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09320

Distr.
LIMITED

ID/WG.300/2
22 May 1979

ENGLISH

United Nations Industrial Development Organization

Regional Seminar on Industrial Applications
of Microbiology in Pharmaceutical Industry

Havana, Cuba, 2 - 9 July 1979

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MICROORGANISMS AND THEIR ROLE IN THE FERMENTATION PROCESSES INCLUDING BIOSYNTHESIS OF ANTIBIOTICS *

by

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id.79-3886

Biosynthesis of vitamins, antibiotics, and other biologically active metabolites is a property widespread among microorganisms. They played an important role in production of different pharmaceutical compounds, antibiotics, steroid drugs, vitamins, aminoacids, enzymes, substances of utmost importance for medicine. Industrial microbiology includes some of the oldest of man's social and domestic activities, for baker yeast, wine-making, vinegar production were carried out by many of the early civilizations.

The microbiological industries as they exist today can be classified into three main groups. There are the alcoholic beverage industries, including yeast production, brewing, beer and wine production which are not only the oldest but also the largest group. In many ways, they differ fundamentally from other microbiological industries.

Another group of microbiological industries concerns with the production of fermented food and food supplements.

In the search for ways of increasing the amount of food produced, microorganisms are involved in protein production (so called single cell protein).

Already some growth-promoting substances for plants, new types of herbicides and insecticides are produced by microorganisms.

Pharmaceutical and, first of all, food industry make use of the capability of microorganisms to synthetize exogenous aminoacids, which are necessary for proper human and animal nutrition. The best producers of aminoacids are species of Micrococcus and Bacillus.

Vitamins are produced basically by microorganisms isolated from soil. The bacteria synthetize in the majority of cases vitamins of the B group e.g. several species of the genus Azotobactor (family Azotobacteraceae) are source of thiamin, pyridoxine and biotin. Production of vitamin B_{12} has been carried out using Bacillus megaterium.

Fungi are also frequently applied for vitamins production. Most of the riboflavin produced microbiologically comes from cultures of Ashbya gossypii and some Candida species (Candida guilliermondii and Candida flareri).

The major part of actinomycetes also shows the ability to the synthetize different vitamins, for example, panthotenic

acid, biotin and nicotinic acid are produced by a strain of Streptomyces rimosus.

The liaison between the pharmaceutical and microbiological industries has become very firm during the past two decades, following the discovery of antibiotics.

The applicability of microorganisms for pharmaceutical industry, especially in drug synthesis, is a result of cooperation of microbiology, biochemistry organic and physical chemistry and biotechnology. More recently, the pharmaceutical industry has found another very profitable use for microorganisms in the production of steroid drugs by microbiological transformation. The production of steroids has become one of the important branches of pharmaceutical industry.

The production of many steroids consists of transforming readily available basic steroids microbiologically to important intermediates, which later on they can be converted chemically to the final product.

Particular position among organisms used in pharmaceutical industry is occupied by antibiotic-producing microorganisms. Antibiotics have widespread application in human and animal therapy, agriculture, food preservation and as tools in biological research etc.

The economical role of the natural products of microorganisms is of utmost importance. Total quantity and value of some natural products produced by microorganisms are given in Table 1.

Hundred of thousands of different microorganisms isolated mainly from soil were screened for their antibiotic-activity. Recently several clinically valuable antibiotics are produced on a commercial scale. Although organic chemistry has succeeded in synthesizing several antibiotics, nearly all of the industrially and clinically important ones, are produced biosynthetically.

In biosynthesis of natural antibiotics the following taxons of microorganisms are the main ones: Actinomycetales, Eubacteriales and Aspergillales.

Up to the end of the 1940's, fungi, and to a lesser extent bacteria, furnished the greatest number of antibiotics discovered. Between 1955 and 1962, however, about 80% of antibiotics found, originated from different genus of the order of Actinomycetales. Now, more than 50% of antibiotics are produced by streptomycetes (Fig. 1). In the past 10 years, again the ratio of antibiotics isolated from Actinomycetales diminished decisively. The ratio of new antibiotics especially those found in fungi, shows now a tendency to increase (Fig. 2).

Great screening program started after 1945, utilized simple methods for the isolation of the large number of strains from soil samples, resulted in hundreds of antibiotics of Actinomycetales origin. The highest number of strains producing antibiotics was found among Streptomycetaceae (Fig. 3). About 90% of antibiotics described up to now, were isolated from Streptomyces species.

Antibiotics isolated from Streptomyces species belong to different chemical groups (macrolides, aminoglicosides polyenes, tetracyclines, peptides, β-lactams). Some Streptomyces species, producing antibiotics of the greatest importance in chemotherapy of infectious diseases are listed in Table 2.

The importance of non-Streptomyces microorganisms belonging to Actinomycetales in an interesting and an intensively studied problem in antibiotic research. The ratio of antibiotics of non-Streptomyces origin amounted to 4-5% up to 1965 and has since risen to 10%. Gentamicin, rifamycin and ristocetin take their origin from Micromonospora and Nocardia species. Some problems are posed by the uncertain identification methods of these groups of microorganisms. Nocardia mediterranei producer of rifamycin

was for a long time supposed to be a Streptomyces species. Probably because of the progress both in new techniques for collecting and processing soil and marine samples for the isolation of microorganisms, and in more sensitive technique for the detection of their antibiotic activities, new antibiotics have been discovered in particular from species belong to the Actinomycetales other than Streptomyces.

Microorganisms belonging to Actinomycetales furnished the greatest part of antibiotics in medical and non-medical use, altogether about 70 of them are utilized today. Despite their declining ratio, Actinomycetales still promise to be the richest source of useful antibiotics in the future. In the past 10 years, alle new antibiotics introduced in practice, altogether 25, were furnished exclusively by streptomycetes, except for fusidic acid.

The antibiotics of fungal origin are frequently used in therapy. Primarily Penicillium and Aspergillus and some other species belonging to fungi imperfecti were of great practical value with respect to antibiotic production. From antibiotics of fungal origin up to 1974

10 have been commercialized: benzy penicillin, penicillin V, penicillin O, cephalosporin, griseofulvin, fumagillin, variotin, fusidic acid, siccanin, and xanthocillin. As yet no new antibiotics of fungal origin, comparable in importance to β-lactam antibiotics, has been isolated. In human therapy of several infectious diseases the semisynthetic β-lactam antibiotics play now a very important role.

Among Eubacteriales and to a smaller extent members of the order Pseudomonadales may be of importance as sources of antibiotics. Within the Pseudomonadales, exclusively Pseudomonas species, and within Eubacteriales only Bacillus species, deserve attention. Up to now about 10 agents of bacterial origin have been introduced: colistin, polymyxins B and M, gramicidin, gramicidin S, tyrothricin, bacitracin, and atterimin from Bacillus species. Pyocyanin and pyrrolnitrin from Pseudomonas species, and finally nisin from other Eubacteriales. Except for pyrrolnitrin, all were discovered before 1950 (Bérdy, 1974).

The systematic screening of a large number of strains belonging to Eubacteriales, Myxobacteriales, Mycoplasmatales, as well as about 50 000 fungal species, neglected up to now, and further screening of alge and lichenes

may lead eventually to the discovery of further new types of antibiotics.

At present the prospect of achieving results in the field of semisynthetic antibiotics seems more promising than in that natural ones. Research in the field of new penicillin and cephalosporin derivatives continues unabated and may result in the synthesis of further new pharmaceuticals of improved properties.

Isolation of microorganisms from the natural environment is now a problem not as fundamental as it was several years ago, though search for new antibiotic sources still remains important and purposeful.

Much more attention is now paid to improvements of the antibiotic yield, by the use of mutants and by making use of the genetic recombination.

Microorganisms with higher antibiotic yields are obtained first of all by induced mutations, and by making use of hybridization and recombination in microorganisms.

Mutation of a specific gene, which occurs under life conditions normal for the organism, is referred to as spontaneous mutation. Its frequency in bacteria is 1 per 10^4 to 1 per 10^{10} cells per generation. In fungi the frequency of spontaneous mutations of the individual

genes was shown to be of the order 1 per 10^5 to 1 per 10^6 nuclei per generation.

Mutation frequency can be increased by treatment of the organism with various physical and chemical agents. Among strong mutagens can be mentioned ionizing radiation (X rays, neutrons, gamma and beta particles) and UV radiation. Strong mutagenic effects are also exerted by chemical agents such as alkylating compounds, nitrous acid, base analogues, and acridine stains.

X rays, UV light, and alkylating compounds (mainly nitrogen mustard and ethyleneimine) are most frequently applied as mutagens in studies on potentiation of the antibiotic yield of actinomycetes, bacteria, and fungi.

The effect of strong mutagens on microorganisms is determined largely by the activity of various repair system capable of acting on single strand gaps formed in the DNA either directly or indirectly. Two such systems, excision repair and post-replication repair, and their effects are described on the basis of work with bacteria. A third system of largely unknown mechanism is error-prone and appears to operate as a minor alternative pathway simultaneously with the two major pathways.

Ultraviolet and ionizing radiations, most alkylating agents cau mutations by misrepair through this pathway.

Some microorganisms do not possess such an error-prone repair system and therefore be essentially immutable by these agents. In these organisms a mutagen must be used that operates by a causing replication errors rather than repair errors (Bridges, 1976).

