Fifty Year's Experience with Actinomycete Ecology

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The 1989 annual meeting of the Japan Actinomycete Society, held July 15, offered opportunity for the writer to describe certain experiences encountered during 50 year's research with actinomycetes. The 50 year span, 1939-1989, encompasses a great change in attitude concerning the ecological significance of actinomycetes. In 1939, debate raged concerning the significance of actinomycetes in soils. In 1989, the issue is settled. Actinomycetes are important members of the soil population, actively growing, metabolizing, degrading resistant substances, capable of producing secondary metabolites useful for man.

Fifty years ago, as a confident young chemist, the author entered Professor Selman A. Waksman's laboratory at Rutgers University in New Jersey, USA. The research project assigned was disappointing, the ecology of soil actinomycetes. It appeared so unimportant. There was abundant literature suggesting that actinomycetes play little part in the soil. A consensus was developing that the many actinomycete colonies which appear on soil dilution plates are derived primarily from inactive spores.

That conclusion was still receiving support in the years following 1939. For example, in one trial, spores of a frequently isolated soil actinomycete were dried on glass cover slips. The slips were buried in soil. After 48 hours, the slips were removed, washed, stained and the percent of spores germinated determined by microscopic examination. Only 5% of the spores on slides were germinated when buried in natural soil. If a readily available nutrient were present in the soil, for example 1% glucose, significant numbers of the spores germinated¹.

A second study by the same authors was based on a publication by Skinner². The study showed that after homogenizing a natural soil at 1400 rpm for 90 minutes, it could be calculated that 98% of the actinomycete colonies appearing on Petri plates originated from spores,

whereas only 2% came from mycelial fragments. In contrast, homogenization of a grass compost with sand showed that half of the actinomycete colonies obtained from plating the compost was derived from active mycelial fragments.

Knowing my dissatisfaction, Waksman suggested initiation of my studies with composts, since the actinomycetes in composts were believed to be active and important.

A visiting investigator from China was in Waksman's laboratory in 1939, planning to compost human feces. He proposed a thermophilic composting process, which would, because of its high temperature, kill all human pathogens present in the feces. Thus, the compost could be used safely by Chinese farmers.

Human feces are not composted in America. Animal feces are. Waksman suggested that my composting studies be performed in companion with the Chinese visitor, employing horse feces, horse urine and straw.

Fig. 1. Mineralization of composts incubated at 28°C and 50°C.

Soon excitement grew. Composting proceeded faster at 50°C than at 28°C³. The ash content increased rapidly, corresponding to the decrease in organic matter (Fig. 1). Even without plating, it was evident that actinomycetes were important. The high temperature composts had a strong actinomycete odor. Platings proved that actinomycetes dominated the population.

The excitement soon turned to dismay. Ammonia released from urine in the raw compost was assimilated quickly by the rapidly growing microorganisms. Thereafter, as the proteins of the feces were broken down, at the 28° temperature the ammonia nitrogen released was immediately converted to nitrate by nitrifying bacteria present in the compost. In the 50°C compost, however, nitrate did not accumulate. Free ammonia did (Fig. 2). It is volatile, wasted, lost to the surrounding air. The high 50 degree temperature had killed the two essential nitrobacteria species which are responsible for converting ammonia to nitrate. The nitrifying bacteria of the compost truly had been killed. The 50°C compost still failed convert ammonia to nitrate, even when the compost temperature was lowered to 28°C.

Fig. 2. Accumulation of NH₄-N in a high temperature compost due to death of nitrifying bacteria.

In this compost experiment, two ecological principles were demonstrated. Actinomycetes can be favored by appropriate environmental conditions in a natural substrate and they will increase in proportion to other microbes. Secondly, unfavorable environmental conditions, if they are sufficiently severe, can entirely eliminate a microbial population from a natural substrate.

