Effects of *Pseudomonas syringae* Phytotoxin, Syringomycin, on Plasma Membrane Functions of *Rhodotorula pilimanae*

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ABSTRACT

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The effects of purified syringomycin on whole cells and a plasma membrane-enriched fraction of the yeast *Rhodotorula pilimanae* were investigated. Syringomycin at concentrations between 0.25 and 3.0 μg/ml inhibited growth and stimulated the cellular uptake of oxygen, tetraphenylphosphonium (TPP) cations, and dimethyloxazolidinedione (DMO). The effects of TPP and DMO uptake were reversed by energy uncouplers and anaerobiosis and were unaffected by KCl. Syringomycin at 2–5 μg/ml stimulated the activity of the plasma membrane-associated ATPase. Results indicate that syringomycin inhibits cell growth by altering the electrical potential and pH difference across the plasma membrane and stimulation of the proton-pumping ATPase.

The toxin syringomycin is a small peptide produced extracellularly by the phytopathogen *Pseudomonas syringae* pv. *syringae* van Hall (12,21). It is one of several factors responsible for virulence or pathogenicity of this organism in susceptible plants (8–10,21). Among the diseases caused by this organism in which syringomycin appears to play a role are holcus spot of maize, systemic necrosis of cowpea, brown leaf spot of bean, Pseudomonas blossom blast of pear, and bacterial canker of stone fruit trees (9,10,21). In addition, low concentrations of the toxin inhibit a variety of fungi. However, a number of prokaryotic organisms are relatively insensitive to it (4,21). Syringomycin can be obtained in pure form (11), and although several of its chemical and physical properties have been described (12,18,21), its complete structure is unknown. A few studies have suggested that one or more membranes of susceptible cells are likely target sites for syringomycin. Microscopic examination of the fungus, *Geotrichum candidum* Link revealed that membrane abnormalities occurred after treatment with syringomycin (3). Radiolabeled toxin was also localized mainly over the plasma and nuclear membranes (3). In another study using syringomycin-treated peach leaves and stems, fluorescent antibodies to the toxin were concentrated at the cell periphery presumed to be the plasma membrane (19). A more recent investigation showed that syringomycin acts in vitro to uncouple oxidative phosphorylation in mitochondria isolated from etiolated maize shoots (23). Whether this particular effect occurs in vivo to elicit disease symptoms is unknown. In summary, the initial site and mechanism of action of syringomycin are largely unknown (8). A membrane site is likely in both fungi and plants, but it is not known which membrane system is the primary site of action and how it is affected. In this report, we describe several observations that show that in the fungus *Rhodotorula pilimanae* Hendrick & Burk, the plasma membrane is a primary site of syringomycin action. In this organism, which is highly sensitive to syringomycin, we suggest that the toxin acts by increasing the plasma membrane electrical potential via stimulation of a proton pump ATPase.

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MATERIALS AND METHODS

Growth of *R. pilimanae*. *R. pilimanae* (ATCC 26423, obtained from T. Emery, Utah State University, Logan) was grown in potato-dextrose broth (24 g/L, Difco) in 1.9-L Fernbach flasks containing 400 ml of medium. The cultures were incubated on a rotary shaker (70 cycles per min) at room temperature for about 20 hr until an absorbance of 1.0 at 600 nm was attained as measured with a Bausch & Lomb Spectronic 20 colorimeter. Cells were harvested by centrifugation at 3,000 *g* for 10 min and washed twice with distilled water. The cells were used immediately to investigate the effects of syringomycin on tetraphenylphosphonium (TPP), dimethyloxazolidinedione (DMO), and oxygen uptake or for cell fractionation.

Purification of syringomycin. Syringomycin was extracted and purified from cultures of *P. s.* pv. *syringae* strain B301D by a method similar to that described by Gross and DeVay (11). A minor modification was the use of fresh potatoes (White Rose, purchased at a local retail market) in the growth medium. They were cut into cubes about 2 cm on each side. Cubes from 200 g of potatoes were added per liter of medium containing 2% glucose and 0.4% casamino acids. Using fresh potatoes in this way provided a larger yield of syringomycin than using boiled potato extracts. In addition, elution of syringomycin in the final CM52 cellulose chromatography step of the purification procedure was done using 0.01 N HCl. Eluted fractions were assayed for syringomycin using *R. pilimanae* in the bioassay described by Gross and DeVay (11). Peak fractions with 230/256 nm absorbance ratios greater than 5 and with the highest biological activities were pooled as the final product. Specific activity ranged between 34 and 50 units of syringomycin per microgram, and the yield was 7–10 mg/L of culture. A unit was defined as the smallest amount of toxin that completely inhibited the growth of *R. pilimanae* or *G. candidum* in the area of application of a 10-μl droplet on potato-dextrose agar bioassay plates. The identity and purity of this preparation were monitored by silica gel thin-layer chromatography and high-voltage paper electrophoresis, which each gave only one ninhydrin spot; high-performance liquid chromatography, which showed one peak; and amino acid composition analyses.

