A STUDY OF CERTAIN MICROORGANISMS OF SOIL ORIGIN EXHIBITING ANTI-MICROBIAL PROPERTIES

by

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TABLE OF CONTENTS

INTRODUCTION

Microorganisms too often are considered solely as detrimental and infectious agents, to be eliminated and destroyed whenever possible. To prove the fallacy of this misconception the soil, which must harbor vast numbers of microorganisms if normal biological changes therein are to progress satisfactorily, may be cited as an example. In the minds of many individuals, the soil or anything associated with it, is regarded as repulsive, unclean, and insanitary. To the soil microbiologist, the picture presented is of an entirely different nature. Long ago he realized that the types of microorganisms present in soil regulate to a great extent, the success or failure of the harvest reaped from the land. It is because of this reason that a certain aggregation of various organisms is desired rather than shunned.

To add to the importance already attached to soil, has been the unchallenged discovery that soil also contains particular microorganisms capable of producing materials beneficial as chemotherapeutic agents, namely, antibiotics. Since the soil is the natural habitat of such a wide variety of microorganisms, it is presently undergoing much investigation in anticipation of isolating new antibiotics capable of supplementing or even surpassing those now available.

This study was undertaken in an effort to obtain factual information relative to the presence of microorganisms in soil

samples taken at random antagonistic toward a selected spectrum of test organisms.

REVIEW OF LITERATURE

The first investigator to recognize the potentialities of microorganisms for therapeutic purposes, according to Florey (1945), was Mosse who in 1852 wrote to the Lancet magazine stating,

> I have now practised in this town (Fareham, Hants) nearly six years, and have had frequent opportunities also here of witnessing the good effect of yeast in these troublesome affections, easily consummating a rapid and complete cure without further recurrence and by a most simple remedy within reach of all.

He was referring to the superficial wounds caused by boils and carbucles and their subsequent treatment through application of an unknown yeast salve. This is apparently the first recorded form of therapeutic antibiosis as we know it today. Tyndall in 1876 further amplified this phenomenon when he described the struggle between bacteria and molds, noting that sometimes one triumphed, sometimes the other.

But it was Pasteur and Joubert who, in 1877, first recorded a clear cut description of bacterial antagonism, when they observed a clearing of anthrax urine solution which had been inoculated with a 'common bacteria' of the air. They later supplemented their findings when they wrote (translated by Pratt and Dufrenoy, 1949),

> It is remarkable that the same phenomenon is seen in the body....leading to the astonishing results that anthrax can be introduced in profusion into an animal,

> which does not develop the disease; it is only necessary to add some 'common bacteria' at the same time to the liquid containing the anthrax bacteria. These facts perhaps justify the highest hopes for therapeutics.

The importance attached to this statement, as witnessed by the results obtained today through use of antibiotics, establish Pasteur and his associates as the founders of modern chemotherapy. In 1879 DeBary further emphasized the significance of the antagonistic relations among microorganisms, noting that when two organisms are grown on the same substrate one overcomes the other sooner or later, and may even kill it.

In 1885 Cantani (Florey, et al., 1949) attempted the first crude practical application of the 'common bacterial' concept through application of "replacement therapy"; i.e., the inoculation of a certain non-pathogenic organism into a patient infected with a pathogen, which had previously been shown to be susceptible to the non-pathogen in vitro. This method was refined in the 1890's and consisted of the administration of a more or less pure extract of an organism which would inhibit or destroy the pathogen involved. Extracts of fungi as well as bacteria were used in these attempts.

It was Vuillemin, in 1889, (Florey, et al., 1949) who first used the term antibiosis. Since then the term has become firmly established and generally means a metabolic product of one microorganism which inhibits or even destroys the life processes of another microorganism. Waksman (1947a) has since elaborated on

the characteristics of an antibiotic in that it,

> ....is a chemical substance, produced by a microorganism, which has the capacity to inhibit the growth of and even to destroy bacteria and other microorganisms. The action of an antibiotic against microorganisms is selective in nature, some organisms being affected and others not at all or only to a limited degree; each antibiotic is thus characterized by a specific anti-microbial spectrum.

In 1887, through a correlation of experiments and observations and the combined efforts of various workers, there emerged from the laboratory of Emmerich and Low results indicating the successful use of 'pyocyanase' against a certain infectious disease. This chemotherapeutic agent is generally recognized as the first antibiotic discovered. It was eventually successfully isolated in a more or less pure form from the bacterium Pseudomonas aeruginosa and though attempts were made to obtain agents from various other organisms, the results, at best, were uncertain and usually disappointing.