Waksman then suggested a different area of ecological research, that of actinomycete pathogenicity. Potatoes are an important agricultural crop in New Jersey. Potato scab is a serious disease caused by an actinomycete which lowers the economic value of potatoes. The pathogen, *Streptomyces scabies*, does not grow under acid conditions. Potato scab disease is controlled by farmers by adding elemental sulfur to soils. A soil microorganism, *Thiobacillus thiooxidans*, oxidizes the sulfur to sulfuric acid, thus producing the desired soil acidity⁴.

Data in Table 1 show typical results of potato culture in New Jersey. Potato yields per acre were equivalent at the natural soil pH level of 6.7 and in sulfur acidified soil at pH 4.2. In the sulfur supplemented soil, almost all the potatoes harvested could be sold, because there was little potato scab infestation. With the untreated soil, *S. scabies* grew well and the potatoes were not salable.

To investigate the ecological relationship of the pathogenic actinomycete, *S. scabies*, and the sulfur oxidizer, *T. thiooxidans*, alkaline soils which had never been used for potato culture were selected. Surely, if inoculated with *T. thiooxidans*, the sulfur added to these alkaline soils would be oxidized more rapidly than would uninoculated sulfur. The soils thus inoculated would more quickly be brought to the acid

Table 1. CONTROL OF POTATO SCAB BY ACID pH.

SOIL TREATMENT	POTATO YIELD BU/ACRE	PERCENT SALABLE POTATOES	SOIL pH
NONE	231	13	6.7
600 LB S PER ACRE	250	98	4.2

state favorable for potato culture.

Figure 3 shows the results. Added inoculum had no value. Soil acidification proceeded equally rapidly in inoculated and uninoculated soils. Another ecological principle was demonstrated. Soil is a wonderful reservoir of microbes. Soil microbes, even if present in small numbers, can rise quickly to meet a metabolic opportunity. The few sulfur oxidizing bacteria present in an alkaline soil multiplied so rapidly to meet the challenge of added sulfur that there was no need for an artificial inoculum.

From this experience with the sulfur oxidizing bacteria, an assumption was made. The large mass of actinomycete spores present in soil has great potential. All required to activate the actinomycete spore mass of a soil is addition of an adequate nutrient challenge. Then, the actinomycete spores of the soil will respond by germination and growth.

By assigning me to conduct personally these two experiments - first, the showing that actinomycetes can undergo massive growth and become metabolically dominant in a substrate under favorable

conditions and, secondly, the showing that under unfavorable environmental conditions a soil actinomycete is decreased in number - Waksman had proven to me by demonstration, much more convincingly than he could by lecture, that actinomycetes can play an active and an important role in nature, and, that they can be manipulated by man.

With eyes opened, I examined more thoroughly the earlier work of Waksman, his associate professor, Robert Starkey, and their graduate students. Examples were present of actinomycete interactions in the soil. One series of literature reports was especially interesting⁵, early publications in 1923, in which Waksman and Starkey noted evidences of antagonism between soil actinomycetes and other soil organisms. They stated "Certain actinomycetes produce substances toxic to bacteria where, around an actinomycete colony on a plate, a zone is found free from fungus and bacterial growth." We, as students, had observed similar evidence of antagonism in laboratory exercises in our soil microbiology course. Nearly every time we plated a soil on a rich agar medium we saw abundant instances of spreading bacteria being inhibited by nearby actinomycete colonies.

Then came the happenings which changed the course of research in Waksman's laboratory. A specific antagonistic agent was isolated by prior student, Rene Dubos, at the Rockefeller Institute in America, and Florey, at Oxford University in England, initiated work with penicillin. Waksman realized that his favorite organisms, the actinomycetes, might be superior producers of antagonists. He gave me my first antibacterial assignment. "Take some soil, put it in pots, manipulate the soil microbes with additives and environmental changes, supplement the soil with a pathogen, see if you can isolate an actinomycete which will produce an antagonistic substance."

