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Impact of microbial diversity on antibiotic discovery, a personal history

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Some 57 years ago, in 1939, I had the good fortune to begin studies in the Soil Microbiology Department of Rutgers University, supervised by professors profoundly concerned with microbial diversity. Graduate students at the university were investigating many types of microorganisms—molds, bacteria, protozoa, and algae; autotrophs, free-living nitrogen fixers, thermophiles, halophiles, and acidophiles; pathogens and saprophytes. Projects under study by students gathered from around the world included microbial oxidation of sulfur in the soil, composting of organic matter at elevated temperatures, soil humus formation, the influence of microbes on soil erosion, nitrogen fixation under acidic conditions, microbial corrosion of pipes in soil, decomposition of cyanide in soil, organic acid formation by soil microbes, the microbiology of marine sediments, and the associative interrelationships among microorganisms growing in soils.

Entering the laboratory of Professor Selman A Waksman was a challenging experience, a great change from the certainty of chemical reactions in which I had been trained as an undergraduate chemistry major, and the study of microorganisms in pure culture which I had experienced in bacteriology courses.

Soon, these wide-ranging experimental experiences of graduate students would change. Breakthroughs were occurring in the investigation of the antagonistic interrelationships of soil microorganisms which would lead to emphasis being given to the microbial products, that is to antibiotics, rather than to the associative processes which occur in nature. As Waksman's student associated with the isolation of actinomycin, the first antibiotic substance obtained crystalline from an actinomycete [20], I inadvertently contributed to the coming departmental specialization. Following our discovery of actinomycin, 17 other antibiotic entities were isolated and described at Rutgers University by Waksman and his students. An additional 11 antibiotics were isolated in the Rutgers laboratories after Waksman's retirement. What effect did this specialization of laboratory endeavor have on the prior departmental research emphasis on the diverse microbes of the soil? This review paper, which was prepared as part of the 50th year celebration of the initial successes in antibiotic discovery at the Agricultural School of Rutgers University, is designed to provide answer to that question and to emphasize the significance of microbial diversity as a factor promoting the early discoveries of antibiotics, thus fulfilling an objective of this special SIM issue.

At the time I entered the Rutgers Soil Microbiology Laboratory, the fact that antagonistic interrelationships occur among microorganisms was well recognized, traced even to Pasteur's reports of the harmful effect of wild yeast on the industrial beer and wine fermentations, as well as to Pasteur's observation that a mixed population of soil microbes which included anthrax spores was less infective for animals than injections of the anthrax organism alone. Waksman, early in his research career, also had noted the antagonistic effects which certain soil actinomycetes exert against other soil microbes and, in association with student Jackson Foster, had published on the phenomenon [15]. The successes of Howard Florey and his associates at Oxford University [1] and of Rene Dubos at the Rockefeller Institute [2] in defining chemical entities as the basis for antibacterial activities led Waksman to initiate a search for other antimicrobial chemicals and the discovery of actinomycin, which was easily crystallized from ether extracts of cultures of *Streptomyces antibioticus*, was the result.

At the time of our initiation of the antibiotic studies, Waksman was a member of the prestigious National Academy of Sciences. He chose to make the first announcement of the discovery of actinomycin at a meeting of the Academy in 1940. Newspapers picked up the story. Reporters descended upon the soil microbiology office and laboratory. Articles appeared, first in the daily press, then in secondary publications, with great flourishes.

Thereafter, newly entering students in Waksman's Soil Microbiology Laboratory were assigned to screening for new antibiotics and the study of interactions among microbes which occur within the soil became a secondary issue. Among departmental visitors was Maurice Welsch, an experienced Belgian bacteriologist. His professor at Liege, Andre Gratia, had previously used the filtrate of an actinomycete culture to cure topical staphylococcal infections [3]. Welsch had worked with Gratia's filtrates. He called them actinomycetin, a name similar to our actinomycin. Although Welsch had started a sabbatical year at

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the Rockefeller Institute in nearby Princeton, because of his interest in antibacterial activities and the success in Waksman's laboratory of isolating an antibiotic chemical, he transferred to Rutgers so he could take part in the developing field. Eventually, Welsch served as co-author with Waksman and myself in publications describing antibiotic screening approaches [17].