By combining mutagenic factors and applying selection, the antibiotic yield of many strains has been increased. However, it should be emphasized that changes leading to increased yield of antibiotic producing strains is a very slow and gradual process. Penicillium chrysogenum Thom NRRL 1951 is a well known example. All industrial strains of Penicillium chrysogenum derive from the Wisconsin family, the results of vast mutation program performed at the Botany Departments of Wisconsin University between 1946 and 1956, which gave at last a fivefold improvement in penicillin yield. The Penicillium chrysogenum NRRL 1951 was a wild strain. Figure 5 illustrates the genealogy of the "Wisconsin family". X-irradiation of its natural variant NRRL 1951 B25 with increased yield gave variant X-1612. Next, UV irradiation (275 nm) gave

highly productive Q-176 strain which was for many years used in the production of penicillin. A further mutant BL_3D_{10}, gave about 25% lower yield, but was unpigmented and produced colorless penicillin, which was much easier to purify. Selection of this strain again increased its yield (NRRL 48-701), and after nitrogen mustard was used as mutagen the high-yield unpigmented NRRL 49-2105 strain was obtained, but this mutant produced a considerable amount of red-brown pigment which was an obstacle in the purification procedure. The line of strains treated with nitrogen mustard gave the unpigmented high yielding mutant NRRL 51-20 (Backus and Stauffer, 1955). Single mutants of Aspergillus nidulands with raised penicillin yields were isolated from descendents of the Glasgow wild-type strain NRRL 194 following ultraviolet light treatment. Each mutation was mapped to its chromosome by parasexual haploidization analysis (McCully and Forbes, 1965).

Improved chlortetracycline-producing strains were obtained in Streptomyces aureofaciens after the treatment by X-rays, UV-rays and other mutagens. Ultraviolet treatment seems to give the best results (Sermonti, 1968).

Together with mutation, the recombination process was basical for obtaining microorganisms whose characters are important in antibiotic production. Recombination results in a combination of parental genes in the progeny. Crossing of strains with defined phenotypes, useful in a given system, leads to accumulation in one genotype as high a number of positive characters as possible.

In eukaryotic organisms the occurrence of regular sexual cycle is a condition fundamental for recombination.

Nuclear fusion and gene segregation can, however, although only occasionally also take place in absence of sexual cycle, by means of processes which have been described as the "parasexual" cycle. In the parasexual cycle the same genetic phenomena occur as in the sexual cycle, namely, complete nuclear fusion followed by chromosome reassortment and crossing-over. However, these processes occur in the somatic cells at a very low rate. This system is only available in the Penicillium chrysogenum, Cephalosporium acremonium and some strains of Aspergillus niger. The life cycle of all these fungi is predominantly haploid, with occasional heterokaryon formation at frequencies varying significantly between species. From these heterokaryon occasional diploid nuclei may be selected as first demonstrated by Roper (1952).

The frequency of heterokaryon formation can be increased by applying the protoplast fusion. The latter makes it also possible to obtain interspecies hybridization (Anne et al., 1976).

Since the discovery of the parasexual cycle, every attempts have been made to use it for the improvement of the production of penicillin. In Penicillium chrysogenum strains carrying spore colour and auxotrophic markers were used as parents in which single-step yield increases were induced by UV. The mutants had yield about 3000 U/ml. Strains carrying different positive mutations were crossed in an attempt to obtain segregants carrying both mutations. Some crosses produced segregants with significantly increased yield. The effects are promising results for the future application of recombination to yield improvement (Ball, 1973, Hopwood and Merrick, 1977).

Recombination is common among actinomycetes, too. Transformation and transduction are of little importance in elevation of the antibiotic yields in strains of the genus Streptomyces. Major part of genetic studies in this field are based on the conjugation process. Conjugation in actinomycetes may lead to heterokaryon formation (actinomycetes are the only example of prokaryotic organisms

in which this phenomenon was observed), to recombination, or to plasmid transfer.

In actinomycetes a cycle analogous to the parasexual cycle in fungi occurs. A fundamental difference, however, consists in instability of the diploid in the heterozygous mycelium.

The conjugation phenomenon was observed in actinomyces strains that produce a number of practically important antibiotics. Unfortunately, it remains hitherto without significant effect on the antibiotic yield.

Only in the case of chlortetracycline-producing Streptomyces aureofaciens and oxytetracycline-producing Streptomyces rimosus, yields higher by 5 to 20% were found in prototrophic recombinants obtained by conjugation of auxotrophic mutants differing in the antibiotic yield (Sermonti, 1968).

In actinomycetes and bacteria, methods applied for genetic improvement of the industrial strains are based first of all on mutations induced by various mutagenic agents. The use of recombination in these microbial groups is limited to a few species only. This process is of importance for improving the antibiotic yield of eukaryotic organisms. May be in future it will appear to be more applicable.

Proper storage of the production strains is an essential problem associated with the use of microorganisms for production of biologically active compounds, including antibiotics. High-yield strains, which are usually obtained by long and troublesome studies, have to be started under conditions that preserve stability of their properties.

Many industrial microorganisms degenerate or "run down" following successive vegetative transfers. Examples of this degeneration, a result presumably of the production of mutant strains of the microorganisms are quite commonly encountered. In a study of this phenomenon in Streptomyces griseus was found that during 100 serial transfers of the organisms on yeast-glucose agar, there was a progressive "degeneration" of the streptomycete. A significant decrease in antibiotic (streptomycin) production was quickly noticed. After 58 transfers, the cultures showed decreased ability to sporulate, and this was followed by the appearance of mealy growth and finally, the complete loss of spores producing ability. It was, found, however, that significant changes in certain other biochemical activities of the streptomycetes, including the ability to produce vitamin B_{12}, did not accompany this loss of antibiotic-producing ability.

There are several methods available for maintaining cultures of microorganisms. The aim of all these methods is store the organism under conditions in which biological activity is reduced to a minimum, while at the same time ensuring that the microorganism remain alive. Recently more and more frequently the methods of deep freezing of the spore suspension (-186°C), and storage in liquid nitrogen are applied. These methods have been worked out for fungi and actinomycetes that produce spores. Storage period amounts to several months.

The most generally used method today is lyophilization or freeze drying. Lyophilization involves a small amount of microorganism in a vehiculum, usually serum, milk or sugar solution, freeze drying this under high vacuum and finally sealing off also under high vacuum. Lyophilization would appear to be the method of choice, particularly for maintaining large collections of cultures.

A techniques which is very valuable in maintaining cultures of spore-forming fungi is to store organisms in sand or soil.

For routine laboratory use, cultures of microorganisms are often maintained in open stock culture on solid media. The cultures are stored at low temperatures in order to

avoid drying up to the agar and to restrict the amount of microbial growth. The cultures need to be transferred at regular intervals. With some microorganisms, frequent transfer has been shown to lead to a loss of morphological characteristics and physiological properties. On the whole, therefore, this is not a method recommended, especially for maintaining collections of microorganisms. It remains, nevertheless, the only method for certain groups of fungi, especially non-sporeforming mould and fungi.

Many cases of phage infection of the antibiotic - producing strains are described in the literature. This may lead to a total destruction of the industrial microbial culture, thus to the production stop, what results in large economic losses.

The phenomenon of lysis in actinomycetes - the main antibiotic producers - has been known for a long time. Schatz and Waksman (1945), by their observations on streptomyoin-producing Streptomyces griseus cultures, contributed to the discovery of the lytic factor. When streptomycin production was stopped in 1947 because of lysis of the production strain mycelium, the factor inducing lysis was found to show features of a phage. The term actinophage

was proposed for phages acting on actinomycetes (Reilly and et al., 1947).

At the same time many reports appeared on the occurrence of specific virulent actinophages during biosynthesis of other antibiotics. Actinophages were demonstrated to appear in the fermentation broth of chlortetracycline-producing Streptomyces aureofaciens, rifamycin-producing Nocardia mediterranei, and novobiocin-producing Streptomyces spheroides. Common occurrence of phage infections in the industrial strains stimulated studies on the causative agents of these infections and on their elimination.

Actinophages able to infect industrially important culture are ubiquitous; they may be found in soil, water, air, or the microbial culture itself.

Although phages are generally designated as lytic or temperate, it must be remembered that lytic phages isolated from the soil may able to lysogenize appropriate recipients and that temperate phages are virulent for numerous indicator strains. Moreover, a temperate phage, through mutation or recombination, may yield lytic variants.

The probability of phage infection of industrial actinomycetes fermentation broth is higher, since lysogenic strains are used in which phage induction occurs under the influence of various factors, often unknown. Because of a widespread distribution of lysogeny among actinomycetes (Rautenstein, 1957; Bradley, 1969), a selection of non-lysogenic strain for production is difficult and sometimes impossible. Production culture should be routinely tested for phage, and new substrains developed as needed by selection. The infection might be due to specific actinophages which get into the fermentors from outside during the biosynthesis process.

Methods aimed at protection of the fermentation broth against phage infection consist in the use of phage-resistant strains, disinfection of the production area (natrium chlorate chloramine). An important problem is sterility of the air accessing the fermentors and fermentation broth.

