5-4-1993

The Syringomycins: Finding Their Role in Nature

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THE 78TH USU Faculty Honor Lecture

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INTRODUCTION

About ten years ago, I began looking for a research problem in the field of plant-microbe interactions. As the name implies, this is the study of how plants and microorganisms associate—either for their mutual benefit or at the expense of one partner. An example is symbiotic nitrogen fixation between leguminous plants and root-colonizing bacteria. Both benefit—the plant gains nitrogen in a useful form and the bacteria gain nutrients and protection. A better understanding of a plant-microbe interaction system could be used to benefit agriculture. For example, boosting nitrogen fixation would mean less petroleum-derived nitrogen fertilizers. These interactive biological systems and their applications were attractive to me. Frequent articles appeared in the prestigious science magazines, Science and Nature, describing new and fascinating problems in plant-microbe interactions, including many that could be addressed at the molecular level. My colleagues in the Department of Biology who were conducting research in this area encouraged me to join them. It was also relevant to the mission of the Utah Agricultural Experiment Station which had been very supportive of my work.

My background is in microbiology and biochemistry with emphasis on phenomena related to cell membranes. All living cells use membranes to regulate which substances are allowed in and out of their various compartments (Fig. 1).

Major Cell Membranes

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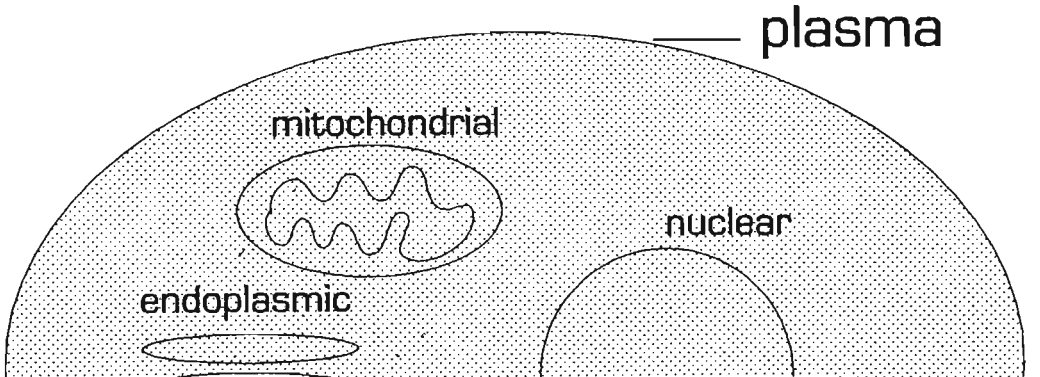
Major Cell Membranes



Like most cellular processes, those of plant-microbe interactions depend on the functions of membranes. Included are membrane effects of toxic molecules produced by microbial pathogens (Daly and Deverall, 1983). One of these toxins was syringomycin. It is produced by a tenacious bacterial plant pathogen and was thought to be the major virulence factor in several diseases (DeVay et al., 1968). Because neither the details of how it worked or its chemical structure were known, I decided to study this plant toxin and learn its characteristics and how it interacted with membranes. As I describe below, however, we learned that syringomycin was not a major plant virulence factor.

SYRINGOMYCIN AND THE BACTERIA THAT PRODUCE IT

Syringomycin is produced by bacteria classified as *Pseudomonas syringae* pv. *syringae* (Gross, 1991). These bacteria are closely associated with a variety of plants and usually on the aerial surfaces. Normally, *P. syringae* pv. *syringae* tries to survive without compromising its plant host. However, when the host is physically damaged (e.g. by freezing) or diseased, certain opportunistic strains of *P. syringae* pv. *syringae* invade the plant and contribute to its death. Because syringomycin is produced by certain opportunistic strains of *P. syringae* pv. *syringae* and appeared to be toxic, it was thought to be a major virulence factor.