Consequently, investigation into the possibilities of this newly created therapeutic field gradually subsided, although it was concurred that an antagonistic action existed between certain microorganisms of the soil (Porter, 1924) and certain of those pathogenic to the animal body (Rettger and Cheplin, 1921). The interest created remained more or less stagnant for a period. Perhaps this lack of enthusiasm can be attributed to the fact that pyocyanase was the only antibiotic to be successfully isolated showing encouraging results and that even this antagonist was apparently losing its power of inhibition----probably because of

deterioration in the quality of the commercial preparation.

It was for Alexander Fleming (1929) to again stimulate interest in this field when he made his classic observation of the inhibitory effect which a Penicillium sp. had upon the growth of Staphylococcus aureus when a nutrient agar plate of the latter was contaminated with the Penicillium. Thus he instigated a revived interest in antibiotics which was immediately taken up by British and American scientists.

With the outbreak of the Second World War, the method for the commercial production of penicillin was especially desired and through the combined efforts of British and American bacteriologists, chemists, and engineers, it soon became available for clinical use (Herrell, 1945). Shortly before the intense research program on penicillin began, the investigations of Dubos (1939) terminated in the isolation of another complex antibiotic, tyrothricin, from culture of the soil organism Bacillus brevis. This antibiotic proved to be toxic when used internally and therefore could only be administered topically. However the possibilities in the field of antibiotics were now recognized, and intense investigations for other antibiotics of less toxic effect were initiated.

The research which has been conducted to the present time is evident from the tremendous amount of literature available on the subject. It is interesting to note that three groups of microorganisms are of special concern in the production of antibiotics, namely, the bacteria, actinomycetes and the fungi. From a review

of the literature, it is apparent that many specific organisms of these groups are undergoing a thorough investigation at the present time. The work of the Oxford group, headed by Chain and Florey, et al. (1940), focused attention on the fungi after the significance of penicillin was established. The previous work of Dubos in isolating the tyrothricin complex brought special attention to the bacteria. And finally, the investigations of Waksman and Woodruff (1940 and Waksman, et al., 1942) drew attention to the actinomycetes which led to the isolation of streptothricin and finally streptomycin, a highly valuable chemotherapeutic agent today.

The majority of the hundred odd antibiotics available in pure form today have proven to be too toxic for clinical use. The five most valuable antibiotics now available include penicillin, streptomycin, aureomycin, chloromycetin, and terramycin. The very latest antibiotic, which is proving to be advantageous for certain infectious diseases, is neomycin. Some are active primarily upon Gram-positive and others are effective against Gram-negative bacteria, whereas still others affect bacteria within each of these groups; some act upon fungi, while others do not; a few are active against rickettsiae. Most antibiotics have very little affect upon vira.

According to Bunn (1948), a substance must possess certain characteristics before it can qualify as a satisfactory therapeutic antibiotic; i.e., its chemical structure must be such that it is easy either to synthesize or extract from the synthesizing

organism; it must have a wide range of anti-microbial action; and must possess properties that insure simple administration. It must not be toxic to the host or interfere with the hosts ability to produce defense mechanisms. The antibiotic should be slowly excreted by the patient and should not be such a nature that pathogenic organisms can readily become resistant to its action. On the other hand Pratt and Dufrenoy (1949) were more exacting in their requirements for an ideal antibiotic. They suggest that if an antibiotic is to be used medicinally it must be soluble in physiological saline, have a broad antibiotic spectrum, and be without toxicity to the host. Also that it should be active against microorganisms at pH values near neutrality, should not be proteinaceous, and should retain its antibiotic activity in the presence of pus, serum, large numbers of bacteria, or other conditions commonly encountered in infectious lesions. Finally, the antibiotic should not stimulate organisms initially sensitive to its action to develop resistance. These two opinions are essentially similar in their over all aspect, though varying somewhat depending upon individual concepts.

There have been various theories expounded regarding the mechanism of antibiotic action. A conclusive statement at this time would be, at best, only speculative because of the amount of current research being conducted and the varieties of opinions which exist. Nevertheless, some of the theories as postulated by the various workers are: (1) exhaustion of nutrients (Fildes, 1940,

McIlwain, 1941); (2) interference with bacterial cell division (Greig and Hoogerheide, 1941); (3) physicochemical changes in medium (Waksman, 1947b); and (4) affect the surface tension of the bacteria, acting as a detergent (Dubos, 1944). From an examination of the literature it would appear that the mechanism of the action of antibiotics, toward certain microorganisms, may vary for each antibiotic. The environment of the antibiotic may also greatly influence its action.