The success of the antibiotics industry had a tremendous impact on the microbiological industries as a whole. First, it showed how co-ordinated effoort between microbiologists, biochemists genoticists and biotechnologists can lead to very rapid advances being made in a very short

period of time. Secondly, it extended the rang of industrial microorganisms to include a hitherto little known group of organisms, the actinomycetes. Finally, it led to the development of a close liason between the pharmaceutical and microbiological industries, which has since brought other developments, notably in the use of microorganisms eg. in biosynthesis of compounds with different biological activities. In this search for new products in the microbiological industries it has become necessary to exploit to the full extent the synthetic capabilities of microorganisms.

Table 1

The quantity and value of some products produced by industrial microorganisms

Product	Quantity of a product manufactured per annum	Value in US $
Beer	550 millions hl	22.5 milliards
Wine and other spiritis	300 millions hl	15.0 milliards
Baker's yeaste	600 000 tons	250 millions
Feeding yeaste	800 000 tons	400 millions
Citric acid	290 000 tons	335 millions
Antibiotics	8 000 tons	1.5 milliards
Glutaminic acid	100 000 tons	300 millions
Corticosteroids		325 millions

Table 2

Some strains of genus Streptomyces producing antibiotics most often used in therapy

Streptomyces strain	Antibiotics
S.griseus	Streptomycin, Chromomycin A
S.venezuelae*	Chloramphenicol*
S.aureofaciens	Chlortetracycline, Tetracycline
S.rimosus	Oxytetracycline
S.erythreus	Erythromycin
S.antibioticus	Oleandomycin
S.narbonensis var. josamyceticus	Leucomycin (josamycin)
S.ambofaciens	Spiramycin (Rovamycin)
S.floridae S.vinaceus	Viomycin
S.orchidaceus S.garyphalus S.lavenduline	Cycloserine
S.noursei	Nystatin (Fungicidin)
S.hachijoensis	Trichomycin
S.albo-niger	Puromycin (Stylomycin)
S.spheroides	Novobiocin
S.orientalis	Vancomycin
S.kanamyceticus	Kanamycins
S.rimosus af.paromomycinus	Paromomycin (Aminosidin, Catenulin, Hydroxymycin)
S.pristinae - spiralis	Pristinamycin
S.lincolnensis var.lincolnensis sp.n.	Lincomycin
S.caproolus sp.n.	Capreomycin
S.nodosus S.sp. M 4575	Amphotericin B
S.caespitosus	Mitomycins

Table 2 (continued)

Streptomyces strain	Antibiotics
S.olivoreticuli	Olivomycin
S.ardus n.sp.	Porfiromycin
S.verticillus	Bleomycin
S.peuceticus S.coreuleorubidus	Daunorubicin
S.tenebrarium Higgens and Kastner	Tobramycin (Nebramycin)

* Produced mainly by chemical synthesis

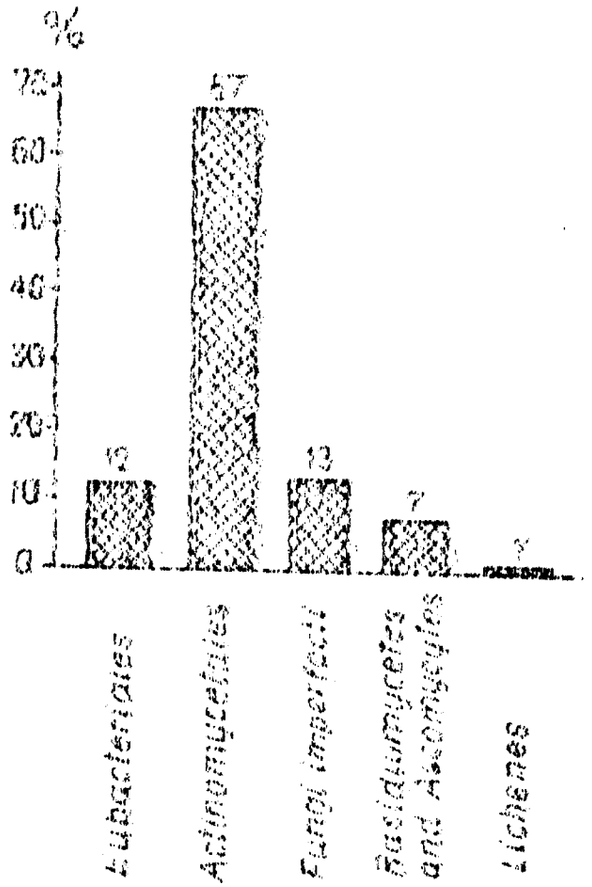
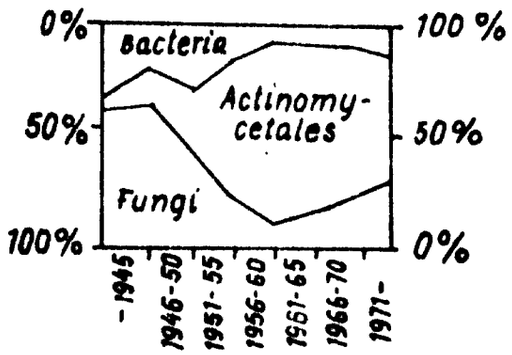


Fig. 1. Origin of antibiotics

Fig. 2. *Percentage distribution of antibiotics according to producer type of microorganisms* (Bérdy, 1974)



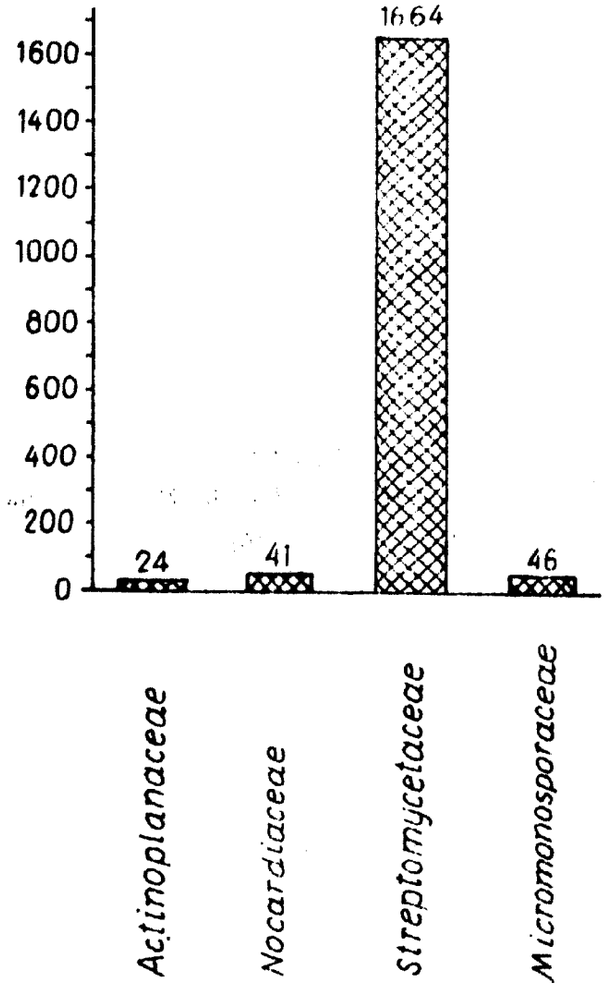


Fig. 3. *Number of antibiotics produced by Actinomycetales*

Fig. 4

Useful antibiotics from Actinomycetales other than Streptomyces (Nara et al., 1977)

Micromonospora	Actinomadura	Actinoplanes	Streptosporangium	Nocardia	Others*
Aminoglycosides	Ansa macrolides	Depsipeptides	Bleo-Phleomycins	aminoglycosides	
[illegible] macrolides	Anthracyclines	Peptides	Chloramphenicol	Ansa macrolides	
Macrolides	Peptides	Polyenes		Chloramphenicol	
Peptides				β-Lactams	
				Macrolides	
				Nucleosides	
				Polyenes	
				Tetracyclines	
				Vancomycin	

* Spirillospora, Planomonospora, Saccharomonospora, Micropolyspora, Microbispora, Chainia, Termoactinomyces, Pseudonocardia, Actinosporangium, Streptoalloteichus