By the early 1980's, there was some evidence suggesting that syringomycin targeted membranes, particularly the plasma membrane. The plasma membrane surrounds the periphery of cells (Fig. 1), and in plant cells, it lies just beneath a tough, outer shell of cell wall material. It was thought, for example, that syringomycin preferentially binds to the plasma membrane of plant cells, based on the reaction of rabbit antibodies directed against a preparation of syringomycin. The antibodies, which presumably bound tightly to syringomycin, were in turn labeled with a fluorescent dye to locate the toxin in infected plant tissues (Paynter and Alconero, 1979).

SYRINGOMYCIN'S POSSIBLE ROLE AS AN IRON CHELATOR

In 1982, two articles reported that a component of syringomycin was δ-N-hydroxyornithine (Gross, 1982; Hemming et al., 1982). All natural molecules possessing δ-N-hydroxyornithine functioned as iron chelators. Microbes produce iron chelators, which tightly bind iron to satisfy the microbes' nutritional requirement for this element. If syringomycin contained δ-N-hydroxyornithine, it

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This discovery was serendipitous. At that time, my laboratory at Utah State University was located in Widtsoe Hall adjacent to the laboratory of Dr. Thomas Emery who was an expert on iron

We reinvestigated the occurrence of δ-N-hydroxyornithine in syringomycin and within a month learned that syringomycin was composed of several amino acids, including 2,4-diaminobutyric acid that the earlier workers misidentified as δ-N-hydroxyornithine (Bachmann and Takemoto, 1989) (Fig. 2). In addition, we found no evidence for iron binding to syringomycin. Emery was correct. Syringomycin is not an iron chelator.

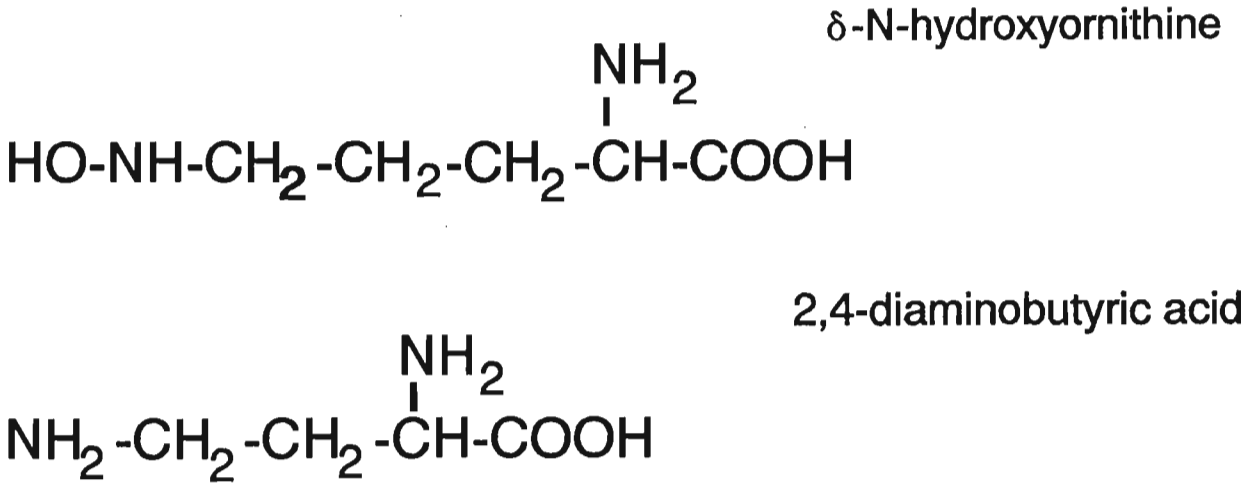
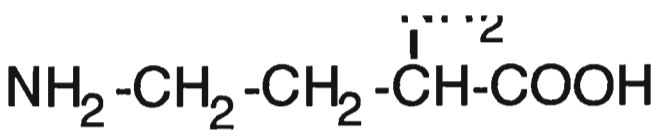


Fig. 2. The structures of two chemically-related amino acids. δ-N-hydroxyornithine was reported to occur in syringomycin, but it was actually 2,4-diaminobutyric acid.



