Protection by sterols against the cytotoxicity of syringomycin in the yeast *Saccharomyces cerevisiae*

CHARNCHAI JULMANOP,¹ YOSHIHIKO TAKANO,¹ JON Y. TAKEMOTO² and TOKICHI MIYAKAWA^{1*}

¹Department of Fermentation Technology, Hiroshima University, Higashi-Hiroshima, 724 Japan

²Department of Biology and Molecular Biology Program, Utah State University, Logan, Utah 84322-5305, USA

(Received 12 February 1993; revised 1 April 1993; accepted 4 May 1993)

A brief exposure (ca 20 min) of the yeast *Saccharomyces cerevisiae* to the phytotoxin syringomycin was sufficient to kill the cell. The protective effect of sterols against this cytotoxicity of syringomycin was investigated. Syringomycin was much more toxic to growing cells than to stationary-phase cells. The cytotoxicity of syringomycin was reduced in an environment containing sterols. Cytotoxicity of syringomycin at $3 \mu\text{g ml}^{-1}$ (ca $2.5 \mu\text{M}$) was completely abolished by the simultaneous presence of $10 \mu\text{M}$ -cholesterol in the medium. Cholesterol acetate had no protective effect. Ergosterol, sitosterol and stigmasterol also protected against syringomycin, but they were less effective than cholesterol. The protective effect of sterols against the action of syringomycin is consistent with our hypothesis that membrane ergosterol is a critical component for syringomycin-binding as suggested by recent genetic studies.

Introduction

The phytotoxin syringomycin, a lipodepsipeptide with an M_r value of 1226, is produced by the bacterial plant pathogen *Pseudomonas syringae* pv. *syringae* and is a major virulence factor in plant diseases caused by this organism (Gross *et al.*, 1977; Sinden *et al.*, 1971). Syringomycin is a significant contributor to a variety of plant diseases (DeVay *et al.*, 1968; Sinden *et al.*, 1971). Application of a partially purified preparation of the toxin to young peach trees gave typical disease symptoms. The diseases caused by this bacterium include necroses of stone fruits and cowpea and holcus spot disease of maize (Gross & DeVay, 1977). In addition to contributing to plant diseases, syringomycin inhibits the growth of several fungi including the yeast *Saccharomyces cerevisiae* (Zhang & Takemoto, 1986, 1987).

As expected from the amphipathic structure of syringomycin, the host plasma membrane is the primary site of action of the toxin. Plant and yeast cells respond to the toxin similarly (Reidl & Takemoto, 1987). Among the effects on the membrane are release of various cations, increase in membrane potential, H^+ -ATPase activity and protein phosphorylation (Bidwai & Takemoto, 1987). By molecular genetic studies of the mechanism of action of syringomycin on *S. cerevisiae*, we

recently found that the yeast gene *SYR1* that complements the phenotype of a resistant mutant *syr1*, is identical with *ERG3* that encodes sterol C-5 desaturase, an enzyme of ergosterol biosynthesis (N. Taguchi and others, unpublished results). This suggested to us that ergosterol, a major component of the plasma membrane accounting for about 15% of the membrane lipids (Longley *et al.*, 1968), is the possible target of the toxin. A null mutant of *syr1* did not contain ergosterol in the membrane, but was viable and resistant to syringomycin. Furthermore, a mutant lacking ergosterol (e.g. *syr1*) had a significantly decreased syringomycin-binding ability. To examine the hypothesis that membrane ergosterol is critical for binding syringomycin, we studied the effect of externally added sterols on the cytotoxicity of syringomycin.

Methods

Yeast strains and growth conditions. Wild-type isogenic diploid *S. cerevisiae* strain RAY-3A-D (MATa/ α leu2ura3his3trp1) was described by Tanaka *et al.* (1988). A syringomycin-resistant mutant YT1-b with a null allele of *SYR1/ERG3* (*syr1-Δ1*) was prepared. In brief, a 300 bp *SalI*–*SalI* region within the open reading frame of *SYR1* was replaced with the *SalI*–*SmaI* fragment of the *HIS3* gene. The one-step gene disruption of the diploid strain RAY-3A-D was performed by transformation with the disrupted DNA fragment, and gene disruption was confirmed by Southern hybridization.