Another laboratory visitor for a short time was Walter Kocholaty from the University of Pennsylvania. Also assigned to screening, he isolated a strain of *Streptomyces lavendulae* which inhibited growth of Gram-negative as well as Gram-positive bacteria. Following Kocholaty's departure, Waksman and I succeeded in isolating the antibacterial principle of the culture. We named it streptothricin [21]. We provided streptothricin concentrate to HJ Metzger of the Rutgers Dairy Department, who had an animal model for investigating *Brucella abortus*, the cause of contagious abortion in cattle and undulant fever in man. Metzger found streptothricin effective in preventing the animal infections [7]. This study was the first success *in vivo* with a Rutgers antibiotic. Delayed toxicity prevented streptothricin's use in man, but soon graduate students Albert Schatz and Elizabeth Bugie with Waksman had isolated streptomycin [9], a clinically effective broad spectrum antibiotic, active also against *M. tuberculosis*, complementing penicillin in its massive impact on clinical medicine and the technology of industrial fermentations.

Hubert Lechevalier, Waksman's successor at Rutgers as the professor specializing in actinomycetes, has written rather disparagingly of his part when as Waksman's student he was assigned to screening endeavors. 'It was a silly-simple project: collect soil samples, plate them out, isolate actinomycetes, test them for antibiotic activity against non-pathogenic strains of mycobacteria and hope that you will find something that will be active against pathogenic strains' [5]. Truly, this is the way we think of screening programs today. However, back in the 1940s, screening was an important entry into a new field. By being observant in his screening approaches, Lechevalier succeeded in finding the next two Rutgers actinomycete antibiotics used successfully to overcome infections, neomycin and candicidin.

The impacts of the reports of the discovery of actinomycin and streptomycin were felt not only at Rutgers. Others, because of Waksman's influence and the exciting media reports, took up the screening approach, using actinomycetes. Paul Burkholder, at Yale, emphasized cultures from far off places and found chloramphenicol, produced by a new type culture obtained from Venezuela. Appropriately, he named the producing organism *Streptomyces venezuelae*. Purification of chloramphenicol was accomplished at the Parke Davis Company, so a laboratory of the pharmaceutical industry was brought into the project. Chloramphenicol had the exciting capability of curing typhoid fever, a long sought need. Benjamin Dugger, a well known mycologist from the University of Wisconsin, who refused to stop laboratory work at retirement age, joined the Lederle Laboratories. Working in association with staff members, including some of his former students, chlortetracycline was discovered, with the exciting result that the spectrum of human cures was extended to rickettsial organisms. Not to be outdone by competitors, researchers at Pfizer and others at the Bristol Laboratories used screening to find oxytetracycline and tetracycline, chemically related substances, but patentably distinct. Next, another class of actinomycete antibiotics, the macrolides, relatively non-toxic and destined to be especially useful in treatment of childhood infections, was discovered. Different members of the macrolide class have received preference in different countries of the world, showing that political considerations can even extend to science.

Merck & Co, Inc, my employer after graduation from Rutgers, lagged behind in the race to find new antibiotic products. At Merck, we microbiologists were fully occupied bringing Waksman and Schatz' streptomycin to the market and soon thereafter we had another actinomycete product, vitamin B12, with which to contend.

Later, Merck did enter the screening field, co-discovering cycloserine and novobiocin in its New Jersey laboratories. These products set the stage for initiation of a large-scale screening laboratory in Madrid, Spain, where more than 20 000 actinomycete isolates were evaluated each year. Important antibiotics obtained from this screen were fosfomycin, introduced in the Latin areas of the world and Japan, based on local physicians' and patients' preference for parenteral therapy, even when applied to minor infections, followed by the discovery of cephamycin and thienamycin, from which the commercial products cefoxitin and primaxin were obtained, as well as the avermectins, anthelmintic agents used for control of round worm infections of farm animals and dogs and for elimination of onchocercosis—river blindness—a debilitating disease of the tropics.