METHODS OF ISOLATING ANTAGONISTIC MICROORGANISMS

Various methods have been devised for the primary isolation of antagonistic microorganisms from natural sources such as soil, composts, stable manure, sewage, water, and food products. These methods, although varying in nature and each possessing certain limitations and advantages, are the same in principal; i.e., that of bringing a living culture of a bacterium or fungus into close contact with a mixed natural population of microorganisms and observing which of these organisms show anti-microbial action. It is the opinion of Brian (1949) that soil, because of the wide variety of species of microorganisms found therein, offers proportionally the most excellent medium from which to isolate potentially antibiotic producing organisms. The term 'test organism' is applied to the organism the growth of which, one wishes to prevent, and the term 'antagonist' to the microorganism producing the

antibiotic material which may or may not inhibit the growth of the test organism. The choice of test organisms is dependent upon the desired end results.

Bacterial Agar Plate Method

To isolate antagonistic microorganisms, the following modification of a procedure first used by Gratia and Dath, is recommended by Waksman (1947b). Washed agar (1.5 per cent) is dissolved in water supplemented by 1 per cent glucose and 0.2 per cent K_2HPO_4. This medium is then tubed, sterilized, and cooled to 42° C. in a water bath. A washed, centrifuged suspension of living bacteria (test organism) is added to the agar tubes and thoroughly mixed. This "bacterial agar" medium is poured into a series of Petri plates containing one-milliliter portions of fresh soil suspensions of dilutions 1:100 to 1:10,000. The plates of seeded media and soil suspensions are thoroughly mixed and allowed to solidify. They are then inverted and incubated at 28° C.

To isolate antagonistic fungi the same general method is employed as for bacteria, with the exception that the bacterial agar is made acid by using KH_2PO_4 in place of K_2HPO_4 to inhibit the growth of bacteria and actinomycetes. Since there are fewer fungi than bacteria naturally present in soil, lower dilutions of the suspension are transferred to the series of Petri plates.

Plates in both cases are observed for characteristic zones

of inhibition after an appropriate length of incubation.

It should be mentioned that this method has the undesirable characteristic (Waksman and Schatz, 1946) that antagonistic organisms have been isolated by this procedure although the original development of the colonies was not accompanied by the formation of a clearly visible zone of inhibition of the test organism. Waksman and Schatz (1946) summed up this point with the statement, "one must, therefore, conclude that the production or lack of production of zones on the bacterial agar plate is no proof at all of the ability of the organism to produce an antibiotic substance."

Soil Enrichment Method

This method (Dubos and Avery, 1931; Dubos, 1939) assumes that any kind of organic matter is more or less quickly decomposed in soil by the mixed micro-flora normally present or artificially added (enriched). Those organisms which thus are able to decompose the organic matter have a decided advantage over those not causing decomposition and therefore multiply at a faster rate. As a result, this procedure presupposes that the addition to soil of living cultures of pathogenic bacteria results in the development of selective antagonists capable of attacking the living cells of these added bacterial species. It is thus assumed that an antagonist, capable of causing the destruction or inhibition of the added bacteria, accumulates in a soil when the soil enrichment method is employed.

In this method fresh garden or field soil is maintained in the laboratory under suitable conditions of temperature, aeration, and moisture to promote aerobic growth of bacteria. The pots or glass beakers containing the soil are placed in an incubator at 28° C. and washed suspensions of the test organism are added from time to time. Samples of the soil are periodically added to a broth mineral medium containing the test organism. This suspension, containing soil and test organism, is incubated at optimum temperature. When lysis of the test organism is initially observed in the suspension, several transfers of this suspension through the mineral medium will usually result in the eventual total elimination of the test organism originally introduced into the soil.

While the enrichment method may have served Dubos (1939) in his isolation of the tyrothricin complex, it may be considered that this organism is not an uncommon soil inhibitant and that the enrichment procedure was not essential for its successful isolation. Foster and Woodruff (1946), while attempting to enrich soil media for the production of antagonistic actinomycetes, observed that there was an actual increase in total number of organisms, but that the proportional number of antagonists remained about the same. From this they concluded, "the chances of random selection of an antagonist from an enriched soil are not better than from a control soil."

