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## Thom Award Address

# Actinomycetes in the Family

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My wife, known to many of you as Midge, and I are greatly touched to have been selected to receive the eleventh Charles Thom Award. We have done many things together and it is a pleasure to be so greatly honored jointly.

I have been accused of doing too many things with my wife. This is considered unhealthy. One of my colleagues told me several years ago: "You work with your wife, you see her all day, you are with her at home, you should not play golf with her. This is too much!." In my defense, I could demonstrate that I do not spend all my time with her and the proof is that she is presently in Mexico, while I have the pleasure of being with you in Saint Paul.

I shall try to give you a summary of what we have done together in the chemical taxonomy of actinomycetes. Please keep in mind that the distribution of effort in this work may not have been equal and that Midge deserves the lion's share of the credit for the results obtained.

We both came to Rutgers to work under the direction of Dr. Selman A. Waksman. I had never seen an actinomycete before 1948 and I presume that she had never seen one either. Dr. Waksman gave Midge many different things to do, she got involved in the study of an antiviral antibiotic, in the taxonomy of the *Streptomyces lavendulae* group, and in the microbial flora of the intestine of earthworms. In my own case, the discovery, isolation, purification, characterization, and other studies of neomycin, candicidin, and relatives plus the innumerable odd jobs that Waksman could always generate were enough to keep me busy until we both graduated in 1951.

By that time, Midge was pregnant and was due to eclipse from the scientific scene but not before Waksman had extracted from her a promise to return to microbiology. This was first done at home, in the Watchung mountains. Midge had developed an interest in fresh-water algae and a friendly salesman, for $25.00, procured us a microscope which had served in the training of innumerable generations of students at Yale University. From fresh-water algae to Actinoplanaceae the chasm is not wide. However, it is on a bisporate actinomycete that Midge concentrated most successfully the resources of her kitchen (Lechevalier and Lechevalier 1957).

From the kitchen, Midge went to the Steroid Preparative Laboratory of E. R. Squibb and Sons where her career as a strictly industrial microbiologist was interrupted in 1961 by a 1-yr stay at the Pasteur Institute in Paris. There we studied the distribution of *Micropolyspora* species in various substrates, including leprous patients. The search for micropolysporae led to the isolation of many of the rarer types of actinomycetes and to the description of *Microellobosporia* with Tom Cross (Cross et al. 1963).

During the winter of 1961-62, we responded to kind invitations from some of our English colleagues and took advantage of our incursion across the channel to meet Dr. Cecil Cummins at the London Hospital. I had been asked to organize a meeting of the French Society of Microbiology in Paris, devoted to actinomycetes. Cummins was invited to review cell-wall composition in the taxonomy of actinomycetes. His summary is given in Table 1.

The results of Cummins showed that there existed two broad groups of actinomycetes, those with lysine and those with diaminopimelic acid (DAP) in their cell walls. In addition, the nocardiae and streptomycetes did not contain the same isomer of DAP as a major constituent. Upon our return to the United States, we started a program of investigation of the cell-wall composition of actinomycestes belonging to various morphological and physiological types. In this we had the cooperation of a graduate student of unusual maturity, Benjamin Becker. The first thing that became obvious, as various types of actinomycetes were analyzed, was that streptomycetes were almost unique in containing major amounts of the L-isomer of diaminopimelic acid. Almost all the other actinomycetes, except for some obvious relatives of the streptomycetes such as strains of *Chainia,* contained major amounts of *meso-* or D-DAP.

One obvious conclusion was that the type of DAP present could be used to establish a separation between the genera *Streptomyces* and *Nocardia.* Classically, the separation between the two genera had been based on the production of chains of spores in streptomycetes and the fragmentation of the mycelium in nocardiae. However, as time passed, fewer and fewer people had confidence in these criteria and the validity of these taxa was being questioned (Bradley 1959). We developed a simple method for the detection of DAP in whole cells of actinomycetes hydrolyzed with HC1 in sealed tubes. The method does not distinguish between the *meso*-form of DAP and the D-form of this acid but recognizes the L-form, thus separating the streptomycetes from the nocardiae. Since the D-form of DAP has not imposed itself as a taxonomic marker, this limitation of the method seems to be of little importance (Becker et al. 1964).