TPP uptake. The experiments were conducted at room temperature. *R. pilimanae* cells (about 2.6 mg dry weight) were suspended in 2 mM Tris-2(*N*-morpholino)ethanesulfonic acid

(MES) buffer, pH 6.5, and 20 mM NaCl (absorbance of 1.5 measured at 600 nm) in a total volume of 2 ml. The suspensions were placed in flat-bottomed glass tubes (1.7 cm in diameter) and aerated by vigorous stirring with a magnetic bar. Measurements began with the addition of 10 μl of ^{3}H-TPP (final concentration and radiospecific activity, 5 μM and 0.25 μCi/ml, respectively). At designated times, samples of 0.2 ml were withdrawn and vacuum-filtered through a glass fiber filter disk (Whatman, 2.4 cm GF/A) prewetted with 5 mM TPP. The disk was washed with 4 ml of the corresponding reaction solution without TPP and syringomycin. Radioactivity was determined using Beckman Ready-solv EP scintillation solution and a Beckman LS100C scintillation counter. To measure the uptake of TPP under anaerobic conditions, a Coy anaerobic glove bag (oxygen concentration less than 10 ppm) was used. The reaction buffer was boiled under an N_2 stream and put into the bag for further deaeration. Cells were resuspended in this deaerated buffer. Each 3-ml cell suspension was transferred to a 120-ml serum bottle, which was then stoppered and sealed. The bottles were filled with N_2 (15 psi) and stirred. Samples were withdrawn by a syringe. The aerobic control in these latter experiments was subjected to all of the above procedures; the stoppers were removed only when the measurements began. The stock solutions of syringomycin and sodium azide (NaN_3) used in these experiments were 2 mg/ml, and 0.1 M in water, respectively. Gramicidin, carbonyl cyanide chlorophenylhydrazone (CCCP), and dinitrophenol (DNP) were dissolved in absolute ethanol to concentrations of 2 mg/ml, 4 mM, and 20 mM, respectively.

DMO uptake. Experimental conditions were the same as those used to determine TPP uptake, except 5 μl of DMO (8 mM in water) and 5 μl ^{14}C-DMO in ethyl acetate were added instead of TPP. The final concentration of DMO was 25 μM and radiospecific activity was 0.25 μCi/ml.

Resolution of plasma membrane and mitochondrial fractions. *R. pilimanae* cells were fractionated according to the method of Fuhrmann et al (5) with modifications. All operations were conducted at 0–4 C. Harvested cells from a 1.2-L culture were washed by suspension and centrifugation in 20 mM triethanolamine, pH 7.0, and 0.4 M KCl (TK buffer). Glass beads (diameter 0.17–0.18 mm) equal to twice the volume of the centrifuged cell pellet were added, and the cells were ground with a mortar and pestle for 10 min. TK buffer (20 ml) was added and the suspension gently stirred. After the beads settled, the supernatant was recovered. This procedure was repeated two more times. The supernatants were pooled and centrifuged at 2,000 *g* for 10 min. The supernatant was recovered and centrifuged at 7,500 *g* for 10 min. The supernatant was removed and centrifuged at 27,000 *g* for 15 min. Pellets from the last two centrifugation steps were suspended in TK buffer, combined, and applied to a linear sucrose gradient (30–60% [w/w] sucrose in TK buffer). The gradients were centrifuged at 120,000 *g* for 4 hr in the Beckman SW41 rotor. Fractions of 1.2 ml were collected.

ATPase assay. The sucrose gradient fractions were assayed for their ability to release inorganic phosphate from ATP (2). The reaction mixture contained 30 mM Tris-MES, pH 6.5 or 9.0, 3 mM $MgSO_4$, 3 mM Na_2-ATP, and 40 mM KCl in a total 1-ml volume. The reactions were allowed to proceed for 15 min at 34 C and were stopped by adding 2.6 ml of a solution containing 0.858 N H_2SO_4, 3.43% ammonium molybdate, and 1.4% ascorbic acid. This solution was made fresh by mixing six parts 4% ammonium molybdate in 1 N H_2SO_4 and one part 10% ascorbic acid. After incubating for 20 min at room temperature, the absorbance at 660 nm was measured. The stock solutions of NaN_3 and vanadate were 0.1 M and 5 mM, respectively.

Succinate dehydrogenase assay. Succinate dehydrogenase was assayed according to the method of King (15). The activity was measured as the succinate dependent reduction of 2,6-dichlorophenolindophenol using phenazinemethosulfate as mediator. A unit of activity was defined as a change of one absorbance unit within 1 min at 600 nm.

Cellular ATP levels. The procedures of Kimmich et al (14) were used to assay ATP by the luciferin-luciferase method. A Beckman LS100C scintillation counter was used to measure the light emitted.

Oxygen uptake. A Yellow Springs Instrument Co. Model 55 oxygen monitor was used. Cells were suspended in 4 ml of 2 mM Tris-MES, pH 6.5, 20 mM NaCl buffer to an absorbance of 1.5 measured at 600 nm and incubated in the monitor chamber with vigorous stirring. Measurements were conducted at room temperature.

Protein assay. The method of Lowry et al (16) was used to measure protein levels using bovine serum albumin as standard.

Chemicals. DMO was purchased from Sigma Chemical Co. and TPP chloride was obtained from J. T. Baker Chemical Co.; ^{3}H-TPP bromide (4.3 Ci/mM) and ^{14}C-DMO (50 mCi/mM in ethyl acetate) were obtained from New England Nuclear Corp.

All experiments were repeated at least four times with reproducible results. The results shown are from representative single experiments.

