ANTIMYCIN A FERMENTATION

I. PRODUCTION AND SELECTION OF STRAINS

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Increase in antimycin A production was achieved through a parallel strain and medium improvement program: a 125-fold augmentation (75 to 9,500 μg/ml) was obtained. The selective system included antimycin A productivity, conidiation, sensitivity to ultraviolet radiation, growth rate and yield, and absence of pigment and actinomycin D production. Among the original strains tested one natural isolate possessed high productivity and several of the above characteristics, and was selected for mutagenesis. Spontaneous and induced variability was then exploited in isolating high-producing strains. The first mutagen used was ultraviolet radiation; it was replaced by ethylenimine when it became no longer efficient in increasing variability. As new, high producers were isolated, the medium was modified to best suit their requirements for still higher productivity. The critical environmental factors were absence of phosphate and organic salts, concentration of the nitrogen source and ratio organic/inorganic nitrogen, ratio ammonium sulfate/calcium carbonate, and addition of slowly utilizable carbon sources, such as lactose and oil; optimum temperature and initial pH were 25°C and 7.0. Aeration/agitation requirements of improved strains were high. Fermentation was characterized by abrupt pH changes which impaired rapid accumulation of the antibiotic. Antimycin A was produced during both the trophophase and idiophase.

Antimycin A was originally described as an antifungal antibiotic[22]. Toxicity precluded its use in humans and animals, but, being a potent succinoxidase inhibitor,[1] it found many applications in enzyme research: antimycin A blocks the passage of electrons by affecting complex III, the sensitive site located between cytochromes b and c_1[28]. Its structure was elucidated in 1961[37]. Antimycin A is more toxic to fish than to any other living form[12], and this property has made it an important tool in fish management[23,38,39].

This paper deals with the development of high-producing strains through the selection of spontaneous[26] and induced[34] variants, the method of choice for obtaining high-yielding strains[5,10,15,17,35]; several authors have reviewed the subject[2,7,8,11,16,20,24]. More recently, conjugation, transformation, transduction, phage conversion have been advocated[4,6,9,32] and some of them applied to antimycin A-producing streptomycetes in a separate program; results will be reported elsewhere.

Throughout the present study emphasis was given to increase the power of the selective system[11,16,24]. Firstly, a rapid, accurate and convenient assay method was developed. Secondly, at each round of mutagenesis, several improved strains were compared not only for productivity but also for early and abundant conidiation on the propagation medium, absence of pigment, growth rate, and only one strain which represented a distinct advantage over the parent was selected for further study. Thirdly, since the environment plays an important role in the

expression of genomic determinants, fermentation medium and conditions were periodically revised to best suit the organism selected for further improvement. Finally, strains that showed higher productivity in shake flasks were evaluated in aerated-agitated fermenters; results of this study are reported in the following paper[20]. Throughout the program a rigid maintenance, propagation and preservation scheme was adhered to.

Materials and Methods

Microorganisms

Original strains of antimycin A-producing streptomycetes are listed in Table 2. Upon reception or isolation from soil strains were transferred onto PRIDHAM's tomato paste oatmeal agar slants[25] and incubated for 1 week at 25°C and 70 % relative humidity. From slants lyophilized[27] and soil stocks[18] were prepared. Transfers from slant to slant were avoided to minimize degeneration; instead, subcultures were made from soil, or occasionally from lyophilized stocks, to PRIDHAM's medium.

Mutagenic treatments

Spore suspensions from ultraviolet irradiation were made by adding H_2O or a 1:10,000 solution of sodium monolauryl sulfate to slant or ROUX bottle cultures and gently scraping the surface with a pipet. Suspensions were shaken with glass beads, then filtered through glasswool and Whatman No. 2 filter paper to break spore chains and remove clumps. Optical density of spore suspensions was read in a Coleman Junior 6A colorimeter at 660 nm (1-cm cuvette), and spore concentration calculated by interpolation from a calibrated curve relating spore count and optical density. Suspensions were diluted to contain *ca* 1×10^8 spores/ml. Fifteen ml of spore suspension were pipetted into a Petri plate, 100 mm in diameter, mounted on the platform of an agitator rotating at 80 rev/min under a Mineralite lamp (Ultra-Violet Products Co., St. Gabriel, California) emitting ultra-violet radiation at 253.7 nm with an intensity of 4.3 ergs/mm^2/sec (258 ergs/mm^2/min) at 30 cm. Irradiation was carried out in subdued light to prevent possible photoreactivation. Samples were taken at time 0 and at time intervals thereafter, diluted in suitable water blanks, and 0.1-ml aliquots of each dilution spread with a glass rod on the surface of PRIDHAM's medium. Counts were made after 1 week of incubation, and survival plotted against ultraviolet exposure (Fig. 1).

For ethylenimine treatment spore suspensions were prepared as above. Ethylenimine (Matheson, Coleman and Bell Co., East Rutherford, N.J.) was added to give a final concentration of 0.1 %; final volume was 50 ml in a 250-ml Erlenmeyer flask. During exposure, the mixture was agitated at 180 rev/min on a rotating agitator and temperature maintained at 25°C. Samples were taken at time 0, and 30, 45, 60, 90 and 120 minutes after the addition of ethylenimine, diluted in suitable water blanks, and 0.1-ml aliquots of each dilution spread with a glass rod on the surface of PRIDHAM's medium. Counts were made after 1 week of incubation. In a typical experiment percent kill at 0, 30, 45, 60, 90 and 120 minutes was 0, 78, 95, 99.8, 99.99 and 100 respectively.

Successive ethylenimine treatment and ultraviolet irradiation[3] were performed as follows: spores were exposed to ethylenimine for 40 minutes to afford *ca* 80 % kill, then a 15-ml sample was submitted to ultraviolet radiation as described above. In a typical experiment percent kill at 2 and 3 minutes was 99.9 and 99.999 respectively. Samples were taken, diluted, and plated on PRIDHAM's medium as before.

Natural isolates and survivors of mutagenic treatments

Four hundred colonies of natural isolates or survivors of mutagenic treatment were transferred to slants of PRIDHAM's medium, incubated for 1 week at 25°C and 70 % R.H. and stored in the refrigerator. Each slant culture was eventually subcultured on two slants, one for inoculating fermentation media to establish productivity and the other for repeating the test if necessary. The original slant culture of the selected isolates or survivors served to

prepare lyophilized and soil stocks from which all further subcultures were derived.