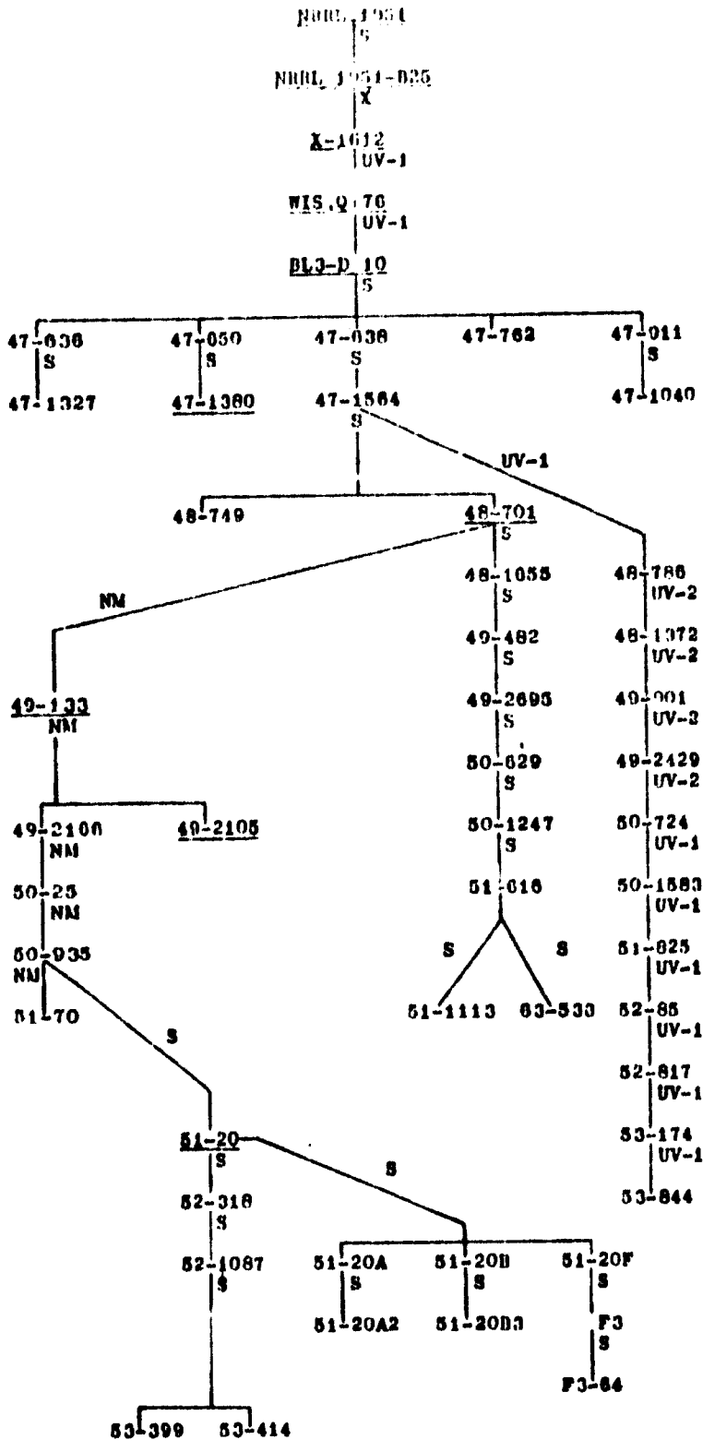


Fig. 5. **Genealogy of Penicillium chrysogenum strains of the Wisconsin family.** UV-1 - selection following UV irradiation 2750 Å, UV-2 - selection following UV irradiation 2537 Å, S - selection without preceding mutation, X - selection following X ray irradiation, NM - selection after nitrogen mustard treatment

METHODS USED IN SELECTION OF YIELDING STRAINS FOR THE PRODUCTION OF ANTIBIOTICS

Microbiology and pharmaceutical industry are now facing a task to maximalize the antibiotic yield of microorganisms, because of continually increasing need for antibiotics, especially those applied as drugs.

The highest possible antibiotic yields can be accomplished in practice by various methods, such as the use of precursors, metabolic inhibitors, enzyme stimulators, precisely formulated production media, and genetic procedures (Berdy, 1974).

Positive results of selection of microorganisms with high capability of producing antibiotics were obtained particularly by various genetic methods. Genetic analysis may contribute to the elucidation of the mechanisms of DNA modifications at molecular level, followed by phenotypic manifestation. As a consequence, by selection of adequate methods, it is possible to control in part the process

of microbial variability toward an increase in the antibiotic yield.

The knowledge of the antibiotic synthesis process is still too little as for the requirements of the contemporary industrial genetics. Microorganisms that produce antibiotics are seldom a model for genetic research of gene-enzyme relation or the regulation of biochemical biosynthesis pathways.

The quality to produce an antibiotic is widely distributed in nature. However, major part of the antibiotics applied in human and animal therapy, or used as tools for metabolic research are produced by microorganisms of the order Actinomycetales and Eubacteriales and by fungi belonging to the class Fungi Imperfecti.

Actinomycetes and Eubacteriae are prokaryotic organisms, while fungi represent Eucaryota. These two groups differ fundamentally with respect to the structure of their genetic apparatus, and specificity of genetic phenomena that occurs in a given groups is determined by this fact. The deoxyribonucleic acid (DNA) of eucaryotes occurs in several separate chromosomes together with structural and regulatory proteins (histone); complex nuclear division

mechanisms (mitosis and meiosis) ensure the exact partitioning of genes to daughter nuclei and progeny; and the chromosomes are retained in the discrete region of the cell by a nuclear membrane. The chromosome of procaryotes, on the other hand, is a single circular DNA molecule with few, if any, protein molecules permanently associated with it, and there is no nuclear membrane. Procaryotes possess plasmids - circular DNA molecules considerably smaller than the chromosome and representing genes dispensable, at least under certain conditions to the organism carrying them. Such genes determine characters - sex, antibiotic production or resistance, pathogenicity etc. Plasmids are carried by only a proportion of populations members. In Eucaryotes genetic research depends on sexual reproduction, involving fusion of haploid nuclei and their formation again by reduction in meiosis. In some fungi parasexual phenomena occur consisting in somatic recombination without sexual reproduction. It is the only system available in the imperfect fungi (Pontecorvo and Sermonti, 1954).

On the other hand, in procaryotes occur several processes transformation, transduction, and conjugation, with the same genetic consequences as sexual reproduction - the

creation of new combination of genes - but differing markedly from it.

After the discovery of antibiotics as natural products, an extensive exploration of natural habitats (mainly soil) is search of new producing strains has started.

Isolation of microorganisms from the natural environment is now a problem not as fundamental as it was several years ago, though search for new antibiotic sources still remains important and purposeful.

Much more attention is now paid to improvements of the antibiotic yield, by the use of mutants and by making use of the recombination variability.

Attempts at increasing the yield by genetic procedures were unexpectedly successful. Studies on Penicillium strains, carried out by Alikhanian (1962) for over 12 years , made it possible to elevate the yield from about 20 to about 8000 penicillin units per ml. Backus and Stauffer (1955) were able to increase the yield of Penicillium chrysogenum strain from some 100 to some 2000 penicillin units per ml. Average yields of Penicillium chrysogenum strains now used for benzyl penicillin production are of the order of several thousands units per ml. As is the case with peni-

cillin, markedly elevated yields were also obtained for strains producing streptomycin, chlortetracycline, oxytetracycline, and erythromycin. The increase in yields of some antibiotics in recent years is illustrated by data in Table I.

Microorganisms with higher antibiotic yields are obtained first of all by induced mutations, and by making use of hybridization and recombination in both prokaryotic and eukaryotic organisms.

Mutations emerge most frequently by gene change into its new allele (gene mutation), by modification of the chromosome structure (chromosome structure mutation), or by modification of the chromosome number (chromosome number mutation). Mutations are expressed as phenotypic effects in prokaryotic and haploid organisms. In diploid organisms the modified gene in the majority of cases is recessive against nonmodified allele, and mutation can be expressed just in the next generations.

Gene mutation consists in a modification of the nucleotide sequence in a given gene, thus it occurs at DNA structure level. In a mechanism called transition one purine base is replaced by another, or a pyrimidine base is replaced by another. Substitution of purine by pyrimidine bas is referred

to as transversion. Deletion or insertion of a single or some nucleotide pairs are also possible.

DNA structural changes result in:

1. Mutations of sense change type - codon for a given aminoacid is replaced by another codon for another aminoacid. In the polypeptide thus formed a given aminoacid may change its position.
2. Mutations of nonsense type - codon for an aminoacid is replaced by a nonsense codon. The polypeptide chain thus formed will be shorter.
3. Translation phase change mutations, consisting in abnormal translation from the mutation point.In the peptide thus formed aminoacids are incorporated improperly.

Mutations are random in character, what means that it cannot be anticipated which gene and in what direction will undergo mutation in a given microbial cell population.

Beside gene mutations, there are also chromosome structural mutations, whose mechanism may be deficiency of a chromosome fragment, integration of an additional homologous chromosome fragment (duplication), translocation of a fragment within a chromosome, or inversion by 180^{0} of a chromosome fragment.

Mutation of a specific gene, which occurs under life conditions normal for the organism, or referred to as spontaneous mutation. Its frequency in bacteria is 1 per 10^{7} to 1 per 10^{10} cells per generation. In fungi the frequency of spontaneous mutations of the individual genes was shown to be of the order 1 per 10^{5} to 1 per 10^{6} nuclei per generation.

Mutation frequency can be increased by treatment of the organism with various physical and chemical agents. Among strong mutagens can be mentioned ionizing radiation (X rays, neutrons, gamma and beta particles) and UV radiation. Strong mutagenic effects are also exerted by chemical agents such as alkylating compounds, nitrous acid, base analogues, and acridine stains.

X rays, UV light, and alkylating compounds (mainly nitrogen mustard) and ethyleneimine are most frequently applied as mutagens is studies on potentiation of the antibiotic yield of actinomycetes, bacteria, and fungi.

Mutagenic effect of X rays first of all consists in inducing multiple ionizations upon penetration of the cytoplasm. The emerging ions, in conjuction with oxygen, give rise to highly reactive free organic radicals which induce mutations by acting on DNA or chromosomes. Indirect effect

of X rays is more essential in inducing mutation than direct action on DNA. Biological effect of X rays is commonly measured in terms of ionization units named roentgens (R). Mutation frequency is related to the number of R absorbed. Mutagenic effect of X rays is higher in oxygen - containing atmosphere, and increases as oxygen contents in the environment increase. According to many authors, mutants with many times higher antibiotic yield can be obtained using doses from 200 000 to 640 000 R, various irradiation doses being optimal for various microorganisms. The mutation index is highest when doses are applied inducing cell death in 70 - 90 percent.