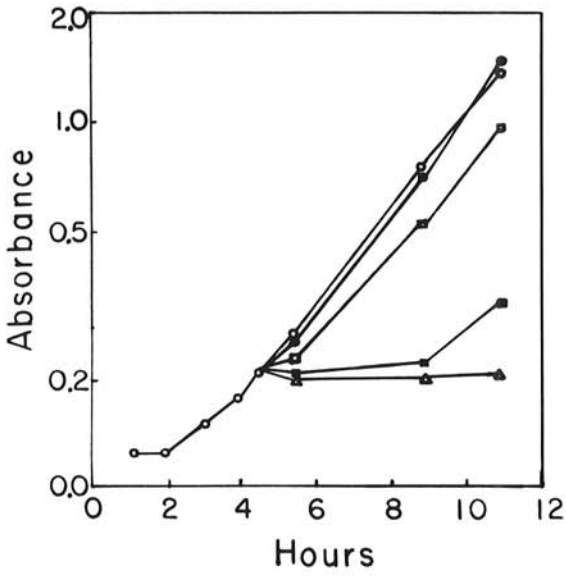

Fig. 1. Effects of syringomycin on growth (A_{600nm}) of *Rhodotorula pilimanae*. Syringomycin was added to the growth medium 4.5 hr after cell inoculation at concentrations of 0.1 (•), 0.25 (□), 1 (■), or 10 (△) μg/ml. No syringomycin was added to the control (o).

RESULTS

Effect of syringomycin on growth of *R. pilimanae*. The growth of *R. pilimanae* in liquid potato-dextrose broth medium was inhibited by syringomycin at concentrations of 0.25 μg/ml and higher (Fig. 1). The inhibitory effect was observed as soon as 1 hr after the addition of syringomycin. At 1 μg/ml, growth completely ceased for about 4 hr and increased slightly thereafter. At 10 μg/ml and higher, the delayed increase did not occur after complete growth inhibition.

Effect of syringomycin on TPP uptake. Syringomycin at concentrations between 0.25 and 3 μg/ml caused a dramatic increase in the rate of TPP uptake into cells. TPP is a membrane-permeable cation (20), so this effect indicated an increase in the electrical potential (interior negative) across the plasma membrane. The effect happened quickly (within 1 min) after the addition of syringomycin. In this concentration range, the uptake rate increased twofold to 15-fold as syringomycin levels increased. At a higher concentration of syringomycin (4 μg/ml), the rate of TPP uptake was initially high, but within 5 min, a rapid efflux of TPP occurred (Fig. 2).

The compounds CCCP, gramicidin, NaN_3, and DNP, which act as uncouplers or lower the cellular ATP levels (Table 1), caused an efflux of TPP after the initial stimulation by syringomycin (Fig. 3). The dependence of the syringomycin effect on cellular ATP levels was also indicated by its lack of effect on TPP uptake by anaerobic cell suspensions (Fig. 4).

The effect of KCl on TPP uptake was examined. Increasing the KCl concentrations in the uptake medium decreased the TPP uptake. No significant differences in the stimulatory effect of syringomycin on TPP uptake were seen with KCl concentrations up to 100 mM (Fig. 5, top).

Effect of syringomycin on DMO uptake. DMO is a nonmetabolized weak acid that is membrane-permeable at pHs below its pKa (6.32 at 25 C) but impermeable at more alkaline pHs (20). Its distribution in the cell interior and external space can therefore indicate the pH difference across the plasma membrane. Concentrations of syringomycin that stimulated TPP uptake also stimulated DMO uptake (Fig. 6). Again, as with TPP, the accumulated DMO was released by uncouplers and electron transport inhibitors (Fig. 7). Increasing KCl concentrations caused increases in rates of DMO uptake, but KCl did not influence the stimulatory effect of syringomycin on DMO uptake (Fig. 5, lower panel). These results indicate that syringomycin caused the cell interior to become more alkaline.

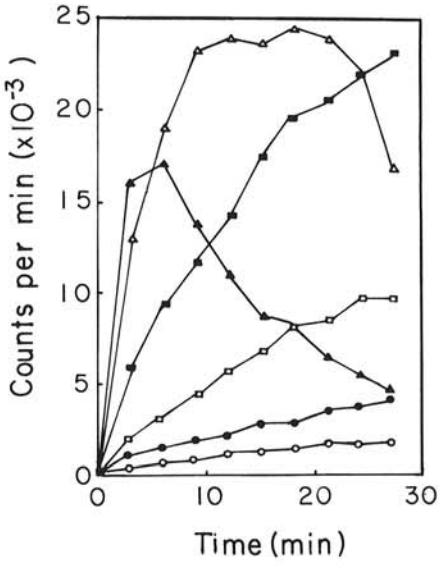

Fig. 2. Effects of syringomycin on tetraphenylphosphonium uptake by cell suspensions. Syringomycin was added at concentrations of 0 (o), 0.25 (•), 1 (□), 1.5 (■), 3 (△), or 4 (▲) μg/ml at zero time.

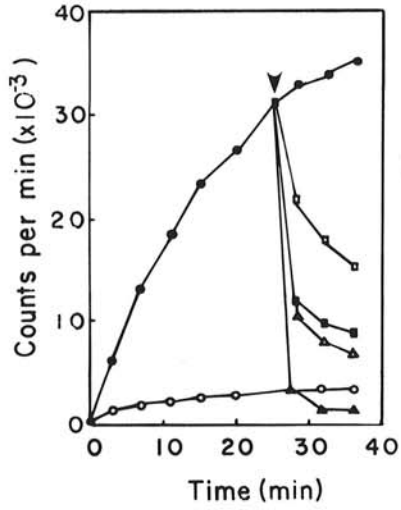

Fig. 3. Effects of uncouplers on stimulation of tetraphenylphosphonium uptake by syringomycin. Syringomycin, 1.5 μg/ml (•), was added at zero time. At the time shown by the arrow, 10 μM carbonyl cyanide chlorophenylhydrazone (▲), 1 mM NaN_3 (△), 20 μM dinitrophenol (■), or 10 μg/ml gramicidin (□) were added. The control (o) contained no syringomycin.