As the work on cell wall progressed, it became obvious that the actinomycetes with cell walls containing DAP could be separated into four groups (Table 2) (Becker et al. 1965; Yamaguchi 1965) and Helmut Prauser noted that actinophages recognized and respected these groups (Prauser 1981). In addition, morphological mutants of strains of actinomycetes retained the parental type of cell wall (Šuput et al. 1967). To us it seemed that by combining morphology with chemistry, it would be possible to separate actinomycetes into workable groups at the generic level. We proposed such a system of classification in 1965 (Lechevalier and Lechevalier 1965) and its principles have been widely accepted.

Cell-wall composition was useful in classifying actinomycetes but the preparation of a pure wall from a given strain is long and tedious. Of course, as we have seen, the determination of cell walls of type I does not require the preparation of cell walls since L-DAP can be recognized in whole-cell hydrolysates. The next question was to determine if cell walls of types II, III, and IV also could be identified from whole-cell hydrolysates. Midge solved the problem by introducing the chromatographic analyses of sugars in whole-cell hydrolysates (Lechevalier 1968).

In that procedure, cell masses are hydrolyzed in an open tube with sulfuric acid, the hydrolysates are spotted on paper and chromatographed with the upper phase of a

TABLE 1. *Composition of the "skeleton" of the cell wall of four different types of actinomycetes.*

| Oxygen Requirements | Name | Main Constituents of the Cell Wall: Sugars | Amino Acids | Amino Sugars |
|---|---|---|---|---|
| Aerobic | *Streptomyces* | Often none. Sometimes glucose and/or galactose. | Alanine, glutamic acid glycine; LL-DAP[a]; or LL + DL-DAP. | Always glucosamine and muramic acid. Sometimes also galactosamine. |
| | *Nocardia* | Arabinose, galactose. (also sometimes glucose or mannose.) | Alanine, glutamic acid, DL-DAP. | |
| Micro-aerophilic or anaerobic | *Actinomyces israelii* (mainly of human origin) | Galactose only. | Alanine, glutamic acid, lysine. | |
| | *Actinomyces bovis* (mainly of bovine origin) | Rhamnose, fructose, other methylated pentoses. | Alanine, glutamic acid, lysine and often aspartic acid. | |

[a]DAP = Diaminopimelic Acid

TABLE 2. *Distinguishing major constituents found in the cell walls of DAP-containing actinomycetes*

| Cell-Wall Type | Major Constituents[a] |
|---|---|
| *Streptomyces* or I | L-DAP, glycine |
| *Micromonospora* or II | *meso*-DAP, glycine, |
| *Actinomadura* or III | *meso*-DAP |
| *Nocardia* or IV | *meso*-DAP, arabinose, galactose |

[a] Alanine, glutamic acid, glucosamine and muramic acid always present.

butanol-water-pyridine-toluene mixture. Detection of the sugars is with acid aniline phthalate. As can be seen in Table 3, the cell-wall types II, III, and IV have corresponding typical whole-cell sugar patterns. One added bonus is the separation of actinomycetes with cell walls of type III into two groups, those with madurose and those lacking this sugar. Madurose was identified as 3-*o*-methyl-D-galactose (Lechevalier and Gerber 1970). One often misunderstood point is that sugars found in the whole-cell hydrolysates, with the exception of those characteristic of cell-wall type IV, are not part of the cell walls of the actinomycetes. There is a correlation between these whole-cell sugar patterns and the cell-wall types but they are not the same thing.