Fermentation

Spore suspensions of the streptomycetes to be tested for antimycin A productivity were prepared from slant cultures and diluted so that a ten-fold dilution gave 50~60 % transmittance at 660 nm (1-cm cuvette). The adjusted suspensions served as inocula and were added to fermentation media at the rate of 1 or 2 %.

The main fermentation media and conditions used are described in Table 1. Media were dispensed into 500-ml Erlenmeyer flasks; flasks were closed with non-absorbent cotton or Johnson and Johnson filter pads, sterilized at 121°C for 20 minutes, cooled to incubation temperature and inoculated. Incubation was performed at 70 % relative humidity on a New Brunswick Scientific gyrotory shaker, model G-53, at 240 rev/min (2″-stroke). At various time intervals samples were taken to determine antimycin A, pH, glucose, lactose, ammonia nitrogen, and mycelium. Fermentation was terminated when pH reached 8.2: at this pH antimycin A is destroyed more rapidly than it is produced.

Antimycin A assay

For a while a microbiological assay[21] using *Saccharomyces cerevisiae* Y-30 as the test organism was followed. However, early in this work a spectrophotofluorometric assay was developed[30,31] which proved invaluable for testing rapidly a large number of samples; samples could be analyzed manually when the number of samples was small; with the automated version samples could be assayed at the rate of 30 an hour. Some strains produced actinomycin D in addition to antimycin A; to detect the presence of these antibiotics an ethanolic extract of fermentation broth was spotted on a TLC plate coated with Silica gel G; the plate was developped in a mixture of ethanol-benzene (20:80). Presence of actinomycin D was detected directly by observing its intense color. Antimycin A was revealed by spraying the dried plate with concentrated H_2SO_4.

Mycelial mass determination

Most media contained particulate matter and the mycelial mass could not be determined accurately; however, an approximate figure could be obtained: 10 ml of broth culture were centrifuged in calibrated conical tubes for 15 minutes in an International model HN centrifuge operating at 2,000 rev/min. Percent of wet, packed mycelium was read directly from the tube. A sample of the medium before inoculation was similarly treated, and the value observed was substracted from that of the unknown to obtain the net mycelial mass present in broth cultures.

Glucose and lactose determination

Glucose was assayed directly by the Glucostat method (Worthington Biochemical Corp., Freehold, N.J.). To determine lactose in the presence of glucose a broth sample was autoclaved at 121°C for 10 minutes in the presence of 1 N HCl; the hydrolyzed sample was then neutralized to pH 7 and total glucose determined by the Glucostat method. The value for native glucose obtained on a separate, non-hydrolyzed sample was substracted from total glucose to evaluate glucose from lactose hydrolysis; therefore, lactose was calculated by difference.

Ammonia nitrogen

The method used was described by UMBREIT *et al*[36].

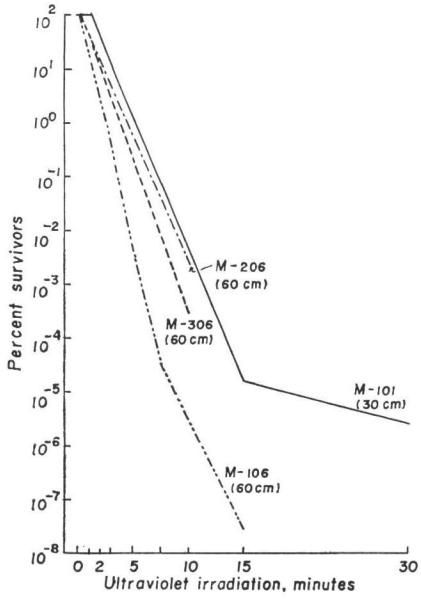
Results

Selection of Strains for Mutagenesis

Streptomyces sp. M-101 (Table 2) is the original strain of LEBEN and KEITT[22]; in STRONG's medium[33] it produced *ca* 25 μg antimycin A/ml. It did not respond favorably to variations introduced in the maintenance medium, the age and size of inoculum and the sources of nitrogen, carbon and energy. Phosphate was inhibitory. Nevertheless, by adjusting pH at 7

Fig. 1. Lethal effect of ultraviolet irradiation at 253.7 nm on various antimycin A-producing strains.

Spore suspensions contained about 1×10^8 viable spores/ml; 15 ml of spore suspension were pipetted into 100-mm Petri plate placed on an agitator rotating at 80 rev/minute. The Mineralite lamp was mounted at 30 cm above the suspension for strain M-101, and at 60 cm for strains M-206 and M-306. The energy emitted at 30 cm was 4.3 ergs/mm²/sec, and at 60 cm 1.075 ergs/mm²/sec. Aliquots were taken at the time intervals indicated, diluted and plated on PRIDHAM's tomato paste-oatmeal agar; incubation: 7 days at 25°C.



before inoculation, incubation temperature at 25°C and agitation conditions as indicated in Materials and Methods, it was possible to obtain reasonably reproducible yields of *ca* 75 μg/ml. Successful fermentation runs were always accompanied by a sharp drop in pH from 7 to 4.5 in 48 hours; pH then rose steadily to reach 8.3~8.4 when maximum yields were observed; on further incubation, antimycin A was rapidly destroyed because of its great lability at pH values above 8. Further attempts to improve the medium were unsuccessful.

Streptomyces sp. M-101 was submitted to ultraviolet radiation with the lethal effect shown in Fig. 1. Survivors were isolated at various exposures. A non-irradiated suspension was also plated out and natural isolates tested. Productivity distributions of natural isolates and survivors in medium 2 (Table 1) are illustrated in Figs. 2 and 3 respectively. Distribution was much broader in survivors than in natural isolates, which reflects the great variability introduced in the population by the mutagenic treatment. The best natural isolates were re-tested in several flasks, and two were found to possess 218 and 206 % the productivity of the parent (Fig. 2). The best survivors were also re-tested, and two produced respectively 328 and 276 % the antimycin A produced by the parent (Fig. 3); both strains had survived a 30-minute exposure (7,740 ergs/mm²). The genealogy and productivity of isolates and survivors of strain M-101 are illustrated in Fig. 4.