Effect of syringomycin on the plasma membrane ATPase. Cells of *R. pilimanae* were fractionated and the plasma membrane-enriched and mitochondrion-enriched fractions were resolved after centrifugation on sucrose density gradients (Figs. 8–10). The lower density fractions contained an ATPase activity that was higher at pH 9.0; the higher density fractions had an activity that was higher at pH 6.5 (Fig. 8). Activity peaks sensitive to NaN_3 and vanadate coincided with these two peaks, respectively (Fig. 9). The mitochondrial marker enzyme, succinate dehydrogenase, was most active in the lower density fractions (Fig. 10). These results show that the plasma membrane, which contained a pH 6.5-stimulated, vanadate-sensitive ATPase, sedimented at the higher density portions of these sucrose gradients. These fractions were well-resolved from the mitochondria, which sedimented at lower densities. Syringomycin at 2–5 μg/ml stimulated the plasma membrane ATPase activity, but had little effect on the mitochondrial enzyme (Fig. 11). The increase in activity varied from 20 to 60% in different experiments.

Effect of syringomycin on cellular ATP levels. Syringomycin added to aerated cell suspensions at 2 μg/ml did not affect cellular

TABLE 1. Effect of syringomycin on cellular ATP levels of *Rhodotorula pilimanae*[a]

Time (min)	ATP levels (nmol/mg dry wt of cells) with additions of				
	None	Syringomycin	CCCP	NaN_3	DNP
0	5.56	5.49	5.49	5.46	5.71
30	5.12	5.10	0.60	0.44	0.41

[a] Cells were suspended in 2 mM Tris-MES, pH 6.5, and 20 mM NaCl buffer to an absorbance of 1.5 at 600 nm. At zero time, 2 μg/ml syringomycin, 10 μM CCCP (carbonyl cyanide chlorophenylhydrazone), or 20 μM DNP (dinitrophenol) was added to the cell suspension. After 30 min, 50-μl samples were withdrawn to measure ATP levels.

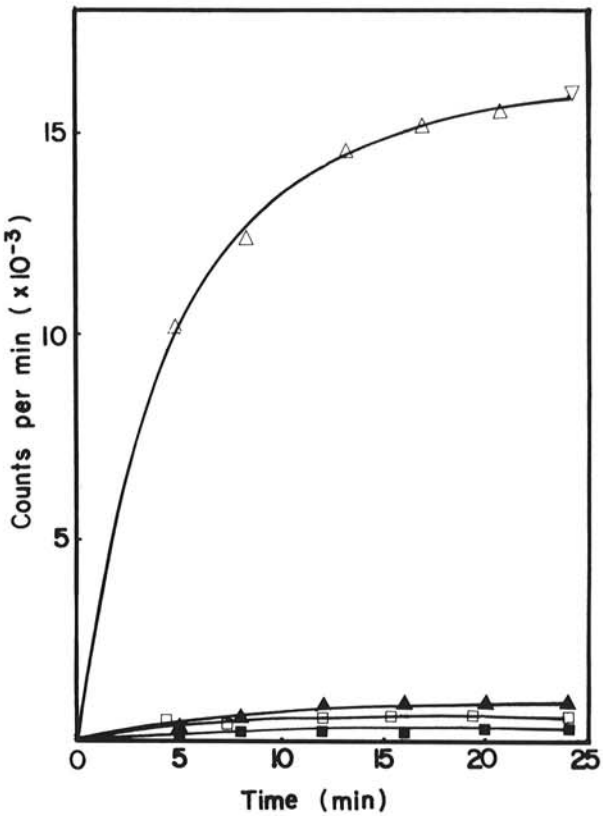

Fig. 4. Effects of anaerobiosis on tetraphenylphosphonium (TPP) uptake. TPP uptake was measured either under anaerobic conditions (■ or □) or aerobic conditions (▲ or △) and with 1.5 μg/ml syringomycin (□ or △) or without syringomycin (■ or ▲) added at the beginning of the measurements.

ATP levels. In one experiment, the ATP levels were measured at 1-min intervals for 30 min after the addition of syringomycin. No differences in ATP levels were observed throughout this period. In a separate set of experiments, ATP levels were determined 30 min after syringomycin addition. Again, no differences were observed (Table 1).

Effect of syringomycin on oxygen uptake. Oxygen uptake by cells was stimulated by either direct addition of syringomycin or by preincubation of cells with syringomycin for 10 min followed by centrifugation and resuspension of the cells in medium without syringomycin (Fig. 12). The rates of oxygen uptake were increased about 40 and 30%, respectively.

DISCUSSION

The experiments described were conducted using purified syringomycin. Purity of the toxin was routinely assessed by thin-layer chromatography on silica gels, high-voltage paper electrophoresis at different pHs, high-performance liquid chromatography, and amino acid composition analyses. In regard to amino acid composition, purified syringomycin was observed to contain arginine, phenylalanine, serine, and diaminobutyric acid in a 1:1:2:2 stoichiometry (R. C. Bachmann, *personal communication*). The diaminobutyric acid components were previously indicated to be unknown amino-containing residues (12).

