGUS R. M.: Strain of *Streptomyces tenebrarius* and biosynthesis of nebramycin. *Folia Microbiol.* **16**, 205 (1971).

TESSMAN J., PODDAR K. R., KUMAR S.: Identification of the altered bases in mutated single-stranded DNA. I. *In vitro* mutagenesis by hydroxylamine, ethyl methanesulfonate and nitrous acid. *J. Mol. Biol.* **9**, 352 (1964).

THOMA R. W.: Use of mutagens in the improvement of production strains of microorganism. *Folia Microbiol.* **16**, 197 (1971).

VANĚK Z.: Arrival of biogenetically tailored antibiotics. In: M. Hejzlar, M. Semonský and S. Masák (Eds.)- *Advances in Antimicrobial and Antineoplastic Chemotherapy* I/2. *Progress in Research and Clinical Application* (Proc. VIIth Internat. Congr. Chemother., Prague 1971), p. 783, Avicenum, Prague 1973.

VANĚK Z., TAX J., KOMERSOVÁ I., ECKARDT K.: Glycosidation of ε-pyrromycinone using the strain *Streptomyces galilaeus* JA 3043. *Folia Microbiol.* **18**, 524 (1973).