Clear mutagenic effect is exerted by UV light, especially at 253.7 nm wavelenght. The curve of mutagenicity to wavelength relation fits perfectly the curve of UV absorption by DNA. It can be inferred, therefore, that mutagenic and lethal effects are due to direct UV absorption by purine and pyrimidine bases. UV - induced reactions in the irradiated cells are complex, since the radiation is absorbed not only by DNA but also by RNA, proteins, and free purines and pyrimidines. The mechanism of UV lethal and mutagenic effects consists in inducing the formation of thymine, cytosine,

and cytosine-thymine dimers. Thymine dimers are formed most frequently.

Exposure to ultra-violet rays (UV)

Various typed of lamps emitting ultra-violet rays of 2537 Å wave-lenght can be used for irradiation. The most commonly used is the "General Electric Germicidal Lamp - 15 watt". With a distance of 60 cm between the irradiated object and the source of irradiation (the above-mentioned type of lamp), the intensity of irradiation amounts to 16 erg/sec/mm^2; other authors calculate that at a distance of 45.72 cm (18 inches), the intensity of irradiation is 20 erg/sec/mm^2.

For this type of work the "Philips" 57413 P/30 TUV 30 W germicidal lamp with quartz filter can also be used. The intensity of emission of ultra-violet rays for the latter lamp at a distance of 60 cm is 40 erg/sec/mm^2.

The intensity of irradiation is in direct proportion to the duration and in reverse proportion to the second power of distance. The duration of irradiation may vary from 1 second to 1 hour and the distance from 5 cm to 140 cm. The doses most frequently used in irradiating Actinomyces spores range from 2000 to 10 000 erg/mm^2.

Irradiation is best performed in a darkroom, in order to avoid reactivation by visible light.

Before starting irradiation with ultra-violet rays a curve should be drawn out depicting the survival ratio of the defined microorganism and given lamp.

The suspension of Actinomyces spores should be prepared immediately before irradiation. Storage of spores is permissible only for hours at $4^{o}C$. Three to four hour incubation of spores before irradiation considerably increases their sensitivity to ultra-violet rays.

The greatest number of mutants is most frequently obtained by lethal or almost lethal doses of ultraviolet rays. Various mutants may show changed morphological and physiological features and may produce less, the same amount or more antibiotic as compared to the initial strains. Irradiated spores do not grow simultaneously and often show delayed growth.

In order to obtain spores for irradiation, a mature culture of a strain on oblique agar should be washed off by a sterile solution of physiological saline (a minority of authors recommends distilled water). In order to obtain separate spores, the suspension should be shaken together with sterile small glass pellets for 2-20 minutes, then

filtered six times through cotton and centrifuged. Next, the density of filtered spores should be measured by the turbidimetric method or counted in a Thoma-Zeiss camera and diluted in physiological saline solution in such a manner as to obtain from 4×10^6 to 2×10^8 spores per 1 ml (various researches give different densities).

The suspension of spores of known density is poured into flat, circular containers made of glass penetrable by ultra-violet rays (quartz or silicate glass, for example), in the amount of, for example, 15 ml of suspension into a 100 ml container. During irradiation the container rotates at a rate of 330 revolutions per minute, in order to secure uniform exposure to ultra-violet rays. A 10 cm Petri plate may also be used, into which 10-15 ml of suspension is poured (the suspension of spores should be approximately 2 mm high). During irradiation the plate is opened and the spores are put in motion by means of a magnetic mixer. At certain defined intervals, parts of the material may be taken for examination during the process of irradiation.

The irradiated suspension of spores is immediately sown on Petri plates with the right agar medium, 0.1 to 0.2 ml per plate spread over the surface with a glass rod.

Other authors dilute the suspension of spores before sowing by means of physiological saline solution to a level of 40-400 spores per ml.

Irradiation of spores may also be carried out on the agar surface in the Petri plate. In order to do this, Petri plates with the right medium for the irradiated strain are prepared. After 2-3 days the slightly dried-up plates are inoculated with a defined amount of suspension of measured density (usually 0.1-0.2 ml of suspension with a density of 250-1250 spores per ml). It is advisable to keep the plates in automatic motion during irradiation. Open plates are irradiated.

The plates with irradiated spores are incubated in a thermostat at 28°C for 5-8 days and the colonies that grow are subjected to examination.

Because of the fact that results are frequently reported in the literature with different units for light intensity the following conversion factors may be found useful:

1 calorie = 4.185 joules

1 joule = 10^7 ergs

1 watt = 10^6 μwatts = 1 joule/sec. = 10^7 ergs/sec.

1 quantum = $\frac{47.6}{\lambda \text{ (in m}\mu\text{)}} \times 10^{-16}$ cal. = $\frac{1992}{\lambda \text{(in m}\mu\text{)}}$ =

= x 10^{-12} ergs.

At λ = 253.7 mμ, 1 quantum = 7.8×10^{-12} ergs.

For a G30T8 germicidal lamp, the effective mitting lenght of which is 81 cm, and the circumference 8 cm, the total ultraviolet emission is about 7 watts, or 10.801 μwatts/cm^2 at the surface, or 108,000 ergs/cm^2/sec. Since most of this is at 253.7 mμ, the emission at the surface is $108.000 \times (10^{12})$ 7.8/quanta/cm^2/sec. = 1.4×10^{16} quanta/cm^2/sec. at 253.7 mμ.

Alkylating compounds, such as nitrogen mustard, diethyl sulfonate, or ethylmethyl sulfonate, are mutagenic by acting of the reactive alkyl groups on guanine of the nucleic acids.

Methyl, ethyl, or a larger group is introduced into position 7 of the guanine ring, what is followed by disruption of the glycoside linkage between guanine and pentose, and this may lead to depurinization of the DNA molecule. Alkylating compounds that possess more than one functional groups may react with guanines in two complementary DNA strands, thus forming cross linkages.

Selection be means of nitrogen mustard (trichlortriethylamine)

1. Preparation of nitrogen mustard solution: Molecular weight of nitrogen mustard = 133. In order to obtain 1.2 M solution of nitrogen mustard it is necessary to weigh out 1.596 g of substance and dissolve it in 10 ml of water.

In order to obtain 0,5 M solution of nitrogen mustard it is necessary to weigh 0,66 g of substance and dissolve it in 10 ml of water.

Caution: the solution retains its effectiveness only for 20 min.

2. Preparation of suspension of spores:

10 ml of distilled water is poured into a test-tube containing a well-sporulating culture of the examined strain. It is then shaken. The suspension obtained in such a manner is filtered in sterile conditions through cotton-wool in order to obtain single spores. The number spores in 1 ml of suspension is then calculated and diluted in such a way as to obtain 1,000,000 spores per 1 ml.

3. Procedure:

5 ml suspension of spores should be added to 5 ml of nitrogen mustard solution, thus obtaining 500,000 spores in 0,25 M solution of nitrogen mustard. From solutions obtained in such a manner samples of 1 ml should be taken after 5, 10, 15 and 20 minutes. The obtained samples (1 ml each) should be diluted decimally i.e. to 1 ml of samples one should add 9 ml of distilled water, well mixed in order to remove the remains of nitrogen mustard and

sown on plates with sporulating medium. The plates should be incubated at 28°C for 10 days.

At the same time, an adequately diluted control suspension of spores (containing 500.000 spores per ml) should be sown in order to calculate the death rate and observe morphological changes taking place in spores exposed to the influence of nitrogen mustard.

Chemical compound used very often for the selection of antibiotic producing actinomycetes besides nitrogen mustard is ethyleneimine. Ethyleneimine reacts readily with carboxyl and hydroxyl groups, and with H_2S.

Ethyleneimine solutions in distilled water of concentration 1:3000 and 1:6000 after 24-48 hours caused marked morphologic variation. Solutions causing about 99% lethality are used for selecting high-yield strains.

Some authors have observed that ethyleneimine gives a higher mutation rate than UV or X rays. The best mutagenic effect was obtained with high concentrations of ethylenimins acting for a short time. The lethal effect was similar under various conditions.

In aqueous solutions ethyleneimine binds molecules of water giving ethanolamine, which exerts a lethal effect equal to that of ethyleneimine, without giving a mutagenic effect.

Supposedly, after longer exposure in solutions of low concentration, the strong lethal effect and weak mutagenic effect are related to the transformation of ethyleneimine into ethanolamine.

Studies on the mutagenic properties of ethyleneimine on actinomycete strains have shown that the maximum of variants with high activity is obtained after shorter exposure to ethyleneimine. Selection by means of ethyleneimine followed by combined exposure to ethyleneimine and UV yielded a stable variant with nearly twofold higher activity than the parent strain.

The effect of strong mutagens on microorganisms is determined very largely by the activity of various repair system capable of acting on single strand gaps formed in the DNA either directly or indirectly. Two such systems, excision repair and post-replication repair, and their effects are described on the basis of work with bacteria. A third system of largely unknown mechanism is error-prone and appears to operate as a minor alternative pathway simultaneously with the two major pathways. Ultraviolet and ionizing radiations, most alkylating agents cause mutations by mis-repair through this pathway.

Some microorganisms do not possess such an error-prone repair system and therefore be essentially immutable by these agents. In this organisms a mutagen must be used that operates by a causing replication errors rather than repair errors (Bridges, 1976).