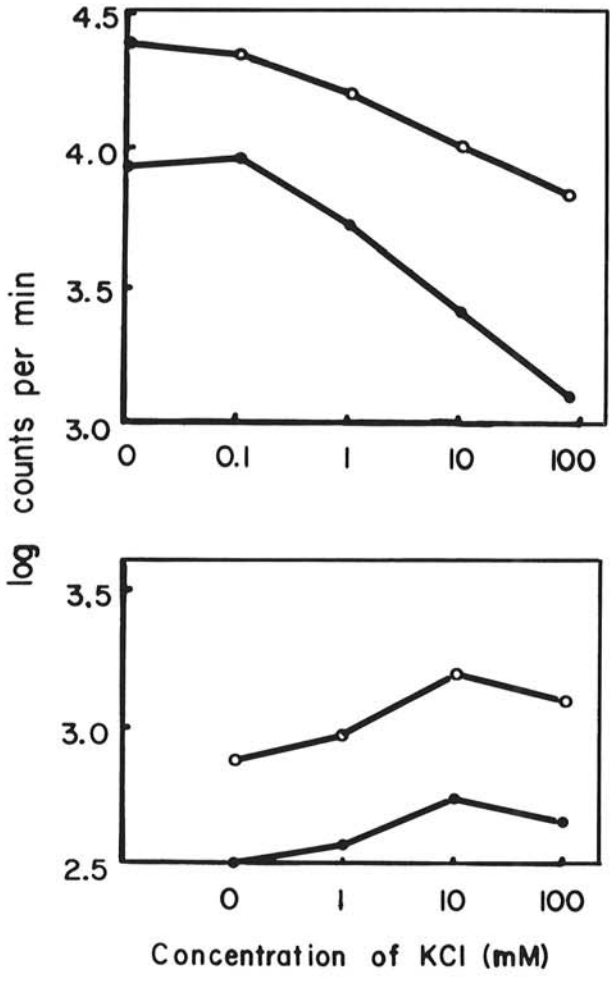

Fig. 5. Effects of KCl on tetraphenylphosphonium (TPP) and dimethyloxazolidinedione (DMO) uptake. Various amounts of KCl were added to cell suspensions with 1.5 μg/ml syringomycin (o) or without syringomycin added (•). The uptake of **top,** TPP or **bottom,** DMO was measured for 19 or 20 min, respectively, after initiation of experiment. For TPP uptake, the cells were suspended in water, and for DMO uptake, in 2 mM Tris-MES, pH 6.5.

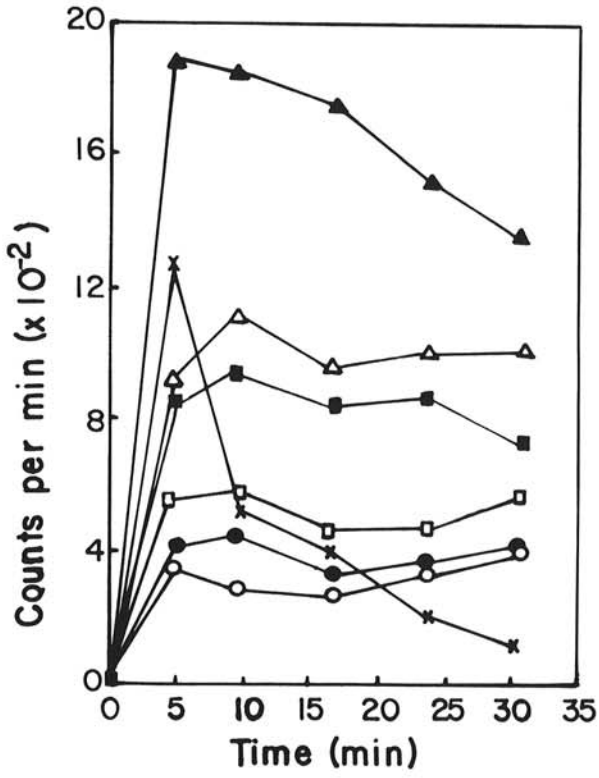

Fig. 6. Effects of syringomycin on dimethyloxazolidinedione uptake. Syringomycin was added at concentrations of 0 (o), 0.25 (•), 0.5 (□), 1 (■), 2 (△), 4 (▲), or 6 (×) μg/ml at zero time.

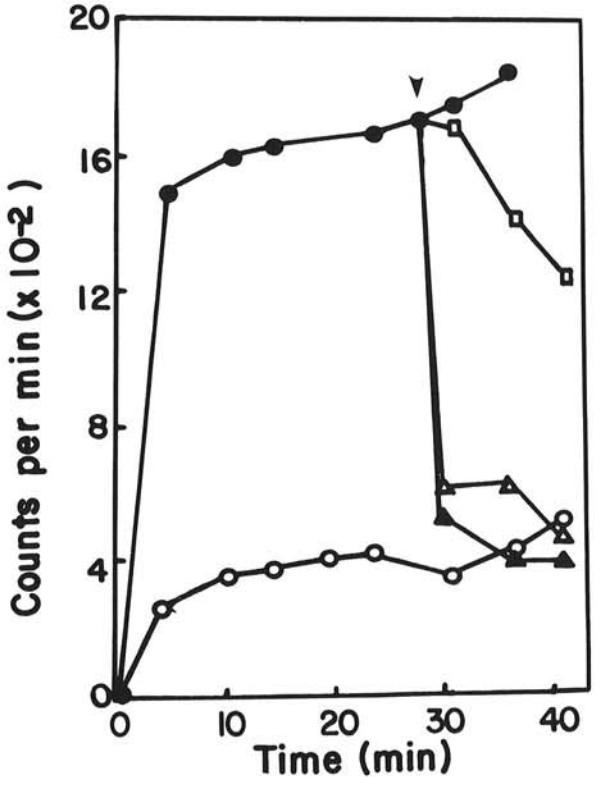

Fig. 7. Effects of uncouplers on stimulation of dimethyloxazolidinedione uptake by syringomycin. Syringomycin, 1.5 μg/ml (•), was added at zero time. At the time shown by the arrow, 10 μM carbonyl cyanide chlorophenylhydrazone (▲), 1 mM NaN_3 (△), or 20 μM dinitrophenol (□) were added. The control (o) contained no syringomycin.