Syringomycin purification. Syringomycin (E-form) was purified to homogeneity as described previously (Bidwai & Takemoto, 1987).

* Author for correspondence. Tel. 81 824 22 7196; fax 81 824 22 7191.

Assay of the growth inhibition of *S. cerevisiae* by syringomycin. Exponentially-growing *S. cerevisiae* wild-type strain RAY-3A was inoculated at a cell concentration of 1×10^6 cells ml^{-1} into YPD medium containing 3 μg syringomycin ml^{-1} and various concentrations of sterols. The cells were grown at 28 °C in aerobic conditions with shaking. The growth of the culture was monitored by measuring OD₆₆₀ every 2 h.

Assay of the cytotoxicity of syringomycin on *S. cerevisiae*. Exponentially-growing *S. cerevisiae* cells were harvested after 8 h cultivation at 28 °C in YPD medium ($\text{ca } 5 \times 10^7$ cells ml^{-1}) and used for assay of cytotoxicity. Stationary-phase cells were harvested after 36 h cultivation ($\text{ca } 1 \times 10^9$ cells ml^{-1}) and used when required. Syringomycin (1–3 μg ml^{-1}) was added to the cell suspension, 1×10^6 cells ml^{-1} in YPD medium or saline (0.85% NaCl) in sterile test tubes. After incubation for the indicated times at 28 °C with shaking, a 0.1 ml sample was taken and immediately diluted in 4.9 ml ice-cold saline and plated on YPD medium after an appropriate dilution. The colonies were counted after incubation for 48 h. To test the effect of sterols and other lipids on cytotoxicity, syringomycin (1 μg ml^{-1}) and indicated amounts of various lipids were mixed. Incubation was initiated by the addition of exponentially growing yeast cells to the mixture. Samples were taken periodically and viable cells were counted as described above.

Results

Effect of sterols on growth inhibition of *S. cerevisiae* by syringomycin

First, we examined the effects of sterols on the growth inhibiting activity of syringomycin. The growth of *S. cerevisiae* cells in YPD medium in the presence of syringomycin and sterols was monitored by measuring the culture OD₆₆₀ (Fig. 1). The growth of wild-type haploid strain RAY-3A was completely inhibited by the presence of syringomycin at 3 μg ml^{-1} ($\text{ca } 2.5 \mu\text{M}$) in the culture medium. Under the same conditions, the growth of an ergosterol-deficient *syrl-Δ1* mutant was unaffected (data not shown). As expected from our hypothesis, the growth of wild-type cells in the medium containing syringomycin was restored by the simultaneous presence of exogenous sterols, such as ergosterol or cholesterol. The protective effect of the sterols was dose-dependent. The effect of cholesterol was more potent than ergosterol. In the presence of 3 μg syringomycin ml^{-1} and 25 μM -cholesterol, the growth of the sensitive cells was restored to a level similar to that without syringomycin. In contrast, ergosterol only partially restored growth even at 50 μM , the solubility limit for this sterol in water. The added sterols had no effect on the growth rate (data not shown).

Cytotoxicity of syringomycin on yeast cells

Since syringomycin very rapidly affects the homeostasis of cellular Ca^{2+} and other ions, it was expected that syringomycin would decrease cell viability shortly after exposure of the cells to the toxin. This possibility was

examined by determination of cell viability after incubation of the cells with syringomycin in YPD medium for various periods of time (Fig. 2). Cytotoxic effects were observed by treating the cells in YPD with concentrations of syringomycin of 1 μg ml^{-1} or higher. The cytotoxic effect was established rapidly on exposure of the cells to syringomycin, and viability was almost lost after about 20 min of incubation in YPD with 2 μg ml^{-1} of the toxin. The cytotoxicity of syringomycin was much higher when it was measured in saline instead of YPD (Fig. 2). The viability of the ergosterol-deficient mutant (*syrl-Δ1*) was unaffected by syringomycin under these conditions (data not shown).