In the meantime, other strains received from culture collections or directly isolated from soil were tested. Productivity of all original strains in Medium 1 is compared in Table 2. *Streptomyces* sp. M-106 appeared superior to all other strains including the best survivors issued from strain M-101.

Productivity of strain M-106 was further increased by modifying the fermentation medium and other conditions. The influence of nitrogenous additives is apparent from Table 3. Highest yield was afforded by a casein hydrolyzate (Casein EHC); ammonium sulfate was almost as stimulatory as casein hydrolyzate and was preferred as a cheaper, more reproducible source of nitrogen. Various ammonium salts listed in Table 4 were then compared: it is noteworthy that the organic salts tested (oxalate and acetate) reduced yields very considerably;

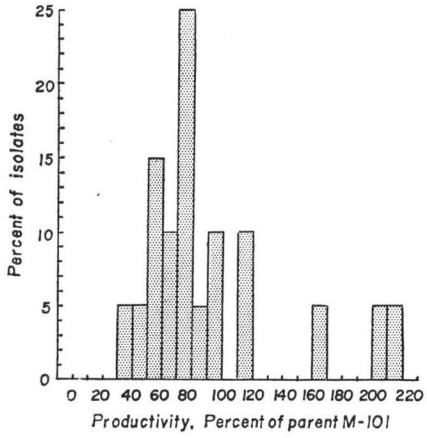
Table 1. Standard combinations of fermentation media and conditions.

Ingredients (g/l) and conditions	Medium and condition combination No.								
	1	2	3	4	4A	5	6	7	8[a]
Soy flour (44% protein)[b]	40	40	40						
Special X soy flour[c]				20	40				
Nutrisoy 220 soy flour[c]						60	60	60	
Cerelose[d]	20	20	20	20	20	20	20	20	
Lactose							5	5	
Glucose									30
Sodium citrate									8.1
Methyl myristate									2.5
Soybean oil							15		
Sperm oil								20	
Fresh yeast						5		5	
$(NH_4)_2SO_4$				6	3	3	6	6	8.1
$CaCO_3$	1.5	1.5	1.5	1.5	1.5	1.5	3	3	1.0
K_2HPO_4									0.5
Trace elements solns[e], ml					0.1				
$MgSO_4 \cdot 7H_2O$									0.25
$ZnSO_4 \cdot 7H_2O$									0.05
$MnSO_4 \cdot 7H_2O$									0.0025
$CuSO_4 \cdot 7H_2O$									0.003
Tap water to, liter	1	1	1	1	1	1	1	1	
H_2O to, liter									1
pH (after sterilization)	6.5	7.0	7.0	7.0	7.0	7.5	7.4	7.4	7.0
Temperature, °C	28	25	25	25	25	25	25	25	25
Volume, ml/500-ml flask	100	100	50	50	50	50	50	50	50

[a]Synthetic medium.
[b]Staley Manufacturing Co., Decatur, Ill.
[c]Archer Daniels Midland Co., Minneapolis, Minn. Special X (50% protein, 6~8% fat), Nutrisoy 220 (43% protein, 22~23% fat).
[d]Corn Products Corporation, New York. "Cerelose" is a pharmaceutical grade of glucose.
[e]Trace elements solutions contained (mg/100 ml) respectively: $Na_2B_4O_7 \cdot 10H_2O$, 88 (10 mg B); $CuSO_4 \cdot 5H_2O$, 393 (100 mg Cu); $MnCl_2 \cdot 4H_2O$, 72 (20 mg Mn); $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 37 (20 mg Mo); $ZnSO_4 \cdot 7H_2O$, 8807 (2,000 mg Zn); and $FeNH_4(SO_4)_2 \cdot 12H_2O$, 1725 (200 mg Fe). Medium 4A contained 0.1 ml of each solution per liter.

ammonium sulfate and ammonium molybdate were most satisfactory; ammonium sulfate was chosen in further experiments. The influence of initial pH and temperature is obvious from the results presented in Tables 5 and 6. All the factors that led to yield increase were considered in the preparation of Medium 4 (Table 1). Original strains were re-tested in this medium, and the results are reported in Table 2: strain M-106 again proved its superiority. Strain M-106 had the further advantages over all other strains that it gave very reproducible yields in replicate flasks and in separate experiments, responded well to modifications of the medium and fermentation conditions (pH, temperature), sporulated abundantly on the maintenance and propagation medium (PRIDHAM's medium), produced a less intense green pigment and not even traces of actinomycin D. This exceptional strain became the ancestor of a family of high antimycin A-producing streptomycetes (Fig. 6).

Fig. 2. Productivity of natural isolates of strain M-101 in Medium 2 (Table 1) after 72 hours of incubation.
Average yield of parent M-101: 74 μg/ml.



Mutagenesis of Strain M-106

Strain M-106 was submitted to ultraviolet radiation according to the procedure described for strain M-101. Survival was extremely low and no survival curve could be drawn. The treatment was repeated with the lamp mounted 60 cm above the suspension (by doubling the distance the ultraviolet intensity was reduced fourfold). The survival curve appears in Fig. 1. At 5-minute exposure percent survival of strain M-106 was 5×10^{-3} (484 ergs/mm^2) as compared to 10° for strain M-101 (1,935 ergs /mm^2); therefore, strain M-106 was $10°/5\times10^{-3}\times1{,}935/484=800$ times more sensitive to ultraviolet radiation than strain M-101.

Fig. 3. Productivity of survivors, selected after ultraviolet irradiation of strain M-101, in Medium 2 (Table 1) after 72 hours of incubation.
Average yield of parent M-101: 74 μg/ml.