We chose *R. pilimanae* as an experimental organism for investigating the mechanism of action of syringomycin. An obligate aerobe, *R. pilimanae* is a pigmented, unicellular organism that grows rapidly in liquid culture and was very amenable to cellular fractionation. In the agar plate bioassay, it was nearly as sensitive to syringomycin as *G. candidum*, which is the more commonly used test organism in studies involving this toxin (3). In liquid media, its growth rate was inhibited by levels of syringomycin as low as 0.25 μg/ml, and growth was completely arrested by 1–2 μg/ml.

The movement of the membrane-permeable cation, TPP, into whole cells was used to measure the electrical potential across the plasma membrane of *R. pilimanae*. The value for growing cells was calculated by the Nernst equation to be 100 mV (cell interior, negative), which is close to the value of 82 mV reported for the related organism, *Rhodotorula gracilis* Rennerfelt (13). TPP uptake was immediately abolished by the addition of DNP, CCCP, or NaN_3. These reagents either lowered the cellular ATP levels by inhibiting oxidative phosphorylation, or they directly eliminated the membrane potential by acting as uncouplers. Dependence on ATP levels was also indicated by the observation that anaerobic incubation completely eliminated TPP uptake. The role of ATP in establishing an electrical potential across the plasma membranes of several fungi is well known (22). Taken together, our observations show that the extent of TPP uptake was a measure of the membrane potential that was probably formed by the hydrolysis of ATP.

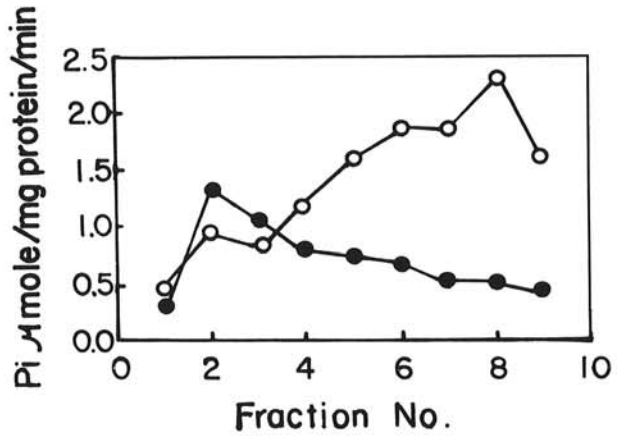

Fig. 8. Sucrose density gradient profiles of plasma membrane and mitochondrial ATPase activities at different pHs. The top of the gradient is on the left, the bottom, on the right. The ATPase activities were determined at pH 9.0 (•) or pH 6.5 (o). Pi = inorganic phosphate.

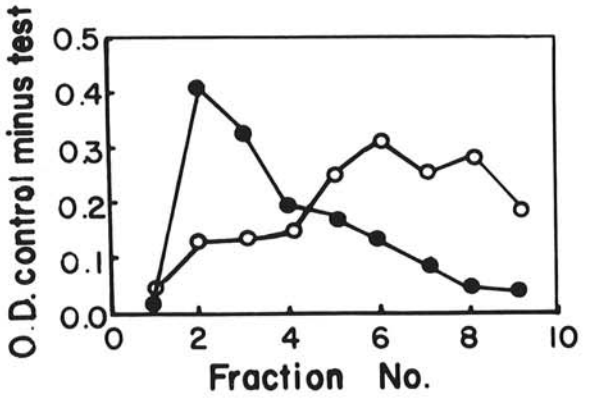

Fig. 9. Sucrose density gradient profiles of azide-sensitive and vanadate-sensitive ATPase activities. The top of the gradient is on the left, the bottom, on the right. Determinations with 1 mM azide (•) and 50 μM vanadate (o) were done at pH 9.0 and pH 6.5, respectively. The values on the *y*-axis were calculated by subtracting the optical density (O.D.) in the assay for inorganic phosphate in the case containing inhibitor from the optical density obtained in the case without inhibitor (control).

Syringomycin quickly stimulated the cellular uptake of TPP at relatively low concentrations (between 0.25 and 3 μg/ml and within 1 min), indicating that a primary effect of this toxin is to increase the plasma membrane potential. These levels of the toxin

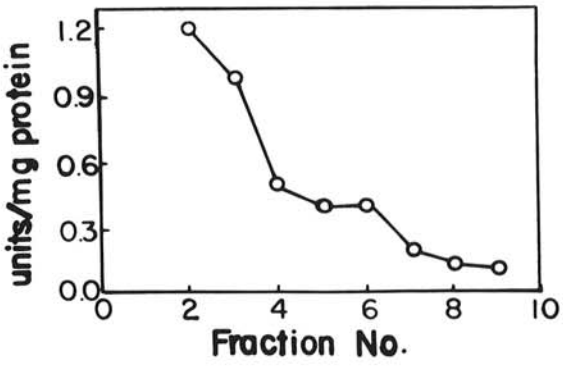

Fig. 10. Sucrose density gradient profile of succinate dehydrogenase activities. The top of the gradient is on the left, the bottom, on the right.