Preliminary to starting the search for actinomycete antagonists, a visit was paid to Dubos. Dubos described addition of the carbohydrate capsule of the pneumococcus to soil and from it isolation of a microbe producing an enzyme capable of hydrolyzing the pneumococcal capsule⁶. Then, Dubos extended the study by adding whole bacterial cells and isolated *Bacillus brevis*, from

Fig. 3. Ineffectiveness of *T. thiooxidans* inoculum on rate of sulfur oxidation in soil.

which he obtained tyrothricin, able to kill the pneumococcus⁷).

Following Dubos' lead, and under Waksman's direction, I chose *E.coli* for addition to soils⁸). *E.coli* could be counted on selective Endo agar and the actinomycetes of the soils on egg albumin agar.

E.coli cells were grown, washed thoroughly and billions were added to pots of rich Rutgers University farm soil held in an incubator. Counts of the surviving *E.coli* and the soil actinomycetes were made weekly. The numbers of *E.coli* decreased, but there were many survivors still present a month after first addition (Fig. 4).

The study was extended for three months. Every week, fresh *E.coli* cells were grown and added to the soil pots. With repeated additions, death of the added *E.coli* became more rapid. By three months, the supplemented soil pots had become highly efficient *E.coli* killing machines. Within a week of addition, all the added *E.coli* cells

were killed. But, the soils had not become toxic. The total population of the soils had increased. The actinomycete numbers in the *E.coli* enriched soils were enhanced by more than 4 fold.

The time had come to search in the *E.coli* enriched soils for a pure culture actinomycete which might be killing the added *E.coli* cells. The enriched soils were plated in agar made turbid with living *E.coli* cells. At an appropriate dilution, colonies of soil actinomycetes appeared, surrounded by hazy zones in which the *E.coli* cells had been killed and partially dissolved. An especially active pure culture actinomycete was isolated. From its broth a highly potent antagonistic substance, named actinomycin, was obtained⁹). With the help of Max Tishler, a chemist from Merck & Co., Inc., actinomycin was crystallized¹⁰).

Waksman decided this class of antagonistic substances deserved a special name. During a luncheon seminar, he proposed the term antibiotic for sub-

Fig. 4. Increase in rate of death of *E. coli* in soil associated with weekly *E. coli* supplementations.

stances produced by one microbe which inhibit or kill other microbes. The word antibiotic entered the scientific literature¹¹).

At first, because of the ease of showing antagonistic relationships among soil microorganisms, it was assumed that many new useful antibiotics would be obtained from soil microorganisms. And at first it was easy. Streptomycin, candididin, bacitracin, chloramphenicol, the tetracyclines were found quickly. Then, little of value was isolated for a long time, in spite of extensive screening. Many soil microbes did produce antibiotics. But, most of them proved toxic.

It became necessary to search far and wide to find useful antibiotic producers. For success in the search, screening specialists were required to take advantage of another important ecological principle. Soils differ very greatly in their actinomycete content, especially the types of actinomycetes which predominate in each soil. If one is to find the rare soil actinomycetes which produce new non-toxic useful antibiotics, the actinomycetes of very many different soils must be surveyed. The search must be concentrated on actinomycetes which have never been isolated before.

An example, drawn from my retirement activities, is the discovery of a potentially useful new natural product produced by an actinomycete isolated from the bank an acidic lake at Goshikinuma. This park area in Japan contains several lakes of volcanic origin which vary in color, in mineral and algal content and in pH. Several are pH 5 or below, most unlikely places to find actinomycetes. To improve chances of obtaining a few actinomycetes from the Goshikinuma lakes, soils taken from the lake edges 3 cm below the water surface were plated in agar at pH 5.5 as well as at neutral pH.

The actinomycetes isolated from the Goshikinuma lake edges grew well on agar at both pH levels. A large variety of actinomycete types were obtained. Obviously, unique, acid tolerant actinomycete populations exist in the Goshikinuma area, a group of cultures unlikely to be included in the usual screening programs.