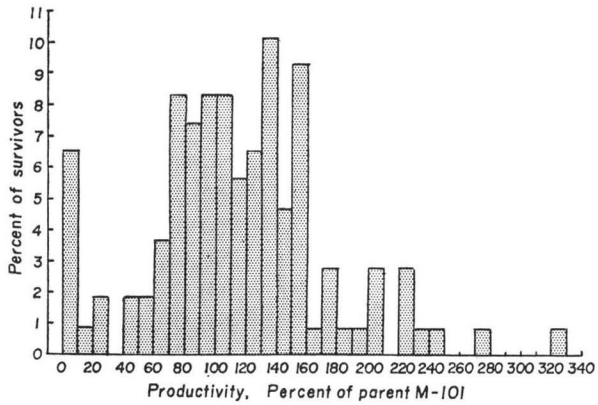
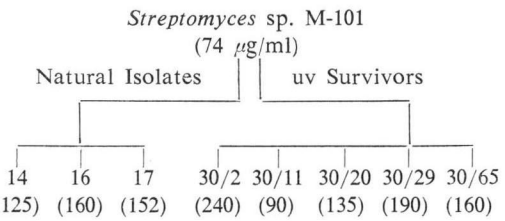


Fig. 4. Family of antimycin A-producing strains of *Streptomyces* sp. M-101 (Table 2).
Yields in μg/ml are given in parentheses.



Survivors from irradiation of strain M-106 were not selected at complete random; only well sporulating colonies with and without pigment, and otherwise identical to parental colonies were selected and tested individually in single flasks. The productivity distribution is illustrated in Fig. 5. Average productivity of the survivors was lower than that of the parent, but several strains produced more than the parent and were re-tested in several flasks. The best strain yielded consistently 160 % the antimycin A produced by the parent and was pigmentless. This strain was assigned the number M-206; it had survived a 10-minute exposure (Fig. 1).

Table 2. Productivity of original antimycin A-producing strains in two media.

Ayerst No. (AY-)	Designation and origin	Antimycin A (μg/ml)*	
		Medium 1	Medium 4
M-101	*Streptomyces* sp. NRRL 2288	74	197
M-102	*Streptomyces* sp. ATCC 11862	73	187
M-103	*Streptomyces* sp. NRRL 2288 (isolate)	100	160
M-104	*Streptomyces antibioticus* NRRL 2411	192	205
M-105	*Streptomyces antibioticus* NRRL 2838	93	257
M-106	*Streptomyces* sp.	235	645
M-107	*Streptomyces* sp.	65	250
M-108	*Streptomyces* sp.	89	250
M-109	*Streptomyces antibioticus*	104	275
M-110	*Streptomyces antibioticus*	74	252
M-111	*Streptomyces antibioticus* NRRL S-1543	<2	75
M-112	*Streptomyces antibioticus* NRRL B-1702	43	252
M-113	*Streptomyces antibioticus* NRRL B-1703	<2	80
M-114	*Streptomyces antibioticus* ATCC 8663	36	262
M-115	*Streptomyces antibioticus* ATCC 10382	103	260
M-116	*Streptomyces* sp. NRRL 2288 (isolate)	114	165

* Maximum yield occurred at 72 hours; final pH 8.3~8.4.

Table 3. Influence of addition of protein hydrolyzates and ammonium sulfate on antimycin A production by *Streptomyces* sp. M-106.

Hydrolyzates added* (10 g/liter)	Antimycin A (μg/ml)
No addition	363
Casein EHC**	843
Cottonseed CTPH**	454
Yeast BYF-50X**	147
Yeast BYF-100X**	138
Meat MPH**	573
Soybean HSP**	259
Corn 600**	306
Corn 826**	281
Corn 816**	268
Cottonseed	362
$(NH_4)_2SO_4$, 6 g/liter	813

* Basal medium (g/liter): Special X soy flour, 20; "Cerelose", 20; $CaCO_3$, 1.5; tap water; pH 7.0; temperature, 25°C; incubation: 72 hours. Volume/flask: 100 ml. Final pH 8.3.

** Amber Laboratories, Inc., Milwaukee, Wis.

In a separate experiment survivors showing great variations in colony morphology (absence of aerial hyphae or conidia, sectors) were isolated and tested. These probably resulted from "major mutations"[2] and were

Table 4. Influence of addition of ammonium compounds on antimycin A production by *Streptomyces* sp. M-106.

Ammonium compounds (g Nitrogen/liter)*	Antimycin A (μg/ml)
No addition	195
$(NH_4)_2SO_4$, 1.272	560
$(NH_4)_2SO_4$, 1.958	560
NH_4Cl, 1.272	400
NH_4Cl, 1.958	400
NH_4OH, 1.272	367
NH_4OH, 1.958	420
NH_4NO_3, 1.272	447
NH_4NO_3, 1.958	447
NH_4-oxalate, 1.272	50
NH_4-oxalate, 1.958	109
$NH_4H_2PO_4$, 1.272	495
$NH_4H_2PO_4$, 1.958	300
$(NH_4)_2HPO_4$, 1.272	390
$(NH_4)_2HPO_4$, 1.958	545
NH_4-acetate, 1.272	42
NH_4-acetate, 1.958	20
$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 1.272	570
$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 1.958	572

* $(NH_4)_2SO_4$, at 6 and 9 g/liter, contains 1.272 and 1.958 g nitrogen respectively. All compounds were tested at the same nitrogen concentrations. For basal medium, see Footnote*, Table 3.

Table 5. Influence of initial pH on Antimycin A production by *Streptomyces* sp. M-106.

Initial pH*	Antimycin A (μg/ml)
4.5	420
5.5	467
6.5	507
7.0	635
7.5	517

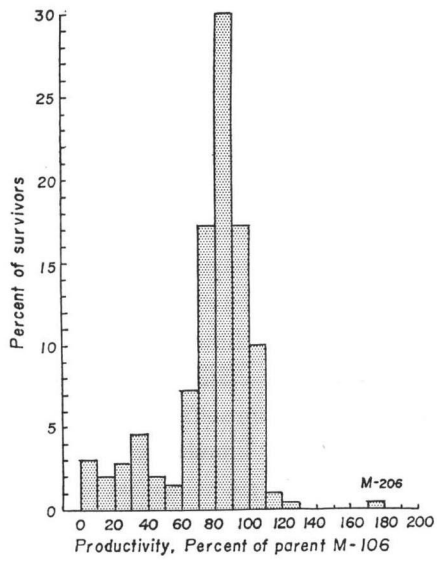
* pH adjusted with NaOH or HCl to give the indicated values after sterilization.
Medium (g/liter): Special X soy flour, 20; "Cerelose", 20; $(NH_4)_2SO_4$, 6; $CaCO_3$, 1.5; tap water; temperature: 25°C; incubation: 91 hours. Volume/flask: 100 ml. Final pH 8.3.