Detailed references to the above antibiotics, as well as to the thousands of others obtained from actinomycetes, are contained in the volume entitled Index of Antibiotics from Actinomycetes [12], as well as in supplements published each year in the Journal of Antibiotics. Early studies on actinomycetes are described in a three volume treatise The Actinomycetes, written by Waksman, who was aided by his student and successor in actinomycete research at Rutgers, Hubert Lechevalier, with respect to Volume III, which contained descriptions of their antibiotics [14]. Recent publications also cover the historical aspects of the penicillin and streptomycin developments [13] and Waksman's research as it relates to the treatment of tuberculosis [8].

Thus, the discovery of actinomycin and streptomycin, which resulted from Waksman's interest in the diversity and interactions of microorganisms of the soil, had significant impact on applied science of the time. But a question may be of interest. Why were the initial discoveries of the actinomycete antibiotics made at Rutgers? What was the instigating factor in their being found in Rutgers' small, two-professor soil microbiology department? Why was the discovery not made at one of the large agricultural universities of the US mid-west, or a major eastern medical school? These are interesting questions. Their answer, I believe, can be traced to a persistent spark of interest held by one man, Selman A Waksman, in the microbial diversity of the soil and, in particular, to his conviction that the actinomycetes of the soil are active members of the soil population and are important.

After completing undergraduate and masters projects on

the fungi of the soil, Waksman transferred to California and extended his studies to include biochemistry. He received the PhD degree in 1918. Returning to Rutgers, he accepted the position of microbiologist in the Agricultural Experiment Station, a place where the field of soil microbiology was already well established under the direction of his former mentor, Jacob Lipman. Waksman was especially intrigued by the group of soil microorganisms he had investigated as a masters degree student, the actinomycetes, which developed on Petri plates as small, hard, slowly growing colonies, forming up to 30% of the colonies present on his agar plates limited in nutrients. These organisms were to become Waksman's lifetime fascination. We can see his enthusiasm in the species names he coined for his newly isolated types, *lipmanii*, named for his professor, *halstedii*, for Byron Halsted his favorite undergraduate teacher of botony, *bobili*, his nickname for his wife Deborah, even *rutgersensis*, where his Master's program on the actinomycetes had originated.

Ever after, Waksman sought evidence that the actinomycetes are important members of the soil population. He studied the actinomycetes extensively in laboratory culture, where they grew well and were very active biochemically. With Arthur Henrici of the University of Minnesota, he was responsible for the presently established generic name *Streptomyces*, applied to the common soil types [16]. He and his students wrote papers on the streptomycetes in the hundreds [23]. He prepared textbooks with extensive chapters on the actinomycetes. He was strongly confident of the part actinomycetes play in the soil, but in obtaining firm proof for this idea he was often frustrated.

In detailed studies with student Robert Starkey, later his co-professor at Rutgers, the response of soil microorganisms following partial sterilization of soil with toluene was studied. The soil protozoa, the soil bacteria and soil fungi multiplied quickly after such treatment. Unexpectedly, however, based on total counts, the actinomycetes often were sluggish in response [18]. Data such as these were discouraging. Workers at other institutions, who did not have Waksman's confidence, concluded that the actinomycetes seen on soil plates have little significance in the soil. The common conclusion of many workers at the time—actinomycetes can be ignored as important soil components.

At the time I entered his laboratory, Waksman grasped at any study which showed soil actinomycetes to be important. I can recall vividly his excitement concerning an experiment in which I was a helper, performed during the first few weeks of my study. A visitor from China was interested in composting human feces at sufficiently high temperature that all human pathogens would be destroyed. We mixed feces with soil and placed the combination in incubators at 50 and 70°C. Composting occurred actively at 50°C, very slowly at 70, as indicated by mineralization of the organic matter. Plating showed a mixed population of soil microorganisms present at 50°C. Actinomycetes were predominant, however, and the compost pot had a strong actinomycete odor. At 70°C, only cultures of actinomycetes were recovered [25]. Waksman was happy. Here was proof that actinomycetes in the soil can be important, when offered favorable opportunity for growth.