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CHEMICAL STRUCTURE OF SYRINGOMYCIN

The iron chelator research initiated the effort to learn syringomycin's chemical structure. There were several reasons why my students and I decided to do this, but two warrant mentioning. **First**, elucidating the structure could provide clues about its mechanism of action, which was our main interest. This had been demonstrated in our work concerning δ–N-hydroxyornithine. **Second**,

progress as these chemists). Deciphering syringomycin's structure was a difficult challenge that involved the identification of unusual and new amino acids. Nevertheless, we succeeded (Segre et al., 1989). Syringomycin's structure is shown in Fig. 3.

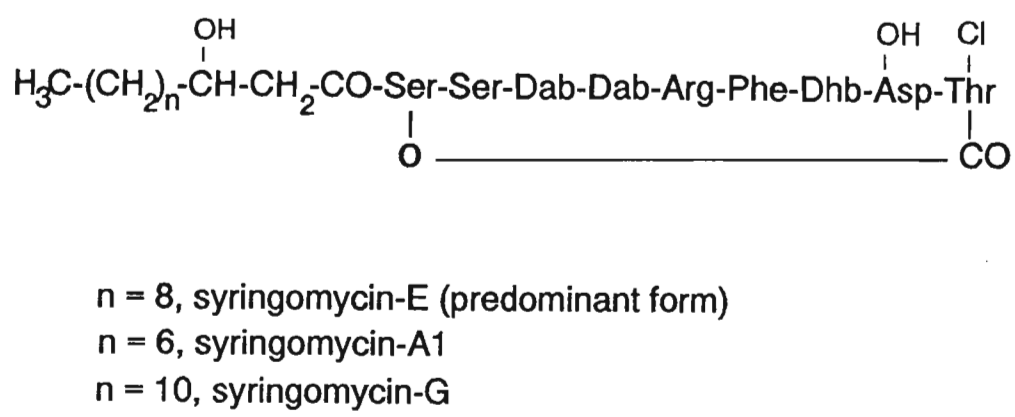


Fig. 3. The structure of syringomycin - its major and minor forms.

n = 8, syringomycin-E (predominant form)
n = 6, syringomycin-A1
n = 10, syringomycin-G

Fig. 3. The structure of syringomycin - its major and minor forms.

The E-form is the most abundant and active of the structural forms. Syringomycin-E is a lipopeptide, a combination of a lipid (actually a fatty acid) and a peptide. The peptide portion contains amino acids arranged in a cycle. Certain of the amino acids are rare. For example, chlorothreonine had not been previously reported, and 2,4-diaminobutyric acid and β-hydroxyaspartic acid do not commonly occur in nature.

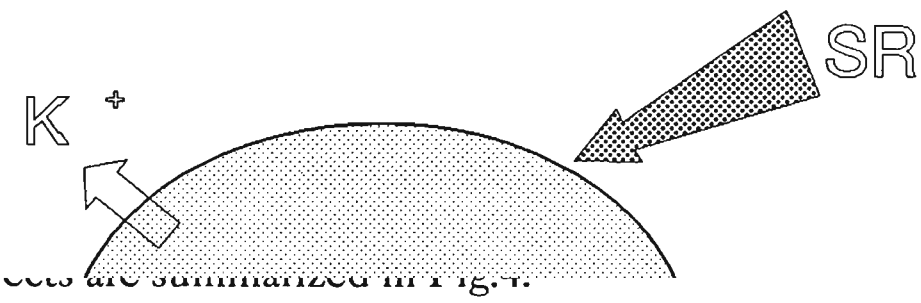
and appear to have similar roles in nature. Together, syringomycin, syringotoxin, and syringostatin are now called the "syringomycins" or the syringomycin family of lipopeptides (Takemoto, 1992). In the remainder of this essay, I will often refer to these molecules as the syringomycins.