Table 6. Influence of temperature on antimycin A production by *Streptomyces* sp. M-106.

Temperature (°C)	Antimycin A (μg/ml)
23±0.5	625
25±0.5	658
28±0.5	460
30±0.5	340

* For Medium, see Footnote, Table 5; pH 7.0.

Fig. 5. Productivity of 297 survivors, selected after ultraviolet irradiation of strain M-106, in Medium 4 (Table 1), after 96 hours of incubation.
Average yield of parent M-106: 620 μg/ml.



invariably poor producers. Since rapid growth and abundant sporulation were considered highly desirable, only those survivors harboring these characteristics were selected for testing and are reported in Fig. 5.

Mutagenesis of Strain M-206

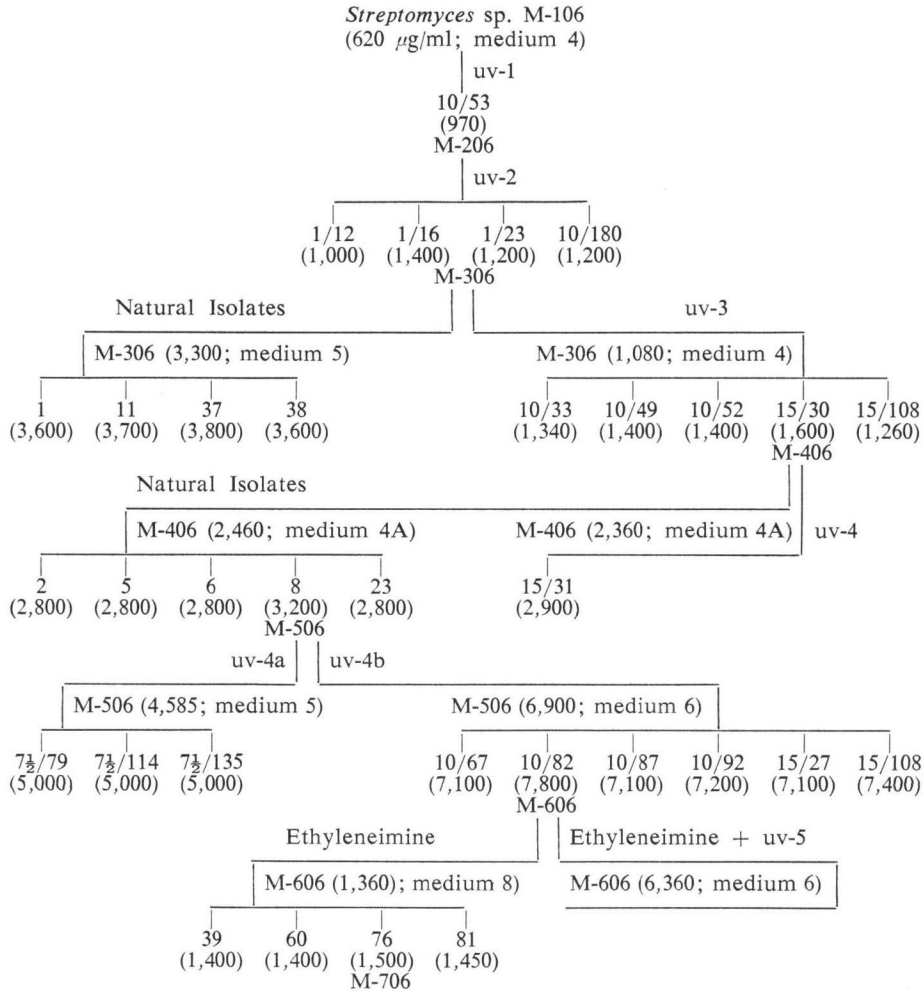
Strain M-206 was irradiated and the survival curve is shown in Fig. 1: strain M-206 was more resistant than its parent, but less resistant than strain M-101 to ultraviolet radiation. Of the 459 survivors tested four were reproducible, pigmentless and abundantly sporulating. These four strains were retested in several media, and one strain, which was assigned the number M-306, was chosen that reproducibly yielded 182 % the antimycin A produced by the parent M-206 (815 μg/ml). Strain M-306 had survived a 1-minute exposure.

Comparison of Natural Isolates and Survivors of Strain M-306

Spore suspensions of strain M-306 were plated directly on PRIDHAM's medium, colonies isolated and tested for antimycin A production. No isolate was found to produce more than 110 % the parent's productivity; several strains produced between 105 and 110 %, and when re-tested later on medium 5 (Table 1) yielded as much as 3,600 μg/ml (Fig. 6). Spore suspensions of strain M-306 were also irradiated; the survival curve is shown in Fig. 1 and indicates that sensitivity of strain M-306 is intermediate between that of strain M-106 and that of strain M-206. Only well sporulating, pigmentless colonies were isolated and tested in medium 4 (Table 1). Of 440 survivors tested, only five strains were pigmentless, heavily sporulating and reproducible. They were re-tested on several media, and one strain which yielded reproducibly 148 % the productivity of the parent was chosen for further irradiation and assigned the number

M-406 (Fig. 6). It had survived a 10-minute exposure.

Fig. 6. Family of antimycin A-producing strains of *Streptomyces* sp. M-106 (Table 2) Yields in μg/ml are given in parentheses.



Fermentation Media and Conditions

Through three successive irradiation rounds it had been possible to improve antimycin A production from 620 μg/ml for strain M-106 to 1,600μg/ml for strain M-406 in medium 4 (Fig. 6). The phenotypic expression of genomic changes obtained through empirical mutation and selection must depend on the selective conditions prevailing. Therefore, it was felt necessary at this stage to re-evaluate the influence of the medium on the productivity of selected strains. The influence of the carbon source on strains M-106 and M-206 is shown in Table 7: used singly only glucose (cerelose) is satisfactory; acetate, citrate and lactate are very inhibitory; sucrose, lactose and starch are poor sources. The addition of magnesium sulfate and/or potassium chloride had little effect on the same strains. Trace elements had a stimulating effect, and zinc was most important in this respect, although all other elements tested were somewhat stimulatory. The net effect of trace elements was to enhance growth and productivity at the beginning of fermentation without increasing significantly total production; therefore, trace elements were

Table 7. Influence of carbon sources on antimycin A production by *Streptomyces* sp. M-106 and M-206.