Crowded Plate Method

In its simplest form, this method consists of plating out suspensions of the sample under consideration, onto Petri plates utilizing the most appropriate medium for the growth of the potential antagonists (Waksman, 1947b). Dilutions of suspensions to be transferred to the Petri plates are optional, the most suitable number of developing colonies being arrived at through a trial and error method. The plates are incubated for an appropriate length of time and at optimum temperature. They are observed from time to time for a clear zone in close proximity to adjacent colonies. This characteristic is indicative of antagonistic action, and the colony is thus "picked off" and maintained on suitable medium in pure culture, for further study against a desired spectrum. This method was successfully used by Stokes and Woodward (1942) who found that with a soil micro-flora, the antagonists were invariably spore-forming organisms, while the organisms attacked were usually bacilli or short gram negative rod-shaped bacteria and occasionally fungi. On the other hand, two objections to this method are to be considered. In the first place the antagonist may be overgrown by the surrounding colonies, if the former develops slowly, and secondly, unless the antibiotic is formed early during the growth of the antagonist, it may exhibit no zone of inhibition. Recommendations offered by Florey, et al. (1949) to overcome these difficulties are: first, to incubate the plate initially under optimum

conditions for maximum growth of the suspected antagonist, and second, to transfer the plate to those conditions best suited for the test organism to develop.

Direct Soil Inoculation Method

This method, first used by Novogrudsky and recommended by Waksman (1947b), is perhaps the easiest and quickest procedure currently employed for the initial isolation of microorganisms exhibiting antagonistic effects. A suitable liquefiable-solid medium is seeded with the desired test organism and allowed to solidify in Petri plates. The plates are incubated for 24 to 48 hours after which time they are "sprinkled" with particles of the fresh or enriched soil sample to be tested. The plates are reincubated at optimum conditions and observed at various time intervals. If antagonistic organisms are present in the added soil sample, this will be indicated by a killing or even the lysis of the original test culture and is characterized by a clear area surrounding the antagonist. The potential antibiotic producing organism is then transferred to a suitable stock medium for further investigation.

The primary difficulty encountered in this method is that obtaining a pure culture of the antagonist may be a somewhat tedious accomplishment because of the variety of non-antibiotic microorganisms which naturally adhere to the particle of "sprinkled"

soil. Also the test organism, which has been allowed to grow first, may reproduce to such an extent that it represses growth of the antagonists present. Nevertheless it has the advantages of making possible an analysis of many different soil samples in a short period of time.

The methods discussed are potentially adequate for isolating great numbers of antagonists. Slight modifications and variations may be applied to each method, depending upon the circumstances and the individual investigator. The field of antibiotics is relatively young and the possibilities encompassed by it are tremendous. Because man is forever improving and building on scientific knowledge already established, it is a fair assumption that newer and improved methods will perhaps augment todays procedures. The advancement and priority enjoyed by antibiotic research is justifiable and these chemotherapeutic agents are truly the "wonder drugs" of today.

EXPERIMENTAL

The methods suggested for isolating antagonistic microorganisms were analyzed and due to certain limitations and advantages of each, none were deemed to be completely satisfactory. It was therefore decided by the investigator, to employ those particular techniques of each method most applicable to the situation arising. Consequently, there is an intermingling of various procedures included in the method employed by the experimenter.

Forty soil samples, from various localities surrounding Manhattan, Kansas, were collected in sterile glass jars. The samples were taken at a depth of approximately six inches from virgin and cultivated soils, from fertile and infertile soils, and from river bank soil. No attempt was made to record the locality from which each sample was secured. It was felt by the investigator that any correlation existing between the abundance of antagonists in the soil and the locality should be disregarded in a study of this type.

The samples were brought into the laboratory and 1 gm of each sample was immediately placed in a 99 ml sterile water blank. Appropriate transfers were made resulting in 1:1,000 and 1:10,000 dilutions of each sample. Transfers of 1 ml per dilution were made to sterile Petri plates, resulting in three plates per sample for a total of 120 plates. The dilutions employed were sufficient to yield the amount of growth satisfactory for exhibiting zones of inhibition by the potential antagonistic colonies. The most advantageous dilution of the samples was determined at the termination of the incubation period.

To each plate was added approximately 25 ml of melted sterile nutrient agar. The plates were thoroughly agitated to facilitate an even distribution of the soil suspension. The medium was allowed to solidify and all plates were incubated at 28° C. in an inverted position.

The plates were observed from time to time for a period of fourteen days. Those developing colonies, which produced a visible

zone of inhibition were "picked off" by means of a sterile straight wire and transferred to nutrient agar slants. There were various sized areas of clear inhibition zones produced on the plates adjacent to the desired colonies. In most cases the clear zone measured from the outer edge of the antagonist was 0.5 to 1.0 mm. For a few colonies it extended to 2.0 mm. From this preliminary isolation procedure, it was observed that a dilution of 1:10,000 produced a larger number of colonies exhibiting zones of inhibition. In the remaining two dilutions used the high concentration of microorganisms resulted in plates crowded with colonies.

At the termination of the fourteen days' incubation period observations were discontinued; sixty-six type cultures which had exhibited definite zones of inhibition were isolated. All cultures were maintained on nutrient agar slants at 28° C. Although growth of a few microorganisms on this medium was slight, it nevertheless was satisfactory for maintenance of stock cultures.