With this breadth of experience with soil actinomycetes, it is not surprising that, when Waksman's interest was sparked by the research studies of Howard Florey and of Rene Dubos, which implied that release of antibiotics could be the explanation for the antagonistic interrelationships he had previously seen among soil microorganisms, Waksman should turn to the actinomycetes as possible sources of such products. To search for soil antagonists, he adopted the enrichment procedure pioneered by Dubos. I was instructed to add each week great numbers of well-washed living *E. coli* cells to a variety of soils and to follow the results. As expected, the *E. coli* numbers in the soils decreased following their addition, slowly at first, then more quickly with each addition, until after four months of additions, within a week or so after an addition, no living *E. coli* could be found [19]. In searching for a cause for the drop in numbers, I initially found a very antagonistic *Pseudomonas* sp, and spent much time in the isolation of and study of the pyocyanase and pyocyanin produced by it. But, Waksman was not satisfied. I must screen further, giving attention to the products produced by the actinomycetes, several of which also showed antagonistic effects in my screening plates. I did so, and the detection of actinomycin resulted.

Thus, my questions concerning why actinomycin was found at Rutgers can be answered. Waksman's awareness of the great diversity of microbes present in soils and his insistence that the actinomycetes of the soil have significance led him to probe every possibility to prove that significance. One of the approaches was directed at testing whether actinomycetes can exert antagonistic effects against other organisms in the soil. This study led to the discovery of actinomycin. Workers elsewhere who did not have the same nagging demands for proof of the significance of the soil actinomycetes did not reach out to find an explanation for their casual observations of antagonism among the actinomycetes. Thus, their opportunity to make a major discovery was missed.

As is well known, because of his antibiotic research, Waksman became famous. He received the Nobel Prize. He received accolades wherever he traveled. I clearly recall on one of my visits to Spain searching out the street named for him during a highly emotional occasion in Madrid when he was showered with flowers by former tuberculosis patients fortunate to be alive because of the availability of streptomycin. Yet, at heart, Waksman was still unfulfilled. I remember visits made to his office during his latter years of retirement, at his urgent phone request, to discuss some important information. Often, the topic of his concern would be forgotten by the time of my arrival. We would review some well known stories, and then he would return to his favorite topic, the actinomycetes. He was disturbed because leaders in the field did not fully accept his opinion of their significance.

Waksman's initial expectation, resulting from the discovery of the many actinomycete antibiotics, that actinomycetes by reason of their ability to produce antibiotics are the controlling members of the soil population, was not fulfilled. It had been shattered by failure to show that antibiotics accumulate or exert their action in the soil. A former student, Jack Stokes, showed that even Dubos' soil enrichment technique for antibiotic discovery was unnecessary.

Antibiotic producers could be obtained with ease from freshly collected soils [11]. Even I had failed him, by recalculating my enrichment data showing that the percentage of antibiotic producers had not increased in my *E. coli* enrichment pots. True enough, the absolute number of antibiotic producers had increased. However, even before enrichment, there were hundreds of thousands of antibiotic producers present per gram [22]. The increase in number of antagonists was more likely due to the increase in total population of all types, antibiotic producers and non-producers, caused by the nutrients released as the added *E. coli* died.

Serious blows to Waksman's hopes for wide acceptance of his belief of the significance of soil actinomycetes were experiments published by respected English investigators. Initially, spores of a commonly occurring actinomycete were dried on glass cover slips which were buried in normal unsupplemented soil. After 48 h, the slips were removed, washed and examined microscopically. Less than 5% of the spores had germinated. Then, taking advantage of evidence that strong maceration with sand destroys actinomycete filaments but has no effect on actinomycete spores, proof was obtained that almost all the actinomycete colonies appearing on soil dilution plates arise from spores. Less than 2% was calculated to arise from mycelial filaments [6, 10]. On this basis, it could be concluded that actinomycetes play little role in the soil.

I owe Rutgers University and Selman Waksman much. I was his student at the Rutgers Agricultural School during an exciting time. Due to his interest in the diversity of soil microbes, my appreciation of microbiology was greatly broadened. I have had a fulfilling life, and much of my excitement can be traced to products of actinomycetes and to demonstrations of their benefit for man. At times I have considered the most meaningful experiment of my lifetime. Looking back, I have decided that experiment does not concern actinomycin, streptothricin, vitamin B12, the amino acids, the nucleotides, nor the many antibiotics studied during my schooling and employment [24], but is a series of experiments conducted in association with researchers at the University of Western Australia, important in defining the significance of the diversity of soil microbes, but of no practical outcome. These cooperative experiments accomplished what Waksman found difficult to achieve in his lifetime, offering convincing proof that actinomycetes are truly active in the soil.