Selective methods for the isolation of mutants are based on differences between the growth rates of the required mutants and their parent strain. The simplest method of all, ie to test a very large number of independent clones with the hope of finding at last one which shows some improvement over the parent strain. Screening in this way is a time-honoured method and without doubt has yielded strains of considerable importance for production processes. It may be the only possible method for selecting useful variants of some of the industrially important microorganisms.

A positive selection, method, using media which restrict the growth of the parent strain and allow the growth of particular classes of mutants, is probably the best method of all. Examples will be discussed of various ways in which the sensitivity of cultures to particular metabolic analogues may be enhanced by varying the growth medium or the growth condition of the inoculum (Clarke, 1976).

Some mutants (resistant mutants) are able to growth on media supplemented by a drug or toxic agent in conditions preventing growth of the wild sensitive cells. The gradient plate method was developed by Szybalski (1952) for isolating mutants resistant to a given antibiotic. Two slanted layers of agar are poured out on a plate, one of them containing the antibiotic. The concentration of the antibiotic is inversely proportional to the thickness of the upper layer of agar. The microorganisms are inoculated on the plate, and the concentration at which their growth is inhibited is noted. Highly resistant microorganisms can be isolated in the zones containing a high concentration of the antibiotic.

Selection method which can identify individual mutant colonies by biochemical reaction which are not given by perent strain are very useful too (Clarke, 1976).

Nutritional mutants (auxotrophs) are the most widely-adopted class of mutant in microbial genetics, and consists of strains unable to grow on minimal media, i.e. on the simplest medium able to support growth of the corresponding wilde type.

The following methods for isolating auxotrophs have been elaborated:

1. Total isolation, developed by Beadle and Tatum (1941), is based on the known fact that wild type colonies grow on complete as well as minimal medium because they possess the capability of synthesizing all the chemical compounds they need. On the other hand, auxotrophic mutants grow only on complete medium. In this method, the irradiated suspension of spores is inoculated first on complete medium, and then subcultured on minimal medium. Microorganisms which grow on complete medium but not on minimal medium are regarded as defective (auxotrophic) mutants and are examined on enriched media.

2. Delayed enrichment method, developed by Lederberg and Tatum (1946) detects defective mutants by partially eliminating prototrophs. The irradiated microorganisms are inoculated on a minimal medium on which only prototrophs grow. After making a note of the colonies, and after a fixed interval, complete medium is added to the same plate, permitting auxotrophs to grow.

3. Penicillin method, developed by Davis (1949), is also based on elimination of prototrophs. The suspension of microorganisms, after being acted on by a mutagenic factor, is inoculated on minimal medium containing 300 units/ml of penicillin. Since only prototrophs grow on minimal

medium, and penicillin inhibits reproduction of cells during growth, the prototrophs are eliminated. Other antibiotics besides penicillin can be used.

4. Filtration method. After exposure to a mutagenic factor, spores are inoculated on liquid minimal medium and incubated until a wild-type mycelium develops. The whole is then filtered through paper which transmits only spores. It is assumed that only prototrophs grow in minimal medium and remain on the filter, while spores of mutants pass to the filtrate. The filtrate is then inoculated on complete medium, on which the mutants develop.

5. "Replica plating" method developed for bacteria (Lederberg and Lederberg, 1952). It may be applied to streptomycetes and fungi giving small colonies. The colonies of the prototrophic strain develop on complete medium and then the open dish is turned over onto a sterile velvet pad held in place by a special support. The dish must be pressed more or less according to the species usually gently for bacteria and moulds, and hard for streptomycetes. Each colony leaves a print on the velvet corresponding to its position in the dish. A dish of sterile minimal agar is then pressed down the velvet pad. The dish of complete medium with the colonies is then put in the refrigerator, and that of the

minimal medium with the printed is put in the incubator. After a day or two a small area of growth will develop on the minimal medium in correspondence with the print of each colony. Only the prints of the auxotrophic colonies will not develope further. By comparing the dish of complete medium with replica on the minimal medium, it is possible to score the colonies which have not replicated. These are the presumed auxotrophs.

By combining mutagenic factors and applying selection, the antibiotic yield of many strains has been increased. However, it should be emphasized that variation leading to increased yield of antibiotic producing strains is a very slow and gradual process. Variation of the Penicillium chrysogenum Thom NRRL 1951 is an example. All industrial strains of Penicillium chrysogenum derive from the Wisconsin family, the results of vast mutation program at the Botany Departments of Wisconsin University between 1946 and 1956, which gave at last of fivefold improvement in penicillin titer. The Penicillium chrysogenum NRRL 1951 was a wild strain. The figure 1 illustrates the genealogy of Penicillium chrysogenum strain of "Wisconsin family". X-irradiation of its natural variant NRRL 1951 B25 with increased yield gave

variant X-1612. Next, UV irradiation (257 nm) gave highly productive Q-176 strain which was for many years in the productio of penicillin. A further mutant F_3D_{10}, gave about 25% lower yield, but was unpigmented and produced colorless penicillin, which was much easier to purifity. Selection of this strain again increased its yield (NRRL 48-701), and after nitrogen mustard was used as mutagen the high-yield unpigmented NRRL 49-2105 strain was obtained, but this mutant produced a considerable amount of red-brown pigment which was an obstacle in the purification of penicillin. The table 2 illustrates the correlation between morphological characters and yield of penicillin in strains used in penicillin production. The line of strains treated with nitrogen mustard gave the unpigmented NRRL 51-20 strain with high yield of penicillin, selection of which gave strains with high but unstabilized yields (Backus and Stauffer, 1955). Single mutants of Aspergillus nidulans with raised penicillin yields were isolated from derivatives of the Glasgow wild-type strain NRRL 194 following ultraviolet light treatment. Each mutation was mapped to its chromosome by parasexual haploidization analysis (McCully and Forbes, 1965).

Better chlortetracycline-producing strains were obtained in Streptomyces [illegible]

UV-rays, or other mutagens. Ultraviolet treatment seems to give the best results (Sermonti, 1968).

Together with mutation, the recombination process is basical for obtaing microorganisms whose characters are more profitable in antibiotic production. Recombination results in a combination of parental genes in the progeny. Crossing of strains with defined phenotypes, useful in a given system, leads accumulation in one genotype of as high a number of positive characters as possible.

In eukaryotic organisms the occurrence of regular sexual cycle is a condition fundamental for recombination. Gene recombination occurs during meiotic division, when upon conjugation of the homologous chromosomes the chromatids break and crossing over takes place. Reassortments of alleles originating from both parents is accomplished. Crossing over occurs in the first meiotic division, when chromosomes enter close contact upon conjugation. Recombination inside a gene, or between linked genes, is not always a result of crossing over. There is a theory that recombination occurs upon chromosome replication. Two chromatids replicate on the template of the original chromatid; if at a specific point the template is reciprocally exchanged, the chromatids

start to replicate on the homologous chromosome template (copy-choice theory). The recombination process probably runs by both crossing over and copy-choice.

Nuclear fusion and gene segregation can, however, although only occoasionally also take place in absence of sexual cycle, by means of processes which have been described as the "parasexual cycle". In the parasexual cycle the same genetic phenomena occur as in the sexual cycle, namely, complete nuclear fusion followed by chromosome reassortment and crossing-over. However, these processes occur in the somatic cells at a low rate. It is only system available in the Penicillium chrysogenum, Cephasloporium acremonium and some strains of Aspergillus niger.

The life cycle of all these fungi is predominantly haploid, with occasional heterokaryon formation at frequencies varying significantly between species. From these heterokaryon occasional diploid nuclei may be selected as first demonstrated by Roper (1952).

The parasexual cycle is accomplishes in the laboratory, and very likely in nature by three succesive steps (Fig. 2).

1. Heterokaryon formations: the hyphae of two different genetically marked strains are joined by a cytoplasmic anastomosis, and a heterokaryotic mycelium is formed.

2. Diploid formation: two different nuclei occasionally fuse within the heterokaryotic mycelium giving a heterozygous diploid nucleus which gives a diploid clone.
3. Mitotic segregation: rare diploid nuclei within the diploid clone undergo mitotic segregation, to give clones which can be either diploid or haploid.

As a result of the parasexual cycle, a differentiated system arises in culture, e.g.:

1. Haploid strain similar to the parental strain,
2. Haploid strain with recombinations of all the chromosomes and chromosomal segments of the parental strain,
3. rarely, diploid strains which are homo-or heterozygous for all possible markers.

Heterokaryotic clones can be detected on culture media partially enriched in the contact zone of two parental strains. These clones grow on minimal medium. Some of the heterokaryotic nuclei in conidia dissociate, giving the original parental strains. The heterozygous diploid can be isolated by inoculating minimal medium with heterokaryotic conidia (10^6 - 10^7 per plate). Colonies with phenotypic characters of wild strain are heterozygous diploids. Next, the spores of the heterozygous diploid are subjected to the mutagenic

factors, and the segregants are examined in detail (Fig. 3).

Diploids strains of Aspergillus nidulans distinguishes from haploids by their larger conidial diameter (Pontecorvo et al., 1953), but this is not always a reliable indicator of ploidy in Penicillium chrysogenum and Penicillium patulum (Macdonald et al., 1963, 1964, 1965).