THE SYRINGOMYCINS TARGET THE PLASMA MEMBRANES OF YEASTS

Researchers often used fungi to identify and study the syringomycins which was unusual because the syringomycins were thought to be plant toxins. Fungi such as the yeasts were very sensitive to the syringomycins, and they were used to assay for their presence. We decided to exploit this sensitivity of fungi to study the syringomycins' mechanism of action. We studied yeasts such as the common baker's yeast, *Saccharomyces cerevisiae.* For molecular biology and biochemical studies, yeasts grow more rapidly and are more easily manipulated in the laboratory than plants. This accelerated research (but did not necessarily mean that the results were easier to interpret). We felt that yeasts and plants were so similar that the syringomycins' actions on yeast would also apply to their effects on plants.

We learned that syringomycin-E does indeed target the plasma membrane of yeasts (Takemoto, 1992). These effects are summarized in Fig.4.

Plasma Membrane Syringomycin Effects



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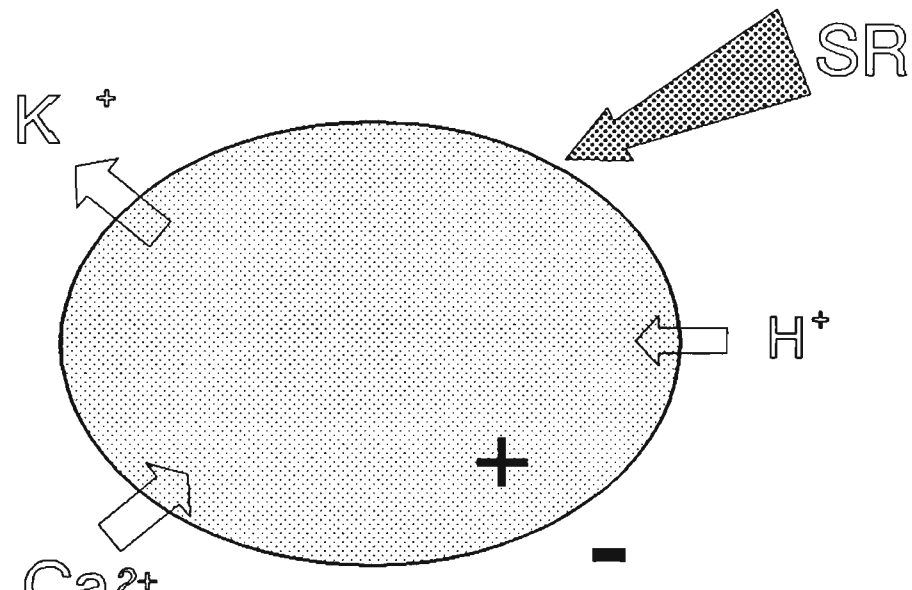

mechanisms of these changes are not known, but it appears that syringomycin binds to a component in the plasma membrane. It also appears that syringomycin alters one or more proteins which function in transporting these ions across the membrane. One such alteration involves the attachment of phosphates—protein phosphorylation which is catalyzed by enzymes called protein kinases. In yeasts, an ATPase enzyme in the plasma membrane transports hydrogen ions. We demonstrated that this enzyme is phosphorylated by a protein kinase, and that syringomycin markedly stimulates the process (Bidwai and Takemoto, 1987; Suzuki et al., 1992). All of these effects occur at syringomycin concentrations that also inhibit the growth of yeasts, which means that these effects on the plasma membrane reflect processes related to cell death.