The purity of each culture was confirmed by conventional methods. Those cultures which were apparently identical or were observed to be mixed cultures or "spreaders" were discarded. Fifty pure cultures of potential antibiotic producing organisms were retained for further study. These fifty cultures were considered as the "antagonists" to be tested against a desired group of "test organisms" in order to determine their actual anti-microbial potency.

The test organisms (or spectrum) decided upon consisted of

seven species, including a mold, yeasts, and bacteria. They were acquired from the stock culture collection of Dr. W. A. Miller, Department of Bacteriology, Kansas State College, Manhattan, Kansas. The group was comprised of Candida krusei, Cospora lactis, Saccharomyces cerevisiae, Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Pseudomonas fluorescens. All cultures were maintained on a suitable slant medium during the period of the investigation.

The technique as first employed by the experimenter, in the test for antibiosis against a spectrum, may be considered as an extension of the "bacterial agar" plate method. But the results obtained were too variable due to the fact that the amount of test organism added to the pour medium each time could not be standardized for the entire group. The time element involved limited a 'trial and error' method from arriving at definite amounts for each test organism. This method, as such, was unsatisfactory for the particular end results desired by the investigator.

Therefore, a variation of the bacterial agar plate method was employed. It has been referred to as the 'spot' inoculum procedure and was considered a more definite and conclusive method. The technique was as follows: 48 hour old antagonist 'spot' transfer was made to the center of solidified poured agar plates. The plates were incubated for 48 hours in an inverted position to allow for growth of the organism and distribution of the antibiotic material into the surrounding area. To the surface of each plate

was added 10 ml of sterile agar medium seeded with a 48 hour old inoculum of the test organism. The plates were allowed to solidify, then inverted and incubated at 28° C. for 48 hours.

At the conclusion of the incubation period, the plates were observed for zones of inhibition. Results obtained indicating the amount of inhibition of each test organism are recorded in Table 1.

Table 1. Action of unidentified antagonistic cultures toward test organisms.

Antagonist no.	E. coli	Ps. fluorescens	Staph. aureus	Can. krusei	B. subtilis	O. lactis	Sac. cerevisiae
	Zone of inhibition in millimeters						
1	0	0	0	0	0	0	0
2	0	0	0	0	3.0-5.0	0	0
3	0	0	2.0-5.0	0	0	0	0
4	0	0	13.0-16.0	0	0	0	0
5	0	0	0	0	0	0	0
6	0	0	3.5-6.0	0	3.0-4.0	0	0
7	4.5-6.0	0	4.0-4.5	0	5.0-6.0	0	0
8	0	0	0	0	3.0-8.0	0	0
9	0	0	0.5-1.0	0	0.5-2.0	0	0
10	0	0	0	0	0	0	0
11	0	0	6.0-13.0	0	7.0-8.0	0	0
12	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0
14	2.0-3.0	0	12.0-14.0	0	0	0	0
15	3.5-4.0	0	0	0	0	0	0
16	7.5-8.0	1.0-1.5	1.0-4.0	0	1.0-4.0	0	0
17	3.5-5.0	0	0.5-3.0	0	7.0-12.0	0	0
18	5.0-14.0	1.5-2.5	6.0-7.0	0	6.5-9.0	0	3.0-4.5
19	5.0-8.0	0	3.5-4.0	0	12.0-14.0	0	0
20	0	0	2.0-2.5	0	3.5-5.0	0	3.0-6.0
21	0	0	0	4.0-5.0	0	0	0
22	1.0-3.0	0	3.0-5.0	0	3.0-10.0	0	0
23	1.5-3.0	1.5-2.0	3.5-6.0	0	4.0-6.0	0	0
24	0	0	0	0	0	0	0
25	1.5-2.0	2.0-3.0	3.0-6.0	0	3.5-7.0	0	0
26	0.5-2.0	1.5-3.0	4.5-5.0	0	4.0-8.0	0	0

Table 1 (concl.).