In early studies with "improved titer" strains of Penicillium chrysogenum most haploid segregants from diploid were of one or the other parental genotype, a phenomenon termed "parental genome segregation" (Elander, 1967). This was probably due the parent haploids differing in chromosomal rearrangements such as reciprocal translocation, which prevented random chromosome assortment. By using closely related "sister" strains as parent, these barriers to recombination were overcome, and random assortment of haploidization groups was achieved (Bull, 1971).

The frequency of heterokaryon formation can be increased by applying the protoplast fusion. The latter makes it also possible to obtain interspecies hybridization.

Heterokaryon formation from Penicillium chrysogenum and Penicillium roquefortii was reported , by protoplast fusion stimulated by polyethylene glycol (PEG). In this

way strains were obtained capable of growing on minimal medium, while parental strains were auxothrophic. The colonies on minimal medium differed in color and morphology from those of the parental strains - they showed jointly the characters of both parents. Among heterokaryon, which produced spores with the characters same as parental strains, colonies were found with diploid mycelium.

The mycelium showed antibiotic activity typical for Penicillium chrysogenum (benzyl, n-amyl, and 2-pentyl penicillin was found in the culture filtrate), and produced prototrophic spores much larger than parental spores. Spores of the diploid strain were green-white, whereas Penicillium chrysogenum spores were white and Penicillium roqueforti spores were green (Anne et al., 1976).

Further studies are in progress, aimed at practical application of the stimulated protoplast fusion in both fungi and actinomyces to improve the production strains (Kurzatkowski et al., 1979).

Every since the discovery of the parasexual cycle, attempts have been made to use it for the improvement of the production of penicillin. In Penicillium chrysogenum

study strains carrying spore colour and auxotrophic markers were used as parents in which single-step titor increases were induced by UV. The mutants had yield about 3000 U/ml. Strains carrying different positive mutations were crossed in an attempt to obtain segreguns carrying both mutations. Two crosses produced as a result of parasexual cycle, segregants with significantly increased yield. The effects of the two mutation were additive, a promising results for the future application of recombination to yield improvement (Ball, 1973; Hopwood and Merrick, 1977).

Recombination is common actinomycetes, too. Transformation and transduction are of little importance in eleration of the antibiotic yields in strains of the genus Streptomyces, and major part of genetic studies in this field are based on the conjugation process. Conjugation in actinomycetes may lead to heterokaryon formation (actinomycetes are the only example of prokaryotic organisms in which this phenomenon was observed), to recombination, or to plasmid transfer. The consequences of the conjugation process in some strains of actinomyces are illustrated in Table 3.

Most investigators believe that the heterokaryon arises from strains of the same species. A heterokaryon from Streptomyces griseus and Streptomyces c aneus has been obtained only in one case, the only known instance of interspecies heterokaryosis.

Obtaining of the heterokaryon made possible the next step in genetic analysis of actinomycetes.

The technique of obtaining recombinants in actinomycetes consist in:

1. incubation of heterokaryons on selective medium,
2. inoculation of spores on selective media on which the original forms grow,
3. isolation and detailed analysis of presumable recombinants.

In actinomycetes a cycle analogous to the parasexual cycle in fungi occurs. A fundamental difference, however, consists in instability of the diploid in the heterozygous mycelium.

The conjugation phenomenon was observed in actinomyces strains that produce a number of practically important antibiotics; unfortunately, it remains hitherto without effect on the antibiotic yield.

In the case only of chlortetracycline-producing Streptomyces aureofaciens and oxytetracycline-producing Streptomyces rimosus, yields higher by 5 to 20% were found in prototrophic recombinants obtained by conjugation of auxotrophic mutants differing in the antibiotic yield (Sermonti, 1968).

As pointed out by genetic analysis of the antibiotic production process in actinomyces, the antibiotic synthesis is controlled by not only genes localized in the nucleoid but also by plasmid genes. Oxytetracycline produced by Streptomyces rimosus, turimycin produced by Streptomyces hydroscopicus, and methylenomycin produced by Streptomyces coelicolor are examples of plasmid-determined antibiotic synthesis. Regulator genes for the structural genes controlling chloramphenicol synthe is by Streptom ces venezuelae are probably also localized in plasmids.

In actinomyces and bacteria, methods applied for genetic improvement of the production strains are based first of all on mutations induced by various mutagenic agents. The use of recombination in those microbial groups is limited to a few species only; this process is of little importance for improving the antibiotic yield of prokaryotic

organisms. May be in future it will appear to be more applicable. On the other hand, recombination is applied in trials on improvement of the antibiotic yield of fungi.

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Table 1

Increase of antibiotic production with years by genetic and cultural improvement (Alikhanian, 1962)

Antibiotic	Initial yield at the time of discovery (units/ml)	Yield obtained in U.S.S.R. after improvement (units/ml)
Penicillin	20 (1941)	8000 (1955)
Streptomycin	50 (1945)	5000 (1955)
Chlorotetracycline	200 (1948)	4000 (1959)
Oxytetracycline	400 (1950)	6000 (1959)
Erythromycin	100 (1955)	2000 (1961)

Table 2

Correlation between morphological characters and yield of penicillin in strains used in penicillin production

Colony type	Strains			
	Wis.Q176	Wis.48-701	Wis.49-133	Wis.51-20
	B	D	D	C
Colony size (in mm)	21	15	14	11
Sporulation rate (comparatively)	4	3.8	2.2	1.5
Penicillin yield (u/ml)	640	1375	2230	2521

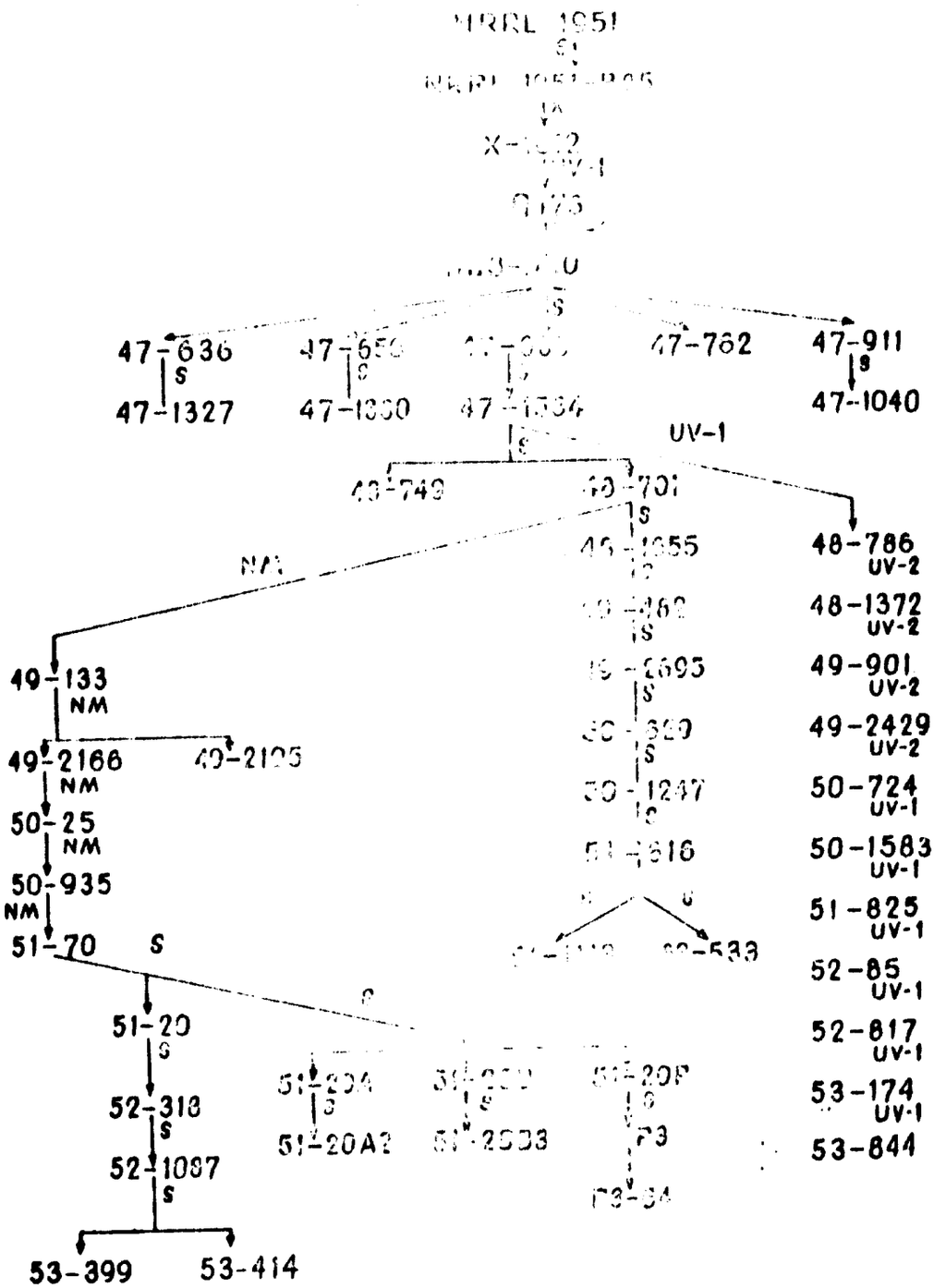
Table 3

Genetic phenomena in streptomycetes other than S.coelicolor A3/2/ (Hopwood and Merrick, 1977)