As useful as cell-wall composition and whole-cell sugar patterns were, they did not solve all the problems of actinomycetic taxonomy. One vexing problem was the separation of the genera of the *Corynebacterium-Mycobacterium-Nocardia* (CMN) spectrum of species. The solution of the problem seemed to rest in the mycolic acids produced by those organisms. Edgard Lederer and Jean Asselineau in France had worked extensively on the lipids of mycobacteria and relatives. Midge went to Dr. Asselineau's laboratory in Toulouse for instruction in the methods of microbial lipidology and, with Ann Horan, a graduate student coming to us from Ruth Gordon's laboratory, began to work on the problem. They were rewarded with success as it became obvious that separation could be made among strains belonging to these three genera on the basis of the size of the mycolic acids that they contained.

Mycolic acids are alpha-branched, beta-hydroxylated fatty acids that can be extracted from microbial biomasses. They can be separated into three major groups, the true mycolic acids with skeletons of about 80 atoms of carbon, the nocardomycolic acids with about 50 carbon atoms, and the corynomycolic acids with about 30 atoms of carbons.

Briefly, the detection of the type of mycolic acid produced by a given organism is carried out as follows: biomasses are first saponified with methanolic KOH and methanol. This solution is discarded for all strains but those suspected to be corynebacteria, and the cells are then extracted with methylene chloride. This extract is

methylated and thin-layer-chromatographed to obtain the mycolate-containing fraction. Pyrolysis of mycolates in a gas chromatograph yields fatty acids originating from the alpha branch and aldehyde fractions representing the rest of the molecules. Gas chromatography permits one to gauge the size of the fragments and from that the size of the original mycolates (Lechevalier et al. 1971, 1973). Other authors have modernized the technique by using mass spectrometry to determine the size of the fragments of pyrolysis (Collins et al. 1982).

The simplified account that I have given you of the mycolates of the CMN group of bacteria does not take into account the diversity of the chemistry of this family of compounds. When studied in detail, mycolates may at times furnish a unique tracer for a given organism. For example, when we investigated the nature of the filamentous bacteria that were responsible for the foaming of many sewage-treatment plants of the activated-sludge type, we concluded that most of the time the culprit was a novel species of *Nocardia, N. amarae.* This organism contained a novel type of nocardomycolic acid whose alpha branch is mono-unsaturated (Lechevalier and Lechevalier 1974). We took advantage of this property to estimate the growth of this organism in the field (H. A. Lechevalier et al. 1977).

Cell-wall composition, whole-cell sugar patterns, and mycolic acid structures have been of assistance in the separation of actinomycetes into genera. During the past

TABLE 3. *Cell-wall types and whole-cell sugar patterns of aerobic actinomycetes containing* meso-*diaminopimelic acid.*

| Cell Wall | | Whole-Cell Sugar Patterns | |
|---|---|---|---|
| Type | Distinguishing Major Constituents | Type | Diagnostic Sugars [a] |
| II | Glycine | D | Xylose, arabinose |
| III | None | B | Madurose |
| | | C | None |
| IV | Arabinose, galactose | A | Arabinose, galactose |

[a] Several nondiagnostic sugars such as glucose may be present.

few years, an effort has been made in our laboratory to evaluate phospholipids as taxonomic markers in the actinomycetes. These compounds actually furnish us with two different markers: the phospholipids themselves, and the fatty acids that they contain.

The method we used was developed in collaboration with Claude de Bièvre of the Pasteur Institute and a third graduate student, Ann Stern, a former protegee of Alma Dietz. This method involved the extraction of early stationary phase cells with a mixture of chloroform and methanol, the separation of the phospholipids from other lipids by column chromatography on activated silica gel and Sephadex, and the

resolution of the individual phospholipids by thin-layer chromatography on silica-gel plate. On this basis, five types of phospholipid patterns could be recognzied as indicated in Table 4. The purified phospholipid fractions when treated with lithium hydroxide in the presence of methanol released their fatty acids as methyl esters. Actinomycetes could be classified into four groups on the basis of the types of fatty acids present as major components of their phospholipids (Table 5) (M. P. Lechevalier et al. 1977; Lechevalier et al. 1981).