Antagonist no.	E. coli	Ps. fluorescens	Staph. aureus	Can. krusei	B. subtilis	O. lactis	Sac. cerevisiae
	Zone of inhibition in millimeters						
27	0	0	1.0-7.0	0	0	0	0
28	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0
31	0.5-3.0	0	1.0-11.0	0	5.0-9.0	0	1.0-4.0
32	0	0	0	0	0	0	0
33	10.0-13.0	1.5-2.5	10.0-15.0	0	5.0-9.0	0	0
34	0	0	7.0-9.0	0	5.0-6.0	0	0
35	0	0	0	0	0	0	0
36	0	0	0	0	0	0	0
37	0	0	0	0	0	0	0
38	3.5-5.0	1.0-1.5	1.0-4.0	0	3.0-5.0	0	0
39	1.5-3.0	0	4.0-6.0	0	3.0-7.0	0	0
40	0	0	0	0	3.0-5.0	0	0
41	3.5-5.0	0	7.0-17.0	0	5.0-9.0	0	0
42	0	0	3.0-4.0	0	0	0	0
43	0	0	1.0-6.0	0	7.0-8.0	0	0
44	0	0	0	0	0	0	0
45	0	0	0	0	0	0	0
46	0	0	0	0	0	0	0
47	0	0	0	0	0	0	0
48	0	0	0	0	0	0	0
49	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0

Those microorganisms which actually inhibited any members of the spectrum were preserved as antagonists while those which failed to exhibit any inhibition were discarded. This resulted in twenty-nine unidentified stock cultures which inhibited various members of the spectrum. They were maintained on nutrient agar slants. Appropriate studies were made toward identification of each antagonist, including morphological, cultural, and physiological observations according to techniques prescribed in the Manual of Methods for Pure Culture Study of Bacteria. The results obtained were correlated with the appropriate classification as described in Bergey's Manual of Determinative Bacteriology (1949). The microorganisms were tentatively identified, as represented in Table 2.

Table 2. Identification of unknown microorganisms.

Antagonist no.	Identified antagonists
2	Bacillus cohaerens
3	Bacillus sp.
4	Bacillus brevis
6	Streptomyces viridochromogenes
7	Nocardia coeliaca
8	Nocardia sp.
9	Actinomycete sp.
11	Bacillus coagulans
14	Bacillus cereus

Table 2 (concl.)

Antagonist no.	Identified antagonists
15	Micromonospora sp.
16	Streptomyces antibioticus
17	Bacillus megatuerium
18	Streptomyces sp.
18	Corynebacterium simplex
20	Micrococcus conglomeratus
22	Micrococcus sp.
23	Bacillus sphaericus
25	Corynebacterium sp.
26	Bacillus sp.
27	Bacillus coagulans
31	Bacillus cereus
33	Streptomyces griseolus
34	Micromonospora sp.
38	Streptomyces flavus
39	Corynebacterium sp.
40	Nocardia sp.
41	Bacillus cereus
42	Micrococcus aurantiacus
43	Bacillus brevis

From these data, it will be observed that of the twenty-nine antagonists isolated, eighteen were true bacteria while eleven belonged to the order Actinomycetales.

SUMMARY OF PRELIMINARY RESULTS OBTAINED

The antagonists isolated indicate that the soil is a natural habitat for potential antibiotic producing microorganisms. The method as employed in the investigation yielded a maximum number of primary antagonists due to the dilution procedure applied at the onset. The time allowed for development of these microorganisms was considered adequate. It should be kept in mind that many variable factors enter into the variety and numbers of antagonists initially isolated. For example, perhaps the soil sample as taken from the field should have been allowed to incubate a day or two before plating commenced; or, the medium which was employed in the poured plates was not utilizable by many antibiotic producing microorganisms; in addition, hydrogen ion concentrations of the substrate may be unsatisfactory for many other potential antagonists. These and other variables may appreciably alter the end results, yielding results which appear negative. Conversely, an organism may be antagonistic in the soil and not produce antibiotic material when cultivated artificially.

According to Table 1, it will be noted that Staphylococcus aureus, a non-spore forming organism was the most susceptible

test organism, being inhibited by 50 per cent of the antagonists; Bacillus subtilis, a spore forming organism, was inhibited by 40 per cent of the antagonists. The closely related susceptibility of these two organisms to antibiotics, indicates that spore presence has little significance. This supports evidence that the mechanism of action is active during the time of cell division.

It is also interesting to observe that Bacillus subtilis was inhibited by several species identified as belonging to the genus Bacillus. The fact that species of organisms belong to the same genus seems to have little bearing on the degree of susceptibility and potency of the individual members toward each other.

The remaining test organisms, Escherichia coli, Pseudomonas fluorescens, Saccharomyces cerevisiae, Candida krusei, and Oospora lactis, were inhibited by 32 per cent, 14 per cent, 6 per cent, 2 per cent, and 0 per cent of the antagonists, respectively. The organism producing the largest single zone of inhibition was Bacillus cereus.

FOOD PRESERVATION

Various attempts are currently being employed by investigators, to demonstrate the possibility of food preservation through use of antibiotics. At the present time, there is no antibiotic available which is capable of substitution for high temperature processing. It is generally agreed that the advantage which would

come from the ability to substitute low temperature for high temperature in the processing of certain products, amply justifies continued attention.