Species	Antibiotic	Genetic phenomena
S.albus		Heterokaryosis
S.antibioticus	Actinomycin	Heterokaryosis and recombination
S.aureofaciens	Chlortetracycline	Recombination
	tetracycline	Interspecific recombination with S.rimosus and S.coelicolor
S.clavuligerus	Cephamycins	Recombination
S.coelicolor (other strains)		Heterokaryosis and recombination
S.erythreus	Erythromycin	Recombination
S.fradiae	Neomycin	Recombination
S.griseus	Streptomycin	Heterokaryosis
	7 others	Recombination
		Interspecific recombination with S.coelicolor
S.hydroscopicus	Turimycin	Turimycin plasmid
S.lipmanii	Cephamycins	Heterokaryosis
S.parvulus	Actinomycin	Receives SCP1 and SCP1' from S.coelicolor
S.venezuelae (and Streptomyces sp. 3022a)	Chloramphenicol	Heterokaryosis

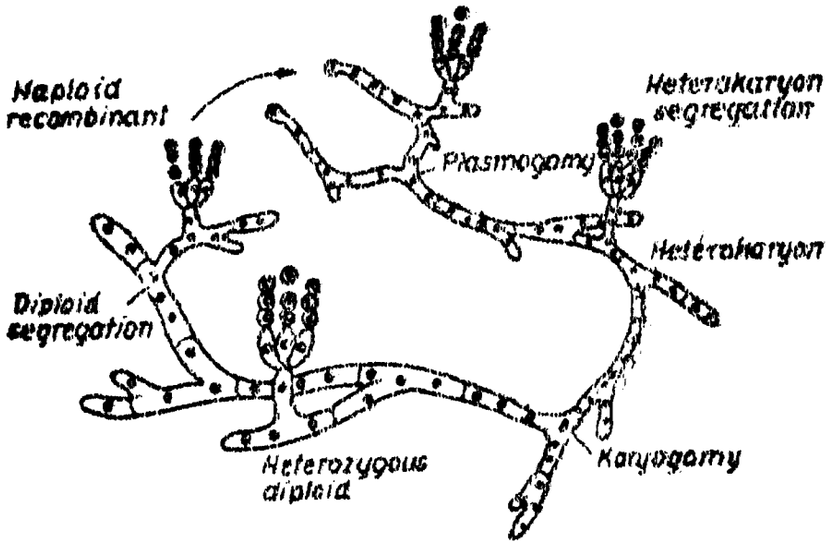
FIG. 1

Genealogy of Penicillium chrysogenum strains of the Wisconsin family.



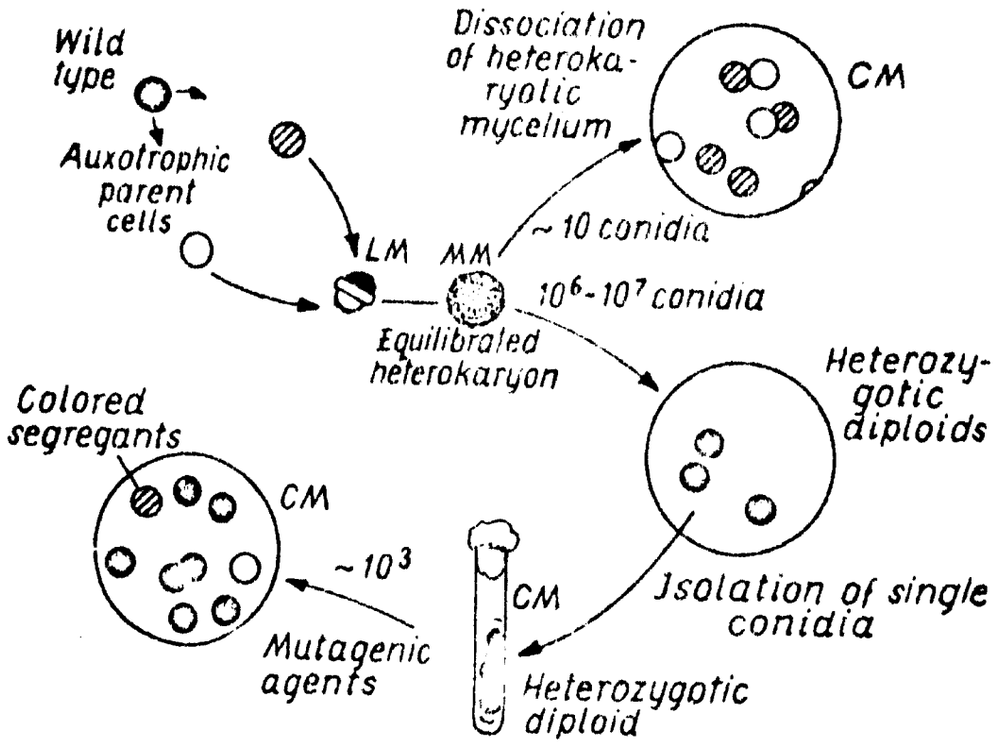
UV-1 - selection after UV irradiation at wavelength 2750 Å
UV-2 - selection after UV irradiation at wavelength 2750 Å
s - selection without prior mutation
x - selection after X-irradiation
NM - selection by nitrogen mustard

Fig. 2



The parasexual cycle in a mould

Fig. 3



A schedule for testing parasexual cycle in Penicillium chrysogenum

We regret that some of the pages in the microfiche copy of this report may not be up to the proper legibility standards, even though the best possible copy was used for preparing the master fiche.

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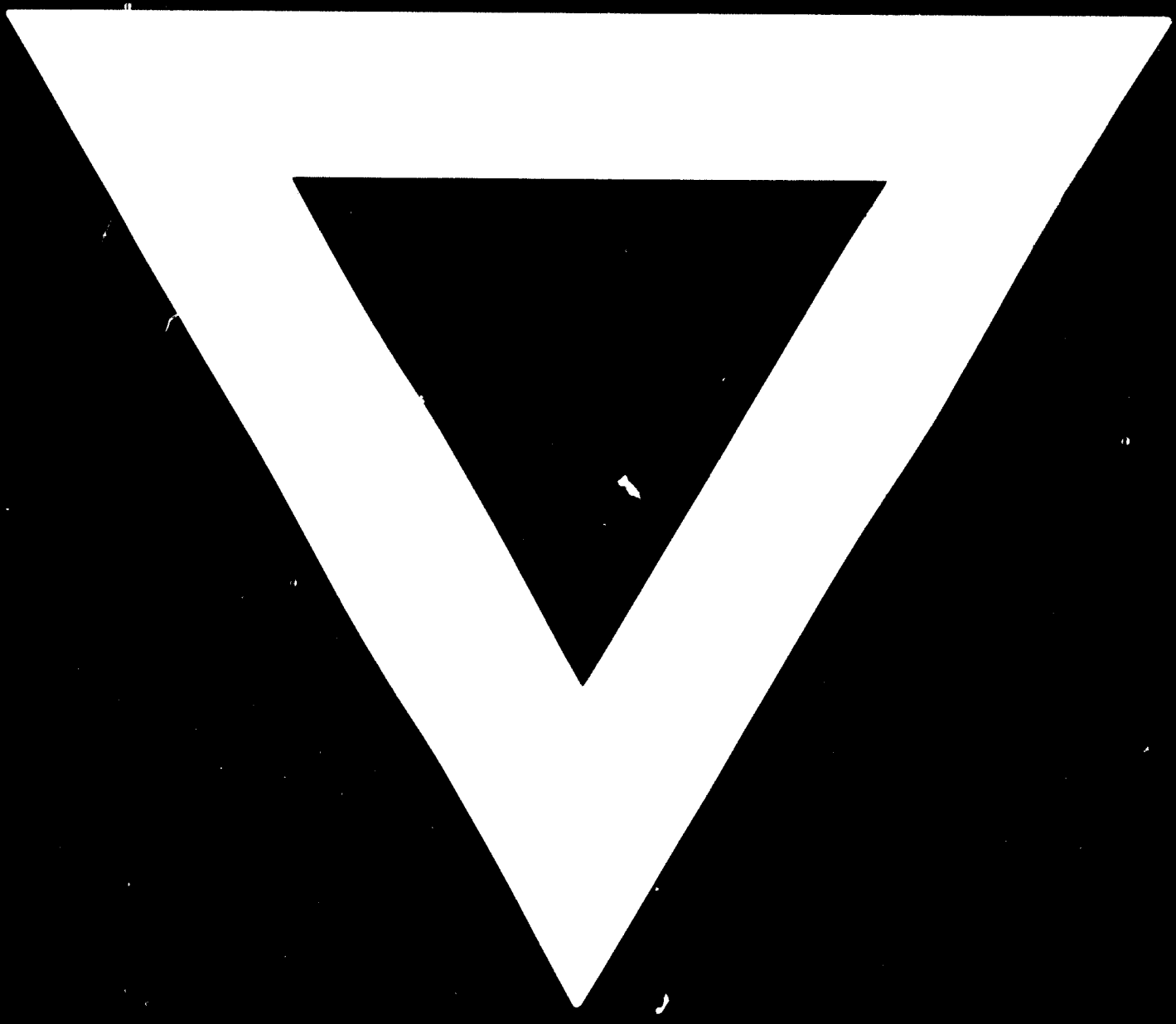

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