Carbon sources* (20 g/liter)	Antimycin A, μg/ml	
	M-106	M-206
"Cerelose"	485	662
Lactose	40	72
Sucrose	40	70
Starch	40	102
Lactate	25	55
Acetate	<2	<2
Citrate	72	50
"Blackstrap" molasses	270	290
Corn steep liquor	67	102

* Basal medium (g/liter): Special X soy flour, 20; $(NH_4)_2SO_4$, 6; $CaCO_3$, 1.5; tap water; pH 7.0; temperature, 25°C; incubation: 72 hours. Volume/flask: 100 ml.

included in medium 4A (Table 1). The ratio organic/inorganic nitrogen was found important for strain M-206: results of Table 8 show maximum yields for the combination of 40 g soy flour and 6 g ammonium sulfate per liter; therefore, this ratio was used in the preparation of medium 4A. The influence of fat content of soybean meal is shown in Table 9: 60~70 g "Nutrisoy 220"/1 led to the highest yields, but peak production was retarded to 168 hours. This observation was considered in the preparation of medium 5 (Table 1). With the introduction of media enriched in total solids it was felt necessary to re-evaluate the aeration/agitation conditions: the type of closure and volume of medium per flask were found important (Table 10), and best conditions were adopted (J. & J. filter

Table 8. Influence of organic/inorganic nitrogen on antimycin A production by *Streptomyces* sp. M-206*.

Special X Soy Flour		$(NH_4)_2SO_4$		Total Nitrogen (g/liter)	Antimycin A (μg/ml)
g/liter	g N/liter	g/liter	g N/liter		
40	1.6	0	0	1.6	360
40	1.6	3	0.6	2.2	815
40	1.6	6	1.2	2.8	820
40	1.6	9	1.8	3.4	830
30	1.2	3	0.6	1.8	690
30	1.2	6	1.2	2.4	755
30	1.2	9	1.8	3.0	655
30	1.2	12	2.4	3.6	700
20	0.8	6	1.2	2.0	610
20	0.8	9	1.8	2.6	540
20	0.8	12	2.4	3.2	585
20	0.8	15	3.0	3.8	505
10	0.4	6	1.2	1.6	<100
10	0.4	9	1.8	2.2	<100
10	0.4	12	2.4	2.8	<100
10	0.4	15	3.0	3.4	<100
0	0	9	1.8	1.8	<100
0	0	12	2.4	2.4	<100
0	0	15	3.0	3.0	<100
0	0	18	3.6	3.6	<100

* Basal medium (g/liter): "Cerelose", 20; $CaCO_3$, 1.5; tap water; pH 7.0; temperature, 25°C; incubation: 96 hours. Volume/flask: 100 ml.

Table 9. Comparison of low-fat and high-fat soybean meals for antimycin A production by *Streptomyces* sp. M-306.

Soybean meals*	g/liter	Antimycin A (μg/ml) at indicated incubation periods				
		72 hours	96 hours	120 hours	144 hours	168 hours
Special X Soy Flour	40	2,281	2,017	—	—	—
	60	2,246	1,794	—	—	—
	80	1,469	1,000	—	—	—
Nutrisoy 220 Soy Flour	40	2,428	2,945	3,306	3,827	3,462
	50	2,188	—	3,769	3,798	4,366
	60	2,883	3,555	3,833	3,972	4,366
	70	2,477	—	3,982	3,995	4,453
	80	1,900	—	3,253	3,318	3,755
	90	1,267	—	2,706	3,143	2,707

* Basal medium g/liter: "Cerelose", 20; $(NH_4)_2SO_4$, 3; $CaCO_3$, 1.5; tap water; pH 7.2; temperature 25°C. Volume/flask: 100 ml.

Table 10. Influence of aeration/agitation conditions in shake flasks on antimycin A production by *Streptomyces* sp. M-306 and M-406.

Conditions*		*S. antibioticus* M-306		*S. antibioticus* M-406	
		72 hours	96 hours	72 hours	96 hours
Type of closure (100 ml medium/flask)	cotton plug	1,106	1,360	1,480	1,513
	J. & J. Filter pad	1,293	1,633	1,600	1,693
Volume medium/flask (closure: cotton plug)	50 ml	2,120	1,733	1,600	2,120
	75 ml	1,726	1,823	1,180	1,920
	100 ml	1,633	1,450	1,040	1,580
	125 ml	1,433	1,206	—	—

* Medium 4A, Table 1.

pads; 50 ml medium/flask) in subsequent experiments.

Natural Isolates and Survivors of Strain M-406

Spore suspensions before and after irradiation were plated on PRIDHAM's medium, and natural isolates and survivors selected according to the usual criteria, sporulation and lack of pigmentation. Results are summarized in Fig. 6. Five out of 29 isolates proved to be superior to the parent, and four of 400 survivors were comparable to the best isolates in medium 4A. Upon retesting in several media the best survivor yielded 106 % and the best isolate 127 % the production of the parent. The natural isolate was designated strain M-506 and chosen for further mutagenesis.

Mutagenesis of Strain M-506

Strain M-506 was irradiated and the survivors tested in medium 5. Trace elements were omitted from the medium, since they had no enhancing effect when the concentration of soy flour was increased to 6 %. Three out of 401 survivors (Fig. 6, uv-4a) produced 110~115 % the antimycin A obtained with the parent. In the meantime, analysis of the fermentation broth revealed that glucose was utilized very rapidly by strain M-506 and could not be detected

Table 11. Influence of addition of lactose and soybean oil on antimycin A production by *Streptomyces* sp. M-506*.

Lactose (g/liter)	Soybean oil (g/liter)	Antimycin A (μg/ml)		
		7 days	10 days	11 days
0	0	5,450	5,650	—
0	10	5,400	8,325	—
5	5	6,300	—	7,000
5	10	6,000	—	7,400
5	15	5,725	—	9,650
10	0	5,375	5,850	—
10	5	5,875	—	7,475
10	10	5,625	—	8,000
10	15	5,475	—	8,500
15	5	5,530	—	7,450
15	10	5,430	—	7,750
15	15	5,180	—	6,900

* Basal medium (g/liter): Nutrisoy 220 soy flour, 60; "Cerelose", 20; $(NH_4)_2SO_4$,6; $CaCO_3$, 3; tap water; pH 7.2; temperature: 25°C Volume /flask: 50 ml.