Our experiments were made possible because of another screening endeavor. In a cooperative study between the University and Merck & Co, hundreds of actinomycetes were isolated from unique soil types in Western Australia. As a complementary research project, David Keast of the Microbiology Department of the University, Edward Stapley of the Merck Research Laboratory and I, then located at the Merck Research Office in Japan, decided not to measure the total number of actinomycetes present in each soil by direct count, but to enumerate the number of distinct morphological types present on soil dilution plates. JW Tukey, Professor of statistics at Princeton University, aided us greatly by devising a mathematical expression for the degree in which two soils differ in types of actinomycetes present. Much laboratory work was required, growing cultures under standard conditions, observing soluble pigments, spore formation, characters which enter into actinomycete classification. In one study, we sampled a fallow field in grid pattern, taking five samples, each 30 cm apart from the other. Surprisingly to us, the actinomycete types did not differ among those soils sampled. In each soil studied, though significantly removed in distance of sampling from the other, the same kinds of actinomycetes were present, to the same degree. It appeared that those who point to an inactive stable actinomycete population in soils might be correct.

Then we moved to a new situation, comparing the soil adjacent to roots of an acacia plant, roots from a species of *Tetragonia* and a soil with no plant growth. In contrast to the former experiment, the three soil populations differed greatly in types of actinomycetes present. Obviously, nutrient background can modify the types of actinomycetes present in soils, regardless of whether they are present as mycelial fragments or spores. The study did not prove that the actinomycetes of the three soils were highly active. The change in types observed could have occurred slowly, requiring many months.

Then, luck intervened in our experiments. Perth, Australia, has a Mediterranean type climate, with extended dry periods, followed by a rainy season. We happened to sample and evaluate a soil for actinomycete content at the end of a dry period. Then the rains came. We sampled five days after rain and again after thirteen days. A major change in the actinomycete types had occurred at the 5-day sampling and an even greater change at 13 days. No great change was seen in the total actinomycete count, so the effect would have been missed by that approach. Our revised technique of studying the types present showed clearly that the actinomycete population of the soil is dynamic. Soil actinomycetes respond to physical factors and associated nutritional factors, and they do so with great speed [4].

So, my respected professor was right all along. Soil diversity is important and the actinomycete population of the soil is dynamic. Actinomycetes have significance beyond their ability to produce antibiotics. They are significant members of the diverse soil population. Actinomycetes respond, they change, they multiply in soil with rapidity. One actinomycete population can replace another. Even though the colonies we recover on our Petri plates may be largely from spores, they can arise from different spores, spores of a new population, one which can develop within hours of an inciting environmental stimulus. Thus, actinomycetes do not differ from other members of the soil biota. They truly are important.

By the manner which I have presented this review, it should be obvious that my admiration and respect for my professor, Selman Waksman, is great. That an experiment in which I played a part has proven him correct in the major thesis of his research life, without doubt, makes it the most satisfying experiment of my career.

The discovery of antibiotics produced by actinomycetes was an important event, occurring more than a half century ago. It is entirely appropriate that celebrations have been held at the sites of the antibiotic discoveries to recognize microbial products which have proven to have great clinical utility. As an important part of the celebrations, however,

we must remember that it was the basic interest of the early leaders of microbiology in the diversity of soil microorganisms which prepared them to design the critical experiments leading to their practical discoveries.

In our consideration of the history of antibiotics, we must take care that the practical events do not overwhelm appreciation for the basis for the antibiotic discoveries. It was appreciation of the diversity of soil microorganisms that led Dubos, Waksman and their followers to choose soils as the source for their experimentations. Many years of study of the varied populations of the soil had prepared these leaders for their productive studies. That fact must not be lost by incoming students of microbiology. In their search for quick accomplishments, they may be all too ready to specialize before appreciation for the importance of microbial diversity has been fully developed. Appreciation of and research on microbial diversity remains an essential background for all studies in microbiology, as important today as it was a half century ago at the dawn of the antibiotic age.

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