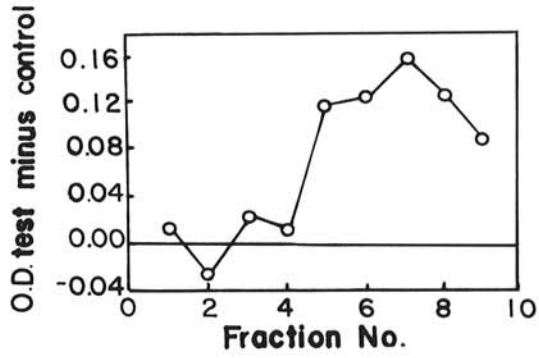

Fig. 11. Sucrose density gradient profiles of the ATPase activities stimulated by syringomycin. The activities were measured at pH 6.5. The syringomycin concentration was 5 μg/ml. The value for each fraction was calculated by subtracting the optical density (O.D.) obtained in the assay for inorganic phosphate without syringomycin (control) from the optical density from the assay with syringomycin (test).

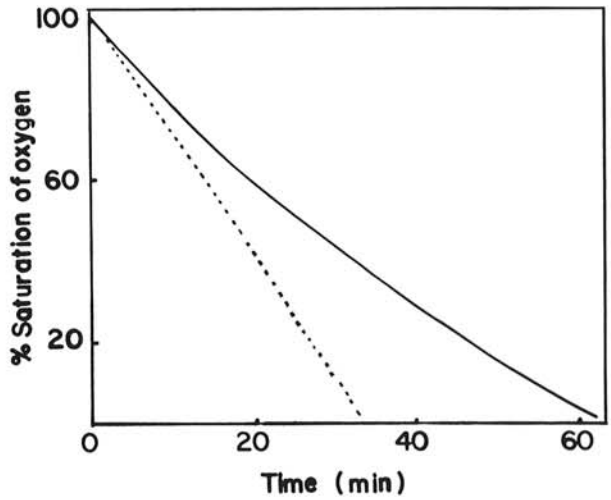

Fig. 12. Effect of syringomycin on oxygen uptake by cell suspensions. Syringomycin (1.5 μg/ml) was added (----) either **top,** 2 min after the measurements began or **bottom,** 10 min before the measurements began. In the latter case, the cells preincubated with syringomycin were centrifuged at 11,000 *g* for 30 sec, then resuspended in fresh medium before measuring the oxygen uptake. The controls were not treated with syringomycin (——). At zero time, the medium was equilibrated with air and considered to be 100% oxygen saturation.

were the same as those required to inhibit cell growth in liquid culture. The effect was eliminated by adding CCCP, DNP, gramicidin, NaN_3, or anaerobiosis, further indicating an influence on membrane potential. In contrast, Surico and DeVay (23) reported that syringomycin decreased the membrane potential of isolated maize mitochondria, suggesting an uncoupling effect. Conceivably, these mitochondria respond differently to syringomycin than fungi. It should be noted that significantly higher levels of toxin (12–24 μg/ mg protein) than those used in the present study (0.4–4.6 μg/ mg protein, assuming half of the cell dry weight is protein) were required to achieve the effect with maize mitochondria.

It is clear that the low levels of syringomycin used in this study did not disrupt the structural integrity of the membrane. A functional and physically intact membrane was absolutely necessary for the increase in the magnitude and maintenance of the membrane potential as observed.

At higher syringomycin levels (4 μg/ml and greater), we observed the rapid uptake of TPP and DMO followed by effluxes of both ions (Figs. 2 and 6). The subsequent effluxes may reflect a loss in the physical integrity of the plasma membrane caused by the higher membrane potentials or a reversal of the potential. Large electrical potentials are known to cause damage to the plasma membrane of *Neurospora crassa* Shear & B. Dodge (7). Therefore, growth inhibition by high levels of syringomycin may also result from membrane damage.

The membrane potential of fungal cells is maintained primarily by the outward extrusion of protons via the action of an ATPase (22). Thus, it was of interest to determine if the syringomycin-initiated membrane potential increase was accompanied by an increased rate of proton extrusion that might be caused by this enzyme. This was observed. Using the uptake of the weak acid, DMO, as a measure of the alkalinity of the cell interior, we observed that the cellular pH increased with syringomycin addition. The intracellular pH was calculated according to the formula (1):

$$pH_{in} = -\log\left[\frac{A_{in}}{A_{out}}\left(10^{-pK} + 10^{-pH_{out}}\right) - 10^{-pK}\right],$$

in which A_{in} is the concentration of DMO inside, A_{out} is the concentration of DMO outside, pK is the pK of DMO, and pH_{out} is the pH of the reaction solution. With the addition of 2 μg of syringomycin per milliliter, the intracellular bulk pH increased about 0.8 pH. The levels of syringomycin required for stimulation and the kinetics of DMO uptake were similar to those observed for TPP uptake (Figs. 2 and 6).

Further evidence for the direct relationship between membrane potential and pH changes with syringomycin was the effect of potassium ions on these phenomena. As the external potassium ion concentration was increased, TPP uptake was diminished and DMO uptake reciprocally increased in a kinetically parallel fashion (Fig. 5). Apparently, external potassium is not required for syringomycin action, because at any given concentration, syringomycin had quantitatively the same effect on the extent of TPP or DMO uptake as was the case with no potassium added (Fig. 5).

Because the plasma membrane ATPase directly affects the membrane electrical potential and cell pH, we investigated the effect of syringomycin on this enzyme. A plasma membrane fraction was resolved on sucrose density gradients and found to possess an ATPase activity that was most active at pH 6.5 and sensitive to vanadate. These properties were previously shown to be characteristic of this enzyme (6). Syringomycin at 2–5 μg/ml stimulated the ATPase, an effect that was consistent with the effects on the membrane potential and pH changes described previously. Further support for the involvement of an ATPase was obtained from the observation that syringomycin stimulated the uptake of oxygen by cells. Such an effect would be expected if syringomycin enhances a system that uses ATP as an energy source.