Are plant plasma membranes also targeted by the syringomycins? This question is not easy to answer. The syringomycins were initially thought to be toxins acting on plant plasma membranes, but the toxicity of the syringomycins may vary with the purity of syringomycin preparations and the amounts used. Some of the effects on plants and membranes were probably due to impurities. For example, we now question the observations based on antibodies described above in which syringomycin preparations used to prepare the antibodies were probably not pure. Excessive amounts of syringomycin were probably used in other plant studies. Only very small amounts (e.g., less than 1 ug per ml) are required to affect yeasts. There are no observable effects when these same amounts are added to plant cells or their plasma membranes. However, the use of higher levels (e.g. 25 ug per ml) results in effects similar to those seen with yeast, e.g. it stimulates protein phosphorylation of plant plasma membrane proteins (Bidwai and Takemoto, 1987). Thus, even though plant plasma membranes do respond to the syringomycins, the large amounts that are required indicate that they may not be major plant virulence factors.

The Syringomycins are Primarily Antifungal Agents

The syringomycins can kill fungi, and do so at very low concentrations. They can kill a broad range of well-known fungi including the yeasts and are "antifungal agents." Is this their main role in nature? Are their roles as plant toxins secondary? It now appears that the syringomycins are primarily antifungal agents based on evidence that I cite below.

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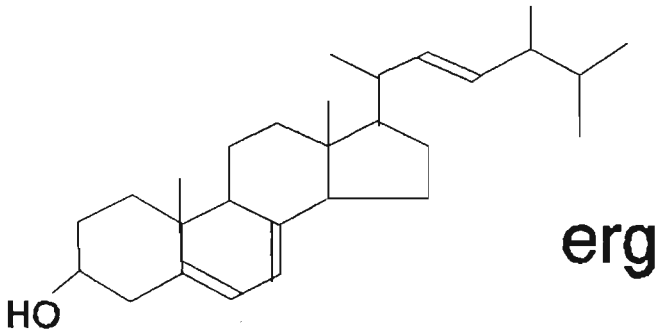
Yeast Molecular Biology and the Importance of Ergosterol

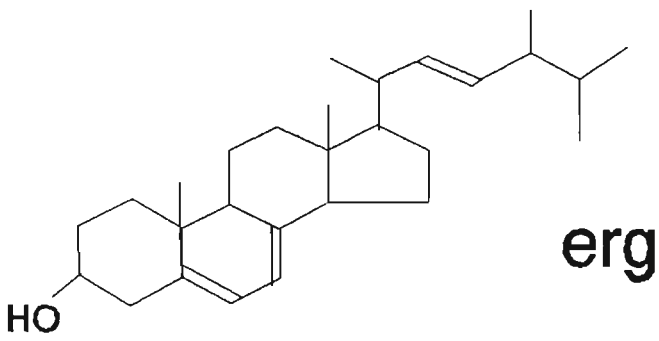
My research group is now isolating and characterizing genes that encode proteins required for inhibition by syringomycin-E (Takemoto et al., 1991). The main goal of this work is to identify the cellular events that occur when syringomycin-E kills yeasts. These events are either catalyzed by proteins or depend on cell components that are synthesized by proteins. The initial step towards

Using this strategy, we cloned genes that encode proteins involved in the syringomycin-E response. One of these genes, SYR1, is important when considering the role of the syringomycins as antifungal agents. We determined the sequence of the nucleotides in SYR1 and deduced the amino acid sequence of the protein it encodes. A computer database of all known protein sequences was searched recently, and we were astonished to find another protein that matched the SYR1-encoded protein. The sequence of this protein appeared in the literature just a few weeks earlier. It was encoded by a gene named ERG3, and was identified as sterol C-5 desaturase, a yeast enzyme which synthesizes a fatty lipid called ergosterol (Fig. 5). Thus, the SYR1 gene nucleotide sequence yielded immediate and precise information about its function: SYR1 allows the yeast to make ergosterol, which is necessary for syringomycin-E to kill it.