In another instance of an unlikely source, a survey was made of soils on

the island of Bermuda. The soils were of the semitropical variety, rich in bacteria with considerable variety in actinomycete types. One sampling was made of a coral bank, one foot above ocean high tide level. There were no plants and no obvious organic matter present. Few microbes were expected, practically no actinomycetes. During sampling, however, a strong actinomycete type odor was noted. Plating showed that the ocean coral bank was unusually rich in actinomycetes. They were practically the only microbes present. The richness of actinomycetes in this unusual soil can be attributed to its constant wetting during storms with ocean foam. Foam is rich in chitinous marine life and chitin is a favorite nutrient for actinomycetes.

Much of the foregoing is anecdotal in nature, yet provides evidence that there is an active ecology of the actinomycetes. Is it possible to raise anecdotal reports in ecology to the level of science? Can one quantify the ecological factors which influence actinomycetes in the soil? If we could do so, the quantitative approaches would be useful for industrial microbiologists engaged in screening. The quantitative methods also would be useful for those attempting to understand the fundamental aspects of ecology.

Research on quantitative approaches for measuring the differences in types of actinomycetes which predominate in soils was undertaken in a cooperative project between microbiologists of Merck & Co., Inc. and the staff of the University of Western Australia. We were led to do so by frustration with deficiencies in the literature on actinomycete ecology. For example, what is the effect of depth of soil on the types of actinomycetes present? There is a depth effect on soil microbes generally. Starting with Waksman's first publication on the actinomycetes in 1916¹²), many workers have shown that the ratio between actinomycetes and single celled bacteria changes with increasing depth of soil, the actinomycetes becoming more prevalent. Characterization of this phenomenon is important. For screening programs, is it sufficient to take a single sample from a soil or should a plating be made of the surface layer and another at depth to be certain to obtain representatives of all types of actinomycetes

present in a soil?

The method devised is dependent on two literature references. The first, from England¹³, showed that for study of actinomycetes it is not necessary to assign full species designations. One can make reproducible morphological groupings based on the consistent appearance of cultures on agar slants. The second, far removed from microbiology, is a statistical procedure found useful by geologists for studying distribution of rocks and pebbles of different geological origin in soils¹⁴.

Based on these two references, a method for evaluating statistically the differences in types of actinomycetes present in two soils was developed^{15,16}. Soils were plated in duplicate on a medium preferred by actinomycetes. Every actinomycete present on Petri dishes was picked, moving from one side of the dish to the other, dividing the cultures during

picking into two sets on an alternate basis, making certain that at least 100 actinomycete isolates were obtained from each plating. The actinomycetes were grown on agar slants, then sorted into 12 distinct morphological groups based on slant morphology and the number falling in each group was counted (Fig. 5).

Statistical terms were calculated, using the method of the Krumbein and Tukey publication¹⁴. f_1 , measured the degree of difference in actinomycete types observed in the alternate picks from a single soil plating, compared with a random distribution. f_2 , compared the differences in actinomycete types observed in the duplicate plating with differences observed in the alternate picks. f_3 , was the degree of difference in actinomycete types obtained from two different soils compared with the controls, that is the duplicate plating and the alternate picks. The f_3 value is

Fig. 5. Development of data for statistical analysis of degree of difference in actinomycete populations of two soils.

important. When the value of f_3 is greater than 4.3, we know with a confidence level greater than 90% that the dominant actinomycete populations of two soil samples differ. The higher the f_3 value, the greater the degree of difference in actinomycete populations of two soils.