This gives us thus five chemical markers which are (1) cell-wall type; (2) whole-cell sugar pattern; (3) phospholipid type; (4) phospholipid-fatty acid type; and (5) mycolic-acid size. The last criterion is applicable only to organisms with a cell wall of type IV; mycolates have not been found in other groups. Some of these markers are very easy to determine, especially those from whole-cell hydrolysates; others require elaborate chemical purifications.

I have already indicated how these chemical markers can be used to cut two taxonomic Gordian knots: #(1) l-DAP to separate soft, bald streptomycetes from nocardiae and *meso*-DAP to separate nocardiae with chains of conidia from streptomycetes, and (2) mycolates to help in the establishment of borders in the genera of the CMN complex. In addition, the lack of mycolates furnishes, by default, a marker for some of the organisms with a type IV cell wall. Among these must be counted the very important species *Micropolyspora faeni.*

I shall try to give you a few other examples. Species of *Nocardioides* are nonsporing actinomycetes with a cell wall of Type I. One could be tempted to believe that they are bald streptomycetes. They differ from streptomycetes, however, in having phospholipids of type PI whereas streptomycetes have phospholipids of Type PII, a difference which is more than just morphological. The genus *Actinomadura* can be separated firmly from the genera *Nocardia* and *Streptomyces* on chemical grounds but is not homogeneous itself even after the removal of the *dassonvillei* group into a genus of its own, *Nocardiopsis*, since some *Actinomadura* species have phospholipids of type PI and others of type PIV.

A genus which lacks homogeneity in certain chemical markers is the genus *Frankia* which is presently the center of Midge's attention. Morphologically, it is a good genus but it is the only genus in the cell wall of Type III group in which various isolates have quite diverse whole-cell sugar patterns. One possible explanation is that different actinomycetes have adapted to the endophytic mode of life with evolution of a morphological-type characteristic of the group. It is not clear why such a morphology is of advantage in symbiotic nitrogen fixation.

The practical lesson in taxonomy, I think, is to avoid dogmas and simply to use what seems to work where it seems to be of assistance. Thus whole-cell sugar patterns may be useful to help characterize genera in some cases and may be of value only in the characterization of species or perhaps even of strains in other cases. After all, sugars found as major constituents in the cells of organisms may not always be part of the same molecules and these different molecules may have very different taxonomic significance.

I think that this essentially covers most of the work of industrial interest on which we have published together. Other joint studies have been more morphological and/or ecological. I would like to terminate with a SIM commercial, if I may, and remind

TABLE 4. *Phospholipid patterns of aerobic actinomycetes*

| Phospholipid Pattern | Characteristic Phospholipids | | | | |
|---|---|---|---|---|---|
| | PE | PME | PC | GluNU | PG |
| PI | - | - | - | - | V |
| PII | + | - | - | - | - |
| PIII | V | V | + | - | V |
| PIV | V | V | - | + | - |
| PV | - | - | - | + | + |

Phosphatidyl inositol always present.

PE = Phosphatidyl ethanolamine, PME = Phosphatidyl methylethanolamine, PC = Phosphatidyl choline, GluNU = Phospholipids of unknown structure containing glucosamine, PG = Phosphatidyl glycerol.

- = Not present; + = Present; V = Variably present.

TABLE 5. *Phospholipid fatty-acid types of aerobic actinomycetes*

| | |
|---|---|
| Type 1 | Principal component: branched-chain fatty acid of the anteiso/iso series. |
| Type 2 | Principal component: a saturated or unsaturated fatty acid other than heptadecenoic acid. Anteiso/iso fatty acids present. |
| Type 3 | Principal component heptadecenoic acid. Anteiso/iso fatty acids present. |
| Type 4 | Anteiso/iso fatty acids absent or if present less than 10% of total fatty acids. |

you that details on the chemical taxonomy of actinomycetes can be found in a book published in 1980 by the Society. It is edited by A. Dietz and D. Thayer (1980) and is entitled *Actinomycete Taxonomy.*

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