Ergosterol synthesis explained why the mutants resisted syringomycin-E. As expected, the levels of ergosterol in the mutants were dramatically reduced, and so was the amount of syringomycin-E that was bound to the mutant cells. In other words, the mutants bound less syringomycin-E than normal yeast, an indication that ergosterol is needed for syringomycin-E to bind to the plasma membrane.



ergosterol



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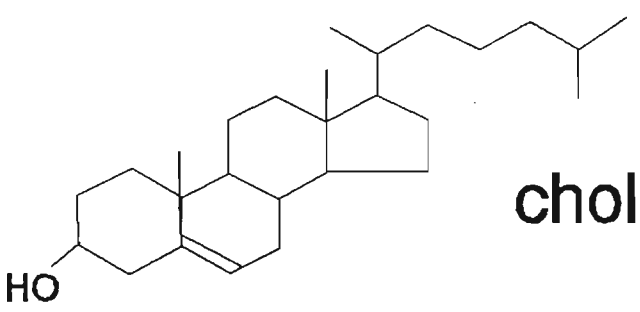

cholesterol

The requirement for ergosterol indicates that syringomycin-E is an antifungal agent. Ergosterol is found only in the fungi, and it is especially abundant in the yeasts. One of its structural analogues is cholesterol (Fig. 5), which is found mainly in animal cells. Plants possess other kinds of sterol lipids. Ergosterol is located exclusively in the plasma membranes of fungi, which could account for syringomycin-E's specific action on this membrane.

A New Bacterial Fungal Antagonist

The syringomycins' antifungal properties are also suggested from recent microbiological studies. Dr. Fatusa Adetuyi (a Nigerian microbiologist visiting my laboratory this year) isolated several new strains of *P. syringae* pv. *syringae* from wheat kernels on a farm in Benson, Utah. One isolate, which Dr. Adetuyi called strain M1, was an effective antagonistic of fungi that produced substantial levels of syringomycin-E. Strain M1 was not pathogenic to wheat or to other plants tested. Thus, *P. syringae* pv. *syringae* is not always a plant pathogen, nor is syringomycin production correlated with plant disease. Dr. Adetuyi is isolating and studying more strains of *P. syringae* pv. *syringae* to clarify the relationships between syringomycin production, fungal antagonism, and plant pathogenesis. The early observations with strain M1, however, indicate that the syringomycins are mainly antifungal agents.

If the syringomycins are not major plant virulence factors, then why are some strains of *Pseudomonas syringae* pv. *syringae* opportunistic pathogens? At present, we can only speculate. Several research groups have found genes in bacterial plant pathogens which are called hrp genes (for hypersensitivity and pathogenicity genes) (Willis et al. 1991). These genes also occur in certain *P. syringae* pv. *syringae* strains. Hrp genes are associated with pathogenicity, and also appear to determine which plants will be susceptible to particular bacterial pathogens. In contrast, Dr. Dennis Gross of Washington State University has identified genes necessary for the synthesis of syringomycin. Comparisons of the two sets of genes show that the syringomycin synthetic genes are not hrp genes. Thus, *P. syringae* pv. *syringae*'s pathogenicity is probably conferred by the hrp genes, and not the syringomycin genes. Further insight is hampered by our ignorance of the proteins encoded by the hrp genes. This is a major focus of research in plant-microbe interactions.

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Syringomycins for Biocontrol of Plant Pathogenic Fungi

If the syringomycins are antifungal agents, what advantage does this confer? As stated above, these bacteria normally exist on plant aerial surfaces and feed on the scarce nutrients provided by the plant. *P. syringae* pv. *syringae* must compete with other microorganisms, such as fungi, for space and food. They are successful competitors, and it is widely believed that the success of these bacteria is partly due to the production and use of antibiotics for eliminating other microbes (Blakeman, 1985).

biocontrol of dwarf bunt of winter wheat, a fungal disease. In the USA, this disease is confined to the Northwest including Northern Utah (Hoffman, 1982). Dwarf bunt has resulted in export restrictions, particularly to China, to limit spread of the disease. As a consequence, Northwest wheat farmers lack access to a huge grain market in Asia.