Table 12. Influence of $(NH_4)_2SO_4/CaCO_3$ ratio on antimycin A production by *Streptomyces* sp. M-506*.

$CaCO_3$ (g/liter)	$(NH_4)_2SO_4$ (g/liter)	Antimycin A (μg/ml)	
		9 days	10 days
1.5	3.0	7,960	8,000
1.5	4.5	6,600	6,950
1.5	6.0	7,250	7,425
1.5	9.0	7,150	7,625
3.0	3.0	7,500	7,500
3.0	4.5	8,400	8,400
3.0	6.0	8,700	9,075
3.0	9.0	7,100	7,425
4.5	3.0	7,450	7,600
4.5	4.5	7,750	7,700
4.5	6.0	8,675	8,300
4.5	9.0	8,775	8,550
6.0	3.0	7,550	7,450
6.0	4.5	7,850	8,000
6.0	6.0	7,750	7,600
6.0	9.0	—	8,400

* Basal medium (g/liter): Nutrisoy 220 soy flour, 60; "Cerelose", 20; lactose, 5; soybean oil, 15; tap water; pH 7.4; temperature, 25°C. Volume/flask: 50 ml.

at 72 hours of incubation. Increasing initial glucose concentration somewhat reduced yields; therefore, addition of slowly utilizable sugar and oil was studied, and the effect is reported in Table 11. The best combination (0.5 % lactose and 1.5 % soybean oil) almost doubled the yields with an increase in fermentation time. The ratio ammonium sulfate/$CaCO_3$ was also found to be an important factor for strain M-506 (Table 12): for a given concentration of $CaCO_3$ there existed an optimum concentration of ammonium sulfate. The role of $CaCO_3$ stems from its buffer and/or complexing properties, and not its calcium content; omission of $CaCO_3$ from the medium reduced yields by 50 %; moreover, the control yield was not restored by supplying equimolar concentration of $Ca(NO_3)_2 \cdot H_2O$ or $CaCl_2 \cdot 2H_2O$. Those results were considered in designing medium 6 (Table 1) which served to test the productivity of survivors of strain M-506 isolated after a second irradiation (Fig. 6, uv-4b). Six out of 396 survivors were superior to the parent (110~120 %). They were retested several times in several media, and one strain was selected that yielded repeatedly 115 % the productivity of the parent, and was designated strain M-606. It had survived a 10-minute exposure.

Mutagenesis of Strain M-606

From the foregoing results it became obvious that the mutagenic treatment gradually decreased in efficiency and yielded less and less strains superior to the parent. It could also be that the test medium was no longer suitable or that the mycelium which retained almost quantitatively the antimycin A produced was saturated. Strain M-606 was submitted to ethylenimine and the survivors tested in medium 8 (Table 1), a synthetic medium; yields were

purposely reduced to detect more easily increased productivity of the survivors. Four out of 311 survivors gave up to 150 % the productivity of the parent. They were re-tested in media 6 and 7, and one survivor produced consistently 10 % more antimycin A than the parent. It was designated strain M-706. In a separate experiment strain M-606 was submitted to consecutive ethylenimine and ultraviolet treatments, and the survivors tested in medium 6: none of the 393 survivors tested yielded 10 % more antibiotic than the parent.

The productivity of selected strains is summarized in Table 13. The respective role of the

Table 13. Average yields of antimycin A-producing strains in various media (μg/ml).

Strain No.	Medium and condition combination No*.						
	1	2	3	4	5	6	7
M-101	65	120		204	316		
M-106	248	503		646	1,938		
↓							
M-206		690		856	3,164		
↓							
M-306		870	1,260	1,960	4,492		
↓							
M-406			1,676	2,070	4,952		
↓							
M-506					5,512	7,180	7,500
↓							
M-606				2,800		7,800	9,500

* Table 1.

strain and the medium emerges clearly; for a given strain yields increase from medium 1 to medium 7, and for a given medium, yields invariably increase from the parent to the most recently improved strain.

Variation of Colony Morphology

When spore suspensions were plated on PRIDHAM's medium all strains appeared homogenous as to their colony morphology; however, their heterogeneity became obvious when they were

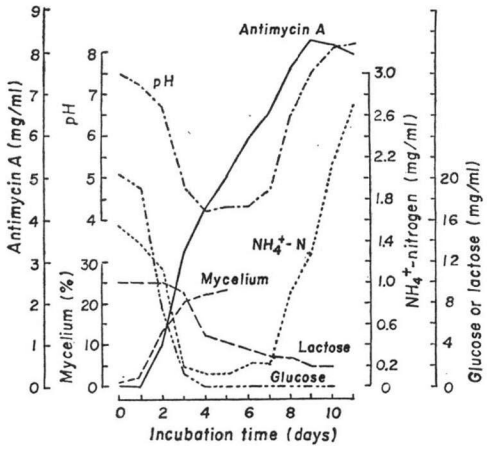
Table 14. Comparative productivity of white and grey colonies of *Streptomyces* sp. M-506 and M-606.*

% of Parent M-506				% of Parent M-606	
white	grey	white	grey	white	grey
39	107	9	107	41	113
49	102	60	108	13	109
49	91	72	109	36	105
50	92	96	107	15	123
46	103	67	111		99
51	105	47	106		103
49	104	39	99		105
54	107	53	110		
48	109	63	110		
49	107	24	96		

* Dilutions of M-506 and M-606 spore suspensions were plated on minimal medium, and white and grey colonies isolated after 1-week incubation. Test medium 7, Table 1.

plated on a minimal medium in an attempt to isolate auxotrophs. On this medium two distinct colony types could be distinguished, a white and a grey types. Twenty colonies of each type were isolated, transferred to PRIDHAM's medium, and tested for productivity. Each type yielded the same type of descendents on minimal medium, white or grey type; both types yielded undistinguishable colonies on PRIDHAM's medium. The productivity of white and grey colonies from strain M-506 is shown in Table 14: white colonies yielded only 50 % as much antimycin A as the control, whereas grey colonies produced slightly more than the parent. The same observation was made with white and grey colonies of strain M-606 (Table 14).