To assess the extent of difference in actinomycete types at different depths in a soil, a fertile laterite loam soil obtained near Perth, Australia was used. Samples were collected at depths of 1, 2, 3, 4 and 5 centimeters. The data in Figure 6 show that the actinomycete populations do change significantly between surface populations and deep populations. There was no significant difference in dominant actinomycete types present in soils taken at the one and two centimeters depth. Then, a change in population occurred. The change in dominant actinomycete types below the second centimeter was highly significant. Clearly, there is a different actinomycete population at the

surface layer of a soil from that below two centimeters of depth, and the change intensifies with increasing depth. With such quantitative data, one need no longer speculate about the effect of soil depth in screening programs. If one wishes to evaluate all types of actinomycetes present in a soil, both a surface sample and a deep sample must be taken at each soil location.

The new statistical approach was used to measure degree of difference in actinomycete populations of an Acacia plant rhizosphere and nearby plant free soil. A true rhizosphere effect on the actinomycete populations was shown (Fig. 7). The population of Acacia plant roots differed very greatly from the actinomycete populations of nearby root free soils. Thus, for efficiency in screening programs, actinomycetes must be selected both from plant free areas of a soil and from the roots of plants growing in that soil.

At a new site, one never previously sampled, how many soil samples should be

Fig. 6. Demonstration of major differences in actinomycete types present in surface and subsurface soil layers.

Fig. 7. Demonstration of major differences in actinomycete types present in a plant rhizosphere and a near-by plant free soil.

plated? To obtain the answer, actinomycete cultures were collected from 5 soil samples taken from plant free areas of a field in a grid pattern, with each sample 30 cm from the next. The result was surprising (Fig. 8). No significant differences were found in the actinomycete types present in the five soil samples. So, screening is simplified. From each general location one need plate at only one plant-free site. To make more such platings would lead to redundancy in a culture collection.

If no differences exist in actinomycete content of soils at distances as great as 30 centimeters apart, does that mean that the geologic origin of a soil has little influence on the types of actinomycetes present? To answer that question, samples were selected from 5 distinct types of soil in Western Australia, ranging from seaside sand, nutritionally poor scrub land, cleared forest, poor outback soil with no prior

contact with man, to a fertile farm field. Selections were made at only a few miles distance from one another, so each soil had had similar rainfall and temperature.

The results show extreme differences in the dominant actinomycete populations of the five soils (Fig. 9). Therefore, when a new area is sampled for screening of soil actinomycetes, advantage should be taken of the geologic differences of the soils present in the area in order to obtain the widest range possible of actinomycetes for the screens.

A variety of other statistical studies have been performed, for example determination of the effect of soil temperature, of rainfall, sun drying of soils, presence of different types of plant cover on the types of actinomycetes present in soils. Clearly, the statistical method has value as a quantitative procedure for studying actinomycete ecology in soils. It has proven

ACTINOMYCETE DIFFERENCES
FIVE SOILS FROM SAME FIELD

Fig. 8. Similarity in actinomycete types present in five soil samples selected in a 30 cm grid patter.

ACTINOMYCETE DIFFERENCES
FIVE SOILS - DIFFERENT AREAS

Fig. 9. Demonstration of major differences in actinomycete types present in five geologically distinct soils.

especially useful as a guide for industrial microbiologists engaged in screening. The method also should be useful for those interested in fundamental aspects of the ecology of the actinomycetes.

During the 50 years in which the author has been engaged in studies of actinomycetes of the soil, much progress has been made in actinomycete ecology. From uncertainty of the significance of the actinomycetes seen on soil dilution plates, we have progressed to a certainty that actinomycetes of the soil truly metabolize and play an important role in the soil. We have learned to utilize the metabolic activities of soil actinomycetes for advantage of man. With new methodology, we now have the opportunity to investigate actinomycete ecology from a quantitative viewpoint.

What of the future? With the newer methodologies of genetics now available, who can predict what the advances will be in the next 50 years. All that is required is interested, well trained investigators. Surely, with support given by organizations such as the Japan Actinomycete Society and with specialized publications available, such as *Actinomycetologica*, in which to record data for all to see and evaluate, the advances of the next 50 years should exceed those of the last.

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