Dwarf bunt is caused by the fungus, *Tilletia controversa* Kuhn (TCK). Spores require cold temperatures for long periods of time, high moisture, and light to reproduce. In wheat fields, these requirements are provided by a prolonged snow cover through the winter and spring, a common condition in Utah's Cache Valley. In late winter, the germinating fungus infects wheat seedlings and develops slowly as the wheat plants grow. When the infected plants mature, it grows rapidly, invades the kernels, and destroys wheat kernels . TCK-infected wheat heads are black, soft, and possess a very unpleasant odor. (The disease is often called dwarf "smut").

Dr. Adetuyi and I realized that our *P. syringae* pv. *syringae* strain M1 was obtained from a wheat field infested with the TCK fungus. We discarded blackened kernels in search of healthy kernels to isolate our bacteria. Strain M1, the strong syringomycin-E producer, was isolated from kernels of healthy plants among diseased plants. Did strain M1 afford protection against the fungus? And, was strain M1's ability to protect conferred by syringomycin-E? These are questions that we are now examining in an effort to determine whether strain M1 and its syringomycin may be used in biocontrol of dwarf bunt.

The experiments are underway. In the laboratory, syringomycin-E and strain M1 do inhibit the fungus as it germinates from spores. Wheat plants and seeds infected with TCK have been challenged with syringomycin and the bacterium, and their effectiveness will be assessed when the plants reach maturity. TCK spore germination in the cold requires 6 to 8 weeks, and the plant experiments will require months. As molecular biologists working with organisms like yeast, we usually conduct experiments that can be completed in a day or overnight. We will need to adjust to the longer experiments of plant pathologists.

MEDICAL APPLICATIONS

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MEDICAL APPLICATIONS

Even though *P. syringae* pv. *syringae* is a plant bacterium, perhaps the syringomycins could be used to inhibit fungi of medical importance. We briefly looked at the ability of syringomycin-E to kill some well-known fungal human pathogens. As we anticipated, it was indeed effective (Table 1).

TABLE I. Growth Inhibitory Activity of Syringomycin-E Against Human Pathogenic Fungi

Cryptococcus neoformans, a yeast-type fungus, was as sensitive to syringomycin-E as our model organism, *S. cerevisiae*. This caught our eye because we knew that *C. neoformans* had recently attracted much attention in the medical community.

C. neoformans is prevalent in bird feces, especially of pigeons, but it also a major human pathogen in opportunistic infectious diseases of immunocompromised individuals (Williams, 1991), such as Acquired Immunodeficiency Syndrome (AIDS). An estimated 20% of individuals with AIDS eventually succumb to *C. neoformans*. Infections begin in the lungs, invade the nervous tissues and the brain, giving rise to a condition known as cryptococcal meningitis. Susceptibility exists whenever the immune system is suppressed—a situation that happens with the infirm and elderly as well as those with certain cancers, including inherited ones.

The prospect that the syringomycins inhibit human pathogenic yeasts such as *C. neoformans* could be important in medicine. Of course, many questions remain about the effectiveness of the syringomycins in treating human disease. All therapies must be employed against AIDS, and we feel that we have a social responsibility to see if the syringomycins could help in this endeavor.

SUMMARY

Our views of the role of the syringomycins have changed more than once over the last few years. They were initially considered to be major plant virulence factors, then iron chelators, and now they appear to be antifungal agents. The syringomycin story is an example of how science should work. Scientific hypotheses are constantly made and challenged, and then abandoned, altered, or retained. The force behind the process is the continual accumulation of knowledge generated by research. My colleagues and I will continue to study the syringomycins. We think we are close to finding their role in nature, but as we have seen, the search may take us in unexpected directions.

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