In an attempt to exhibit practical application of the antagonists isolated, in the preservation of foods, broth cultures of the various antagonists were incorporated into a vegetable in test tubes. This was followed by a low temperature heat processing treatment.

The method used was as follows: each antagonist identified as belonging to the order Actinomycetales was grown on 60 ml of soybean broth medium as prepared by Savage (1949). Growth was allowed to progress in a thin layer in 300 ml Erlenmeyer flasks for seven days at 28° C. At the termination of the incubation period, it was assumed that sufficient antibiotic material was liberated into the broth to warrant proceeding with the experiment. A package of "Snow Crop" frozen green peas was secured and allowed to thaw, and distributed in large test tubes (12 peas per tube). Enough tap water was added to each tube to cover the peas. From the broth cultures previously prepared, the following amounts of broth from each Actinomycetales culture were added to a series of tubed peas; i.e., 0.1, 0.5, 1.0, 2.0, and 4.0 ml. Ten control tubes were also prepared containing only peas and water.

The mixtures and controls were subjected to a heat treatment in a conveniently adapted water bath. At the completion of the heat treatment, the tubes were removed from the water bath and immersed in cool water for five minutes. This was followed by

incubation at 28° C. Observations were made at the conclusion of seven days.

The group of eleven Actinomycetales antagonists was assayed in two units. Tubes of peas containing anti-microbial material from the first group of five microorganisms was subjected to a processing treatment of 60° C. for a period of thirty minutes. This process of pasteurization was not satisfactory, as all cultures observed at the termination of seven days were spoiled due to the presence of non-spore forming lactic acid bacteria. Peas containing anti-microbial material from a second group of six microorganisms were therefore treated for an extended period at a higher temperature; i.e., for one hour at 70° C. Tubes observed after seven days, displayed a different type of growth with a characteristic musty to putrid odor and a prominent pellicle present in all cultures. All controls without the antibiotic material showed similar spoilage.

The results obtained were of a negative nature, indicating that these antagonists, as assayed, failed to inhibit growth of spore forming organisms and lactic acid bacteria naturally present on frozen peas.

Those antagonists which were previously characterized as belonging to the true bacteria were investigated in a similar manner. However, slight variations were necessary due to generic differences. Also, these broth cultures (beef extract, peptone, and distilled water) were centrifuged and filtered by means of Seitz filters. This step was necessary to remove the spore forming

organisms present which are heat resistant and would result in erroneous conclusions. The resulting filtrate was the antibiotic material added in the various amounts to the respective series of tubed peas.

The results obtained with this group of microorganisms were, for the most part, also of a negative nature. Unlike the Actinomycetales, two organisms belonging to the genus Bacillus and the Corynebacterium genus exhibited a positive degree of inhibition which appeared favorable. The antibiotic substances which produced these results were derived from antagonists three and twenty-five, as represented in Table 2. All controls showed definite spoilage within 48 hours after incubation.

The 4.0 ml quantity of filtrate from the organism identified as Bacillus sp. inhibited growth of spoilage organisms for a period of five days. Nevertheless, characteristic spoilage appeared at the termination of the seven days incubation period. Physical observations were limited to pellicle formation accompanied by a musty, putrid odor.

The antagonist belonging to the genus Corynebacterium showed more promising results. At the end of 48 hours of incubation, the series of pea cultures containing the 1.0, 2.0, and 4.0 ml inoculum were noted to be free of pellicle formation, as compared with the remaining series. There was also a lack of "off" odor, indicating a definite degree of inhibition. The tubes were observed from time to time for any significant changes. At three days, a slight

pellicle formation was visible in the tubes containing 1.0 and 2.0 ml of the antibiotic material, while the 4.0 ml inoculum was persistent in preventing spoilage. At the conclusion of seven days, spoilage was evident in all tubes but only to a moderate degree.

DISCUSSION AND SUMMARY

Despite the inactivity displayed by the various antagonists in vegetable preservation, it must be borne in mind that loss of anti-bacterial properties is not uncommonly encountered. Members of Actinomycetales are especially notoriously characteristic in this short-coming. The antagonism, which all organisms initially exhibited with the "spot" inoculum technique, indicated the presence of this characteristic to some degree. Apparently it is necessary to subject these antagonists to more compatible conditions for them to display their antibiotic properties. A synergistic effect may be operative in the case of various members of these isolates, in which case a combination of certain filtrates might be advantageous.

The pasteruization treatment may have destroyed the preserving capacity of the respective antibiotic agents. While this is possible, filtrates, of necessity must be heat stable if they are to be satisfactory for food preservation.