Fig. 7. Time course of antimycin A fermentation by strain M-506 in Medium 6 (Table 1).



Fermentation Characteristics

Time course of a typical antimycin A fermentation by strain M-506 in medium 6 is illustrated in Fig. 7. Three distinct phases are recognized: during the first phase, from inoculation to the third day, the organism grows to almost its maximal value, glucose and ammonia nitrogen are utilized very rapidly and almost completely, and pH drops to 4.3: antimycin A is also produced during this phase, rather slowly up to the second day, but at fastest rate between the second and third days, which corresponds to the late growth period. In the second phase, from the third to the seventh days, the mycelial mass reaches its maximal value, lactose begins to be utilized when glucose has almost completely disappeared, a typical "diauxie", ammonia nitrogen remains low, and pH does not change appreciably. From the third day on, antimycin A is produced at a slower but very steady rate. During the third phase, from the seventh day to the end, some lactose is still present and slowly utilized, and ammonia nitrogen is excreted into the medium to reach after eleven days a value higher than at the beginning of fermentation. This probably corresponds to a phase of autolysis, even though no morphological changes were observed in the mycelium; during this period the pH curve parallels that of ammonia nitrogen. Antimycin A is produced steadily at the same rate as that observed in the second phase and reaches a maximum after nine days. Much higher yields would probably be obtained at eleven days if the pH could be maintained below 8, since antimycin A is rapidly destroyed at pH values higher than 8.

Discussion

The decisive factors involved in strain improvement for antibiotic production are the organism itself, its responsiveness to the mutagenic treatments used, and a happy, repeated choice of spontaneous and induced variants through a powerful selective system[2,7,11,20,24]. The program is empirical, but its issue is generally successful; the objectives are more rapidly attained when the physical chemical properties of the antibiotic, and the fermentation characteristics are known.

Antimycin A consists of several fractions the biological activity of which is identical[21];

therefore, an assay method for total antimycin A was suitable. (An alternate assay based on inhibition zones developing around isolated colonies top-layered by the test organism[14] would eliminate the tedious shake flask work, but could not be adapted successfully using *Saccharomyces cerevisiae* as the test organism). In the course of strain selection only one change in composition was observed: it involved a 50 % increase in the A_1 fraction and took place in strain M-406[21].

This advantage of an accurate and convenient assay for antimycin A is counter-balanced by the drastic pH changes during fermentation: growth was impaired by a sharp pH drop at the beginning of fermentation, and the antibiotic was rapidly destroyed at the high pH values attained at the end of fermentation; the addition of a slowly utilizable sugar during the course of fermentation led to high yields, but prolonged the process considerably and decreased the validity of corrections for evaporation. This tended to decrease the effectiveness of the selective system. Automatic pH control is impractical when a large number of flasks is involved, and addition of buffer, especially phosphate, at any stage of fermentation, halted production abruptly.

For comparing efficiently spontaneous and induced variants all other factors must be kept constant. Maintenance, propagation and preservation were rigidly controlled, as well as incubation conditions (temperature, initial pH, aeration, and agitation) once their optimum values were known. Medium composition was also kept constant in a given experiment, but was changed periodically to meet the nutritional requirements of selected strains. It is obvious from Table 13 that in this dual program medium improvement contributed as much as strain selection in the overall yield increase. Used alone medium changes are a waste of time, as shown for strain M-101 at the beginning of this work; combined with mutation and selection they speeded up production increase. From Table 13 it is noteworthy that yields climbed from 248 to 9,500 μg/ml through only five steps of selection from strain M-106.

A relatively high number of isolates or survivors must be tested to obtain several high-producing strains to choose from[11]; this precaution also allows for applying other selective criteria, such as conidiation[24] and lack of pigment production. It is shown in Figs. 4 and 6 that at each selection step several desirable strains were available: the strain selected for further work was rarely much more productive than its sisters, but it was never found necessary to return to a sister strain, since the selected strain never proved disappointing. In antibiotic production the usual yield for an original strain lies between 25 and 50 μg/ml[20], hence the interest in isolating better strains[26]. Strain M-106 was an exceptional producer for an unimproved strain: it produced more than any spontaneous and induced variant obtained previously from strain M-101, was much more sensitive to ultraviolet radiation (Fig. 1) and more reproducible (symetrical distribution in Fig. 5). There is no doubt that ultraviolet radiation increased variability (compare Figs. 2 and 3 for strain M-101), and that the largest number of high-producing variants was observed for a particular exposure; in Figs. 4 and 6 a two-number designation was used for every survivor of mutagenic treatment: the first figure refers to the time of irradiation, the second one to the isolate number in the order of its isolation. For example, 1/16 is the sixteenth isolate obtained at 1-minute exposure. In Fig. 6 three strains were isolated at 1-minute exposure and one at 10-minute exposure of parent M-206; three strains were isolated at 10-minute and two at 15-minute exposures of parent M-306. However, in the latter case, the selected survivor was from the exposure that yielded the least number of high producers. Selected strains were generally from a high kill[11], sometimes beyond the exponential part of the curve[35].

The sensitivity to ultraviolet radiation was much higher for strain M-106 than for M-101, but did not change among the descendents of M-106 even through several rounds of irradiation (Fig. 1). The same degree of variability was introduced by this mutagen in strains M-106, M-206 and M-306, but strains M-406 and M-506 were more stable. No high producer could be isolated among the survivors of M-406, and two successive irradiations led to the same distribution patterns for strain M-506. Ethylenimine treatment of strain M-606 restored variability, but the combined treatment was unsuccessful. From those results it is not possible to say that a mutagen was better than another; when desired variability is not obtained with a

given mutagen, a new mutagen should be used[85].

The presence of white and grey colonies was detected by chance *i.e.* when a presumably pure culture of strain M-506 was replicated on minimal and complete media to isolate auxotrophic mutants[18,19]. The presence of white variant in the grey culture did not impair yields, but grey colonies were much better producers than white ones. This major variation of grey to white, in contradistinction to the selection of strain M-206 (pigmentless), led to lower productivity[16].

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