Despite its effect on the plasma membrane ATPase, syringomycin did not alter the cellular ATP levels of *R. pilimanae*. Possibly this organism, like *R. gracilis*, relies on the metabolism of large lipid reserves to maintain stable cellular ATP levels (17). Cell growth inhibition therefore occurred because of the membrane potential or pH changes and not as a consequence of altered ATP levels.

Our results strongly suggest that the plasma membrane is the primary target site of syringomycin in fungi. In contrast, the mitochondria of etiolated shoots of maize have been shown to be a site of syringomycin action (23). However, preliminary results in our laboratory show that a mitochondrial petite mutant of *Saccharomyces cerevisiae* responds identically to syringomycin both in bioassay and in TPP uptake when compared with the parent wild-type strain (*unpublished*). This suggests that mitochondria have no direct role in the sensitivity of fungi to syringomycin. Further studies will determine if the primary sites for the toxic effects of syringomycin are different in plants and fungi.

LITERATURE CITED

1. Addanki, S., Cahill, F. D., and Sotos, J. F. 1968. Determination of intramitochondrial pH and intramitochondrial-extramitochondrial pH gradient of isolated heart mitochondria by the use of 5,5-Dimethyl-2,4-oxazolidinedione. J. Biol. Chem. 243:2337-2348.
2. Ames, B. N. 1966. Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol. 8:115-118.
3. Backman, P. A., and DeVay, J. E. 1971. Studies on the mode of action and biogenesis of the phytotoxin syringomycin. Physiol. Plant Pathol. 1:215-233.
4. DeVay, J. E., Gonzalez, C. F., and Wakeman, R. J. 1978. Comparison of the biocidal activities of syringomycin and syringotoxin and the characterization of isolates of *Pseudomonas syringae* from citrus hosts. Pages 643-650 in: Proc. Int. Conf. Phytopath. Bact., 4th. Angers, France.
5. Fuhrmann, G. F., Wehrli, E., and Boehm, C. 1974. Preparation and identification of yeast plasma membrane vesicles. Biochim. Biophys. Acta 363:295-310.
6. Goffeau, A., and Slayman, C. W. 1981. The proton-translocating ATPase of the fungal plasma membrane. Biochim. Biophys. Acta 639:197-223.
7. Gradmann, D., Hansen, V.-P., Long, S. W., Slayman, C. L., and Warncke, J. 1978. Current-voltage relationships for the plasma membrane and its principal electrogenic pump in *Neurospora crassa*: I. Steady-state conditions. J. Membr. Biol. 39:333-367.
8. Gross, D. C., and Cody, Y. S. 1985. Mechanisms of plant pathogenesis by *Pseudomonas* species. Can. J. Microbiol. 31:403-410.
9. Gross, D. C., and DeVay, J. E. 1977. Population dynamics and pathogenesis of *Pseudomonas syringae* in maize and cowpea in relation to the in vitro production of syringomycin. Phytopathology 67:475-483.
10. Gross, D. C., and DeVay, J. E. 1977. Role of syringomycin in holcus spot of maize and systemic necrosis of cowpea caused by *Pseudomonas syringae*. Physiol. Plant Pathol. 11:1-11.
11. Gross, D. C., and DeVay, J. E. 1977. Production and purification of syringomycin, a phytotoxin produced by *Pseudomonas syringae*. Physiol. Plant Pathol. 11:13-28.
12. Gross, D. C., DeVay, J. E., and Stadtman, F. H. 1977. Chemical properties of syringomycin and syringotoxin: toxigenic peptides produced by *Pseudomonas syringae*. J. Appl. Bacteriol. 43:453-463.
13. Hauer, R., and Hofer, M. 1978. Evidence for interactions between the energy-dependent transport of sugars and the membrane potential in the yeast *Rhodotorula gracilis* (*Rhodosporidium toruloides*). J. Membr. Biol. 43:335-349.
14. Kimmich, G. A., Randles, J., and Brand, J. S. 1975. Assay of picomole amounts of ATP, ADP, and AMP using the luciferase enzyme system. Anal. Biochem. 69:187-206.
15. King, T. E. 1967. Preparation of succinate dehydrogenase and reconstitution of succinate oxidase. Methods Enzymol. 10:322-332.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.
17. Misra, P. C., and Hofer, M. 1975. An energy-linked proton-extrusion across the cell membrane of *Rhodotorula gracilis*. FEBS Lett. 52:95-99.
18. Mitchell, R. E. 1981. Structure: Bacterial. Pages 283-285 in: Toxins in

Plant Disease. R. D. Durbin, ed. Academic Press, New York.
19. Paynter, V. A., and Alconero, R. 1979. A specific fluorescent antibody for detection of syringomycin in infected peach tree tissues. Phytopathology 69:493-496.
20. Rottenberg, H. 1979. The measurement of membrane potential and pH in cells, organelles, and vesicles. Methods Enzymol. 55:547-569.
21. Sinden, S. L., DeVay, J. E., and Backman, P. A. 1971. Properties of syringomycin, a wide spectrum antibiotic and phytotoxin produced by *Pseudomonas syringae*, and its role in the bacterial canker disease of peach trees. Physiol. Plant Pathol. 1:199-213.
22. Slayman, C. L. 1985. Plasma membrane proton pumps in plants and fungi. BioScience 35:34-37.
23. Surico, G., and DeVay, J. E. 1982. Effect of syringomycin and syringotoxin produced by *Pseudomonas syringae* pv. *syringae* on structure and function of mitochondria isolated from holcus spot resistant and susceptible maize lines. Physiol. Plant Pathol. 21:39-53.