All antagonists isolated were inhibitory to various degrees for certain members of the spectrum. Two microorganisms gave results which suggested their possible significance in the

preservation of certain foods. It is interesting to speculate on the potentialities which are apparently inherent in these two antagonists.

An extensive systematic examination of an antagonistic microorganism should be conducted if its potential possibilities as an antibiotic producer are to be realized. This is especially true for microorganisms classified as Actinomycetales.

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A STUDY OF CERTAIN MICROORGANISMS OF SOIL ORIGIN EXHIBITING ANTI-MICROBIAL PROPERTIES

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This study was undertaken in an effort to obtain factual information relative to the presence of microorganisms in soils which were antagonistic toward a selected spectrum of test organisms.

Methods, as suggested in the review of literature, for isolating antagonistic microorganisms were analyzed and due to certain limitations of each, none was deemed to be completely satisfactory. It was therefore decided to employ those particular techniques of each method most applicable to the situation arising. Consequently, there is an intermingling of various procedures included in the methods employed by the experimenter.

Forty soil samples were collected in sterile glass jars. The samples were brought into the laboratory and subjected to preliminary techniques which resulted in the isolation of sixty-six cultures which had exhibited definite zones of inhibition on nutrient agar poured plates.

The purity of each culture was confirmed and those which were apparently identical, mixed cultures, or "spreaders" were discarded. Fifty pure cultures of potential antibiotic producing organisms were retained for further study.

These "antagonists" were subjected to a "spot" inoculum technique for the purpose of displaying anti-microbial properties against a selected group of "test organisms". The test organisms (or spectrum) decided upon consisted of seven species, including one mold, two yeasts, and four bacteria. They were acquired from the stock culture collection of Dr. W. A. Miller, Department of Bacteriology, Kansas State College, Manhattan, Kansas. The group

was comprised of Candida krusei, Oospora lactis, Saccharomyces cerevisiae, Escherichia coli, Staphylococcus aureus, Bacillus subtilis, and Pseudomonas fluorescens.

Data were obtained indicating the millimeters of inhibition of certain test organisms. Twenty-nine antagonists which displayed some degree of spectrum inhibition were retained for further study.

The tentative identity of each antagonist was established by appropriate combinations of study. There were eleven microorganisms classified in the order Actinomycetales while eighteen organisms were identified as true bacteria.

From the data accumulated it was observed that the test organism Staphylococcus aureau, a non-spore forming organism, was the most susceptible test organism, being inhibited by 50 per cent of the antagonists; Bacillus subtilis, a spore-forming organism, was inhibited by 46 per cent of the antagonists. The closely related susceptibility of these two organisms to antibiotics, indicates that the presence of spore has little significance. This supports the concept that the mechanism of action is active during the time of cell division.

Bacillus subtilis was inhibited by several species identified as belonging to the genus Bacillus. The fact that species of organisms belong to the same genus, seems to have little bearing on the degree of susceptibility and potency of the individual members toward each other.

The remaining test organisms, Escherichia coli, Pseudomonas fluorescens, Saccharomyces cerevisiae, Candida krusei, and Oospora lactis, were inhibited by 32 per cent, 14 per cent, 6 per cent, 2 per cent, and 0 per cent of the antagonists respectively. The antagonist producing the largest single zone of inhibition was Bacillus cereus.

In an attempt to demonstrate possible practical application of the antagonists isolated to the preservation of foods, sterile filtrates of broth cultures of the various antagonists were added to frozen peas in test tubes. This was followed by a low temperature heat processing treatment. The advantages which would come from substituting a lower temperature in the processing of certain food products would justify attention to this study.

The group of eleven antagonists identified as Actinomycetales displayed negative results, indicating that these microorganisms as assayed, failed to inhibit growth of spore-forming bacteria and lactic acid bacteria naturally present on frozen peas.

The antagonists characterized as belongong to the true bacteria showed similar negative results. However, two organisms, each belonging to the genus Bacillus and a Corynebacterium sp. delayed spoilage in peas.

The filtrate from the antagonist identified as a Bacillus sp. inhibited growth of spoilage organisms for a period of five days.

The antagonist belonging to the genus Corynebacterium prevented spoilage of peas for seven days.

Despite the inactivity displayed by the various antagonists in vegetable preservation, it must be borne in mind that loss of anti-microbial properties is not uncommonly encountered.

A synergistic effect may be operative in the case of various members of these isolates, in which case a combination of certain filtrates might be advantageous.

An extensive systematic examination of the antagonistic microorganism should be conducted to realize the highest potential possibilities of antibiotic producers.