We next compared the effect of syringomycin on exponentially growing cells and stationary-phase cells to

Fig. 1. Protection by sterols against the growth inhibition of *S. cerevisiae* cells by syringomycin. The growth of wild-type *S. cerevisiae* cells in YPD medium at 28 °C in the absence of syringomycin (○), or in the presence of 3 μg syringomycin ml^{-1} ($\text{ca } 2.5 \mu\text{M}$) and various concentrations of ergosterol (a) or cholesterol (b) was monitored by measuring OD₆₆₀. The experiment was done 4 times with reproducible results. Results presented are from a representative single experiment. The concentrations of the sterols added were: 0 (●), 5 (▲), 10 (△), 25 (□) and 50 (■) μM .

Fig. 2. Effect of incubation time on cytotoxicity of syringomycin. The cells were incubated at 28 °C for various periods of time (0–30 min) in YPD medium (a) or saline (b) containing 1 (●, ○), 2 (■, □) or 3 (▲, △) μg syringomycin ml^{-1} . At the end of incubation, the cells were immediately diluted with ice-cold saline, and viable cells were determined by plating the cells on YPD plates. The experiment was done twice with reproducible results. Results presented are from a representative single experiment.

Fig. 3. Comparison of cytotoxicity of syringomycin on exponentially growing cells and stationary-phase cells. Exponentially growing cells (a) and stationary-phase cells (b) were incubated for various periods of time (0–30 min) at 28 °C in YPD medium containing 1 (●, ○), 2 (■, □) or 3 (▲, △) µg syringomycin ml⁻¹, and viability of treated cells was determined as described in the legend to Fig. 2. The experiment was done three times with reproducible results. Results presented are from a representative single experiment.

Fig. 4. Protection by sterols and other lipids against cytotoxicity of syringomycin. Cells were incubated at 28 °C with 1 µg syringomycin ml⁻¹ and the indicated concentrations of the lipids for 30 min in saline, and viability of treated cells was determined as described in the legend to Fig. 2. The lipids added were as follows: cholesterol (○), ergosterol (●), sitosterol (□), stigmasterol (■), cholesterol acetate (△), oleic acid (▲) and phosphatidylcholine (▽). The experiment was done three times with reproducible results. Results presented are from a representative single experiment.

see if the cytotoxicity depends on growth phase (Fig. 3). Exponentially growing cells in YPD medium were much more sensitive to syringomycin than stationary-phase cells. However, the higher sensitivity of the exponential-phase cells was not dependent on cell division. Exponentially growing cells starved for 30 min at 28 °C in saline still remained highly sensitive to the toxin (data not shown).

Effect of sterols on the toxicity of syringomycin

To see if externally added sterols protect the cells from the cytotoxicity of syringomycin, cells were treated with 1 µg syringomycin ml⁻¹ in saline for 30 min in the presence of various concentrations of sterols before measuring cell viability (Fig. 4). Among the sterols tested, cholesterol had the most potent effect, and the

Fig. 5. Reversing effect of cholesterol against cytotoxicity of syringomycin. Syringomycin (1 µg ml⁻¹) was added to cell suspensions in saline, and cholesterol (25 µM) was added at different times during the incubation. The incubation of the cells with syringomycin was for 30 min at 28 °C. Cell viability of treated cells was determined as described in the legend to Fig. 2. The experiment was done three times with reproducible results. Results presented are from a representative single experiment.

cytotoxicity of syringomycin was completely abolished by the presence of concentrations of cholesterol of 10 µM or higher. Cholesterol acetate had almost no protective effect. Ergosterol had a protective effect against syringomycin at concentrations between 1 and 10 µM. However, the effect of ergosterol was partial, restoring viability only in 40–60% of the treated cells. Higher concentrations of ergosterol (above 50 µM) had little protective effect, possibly due to a solubility problem. Sitosterol and stigmasterol also protected against cytotoxicity, but their effects were weaker than cholesterol, restoring viability in 50–70% of total cells. Other lipids such as phosphatidyl choline and oleic acid also had a weak protective effect, but only at very high concentrations (ca 100 µM).

We next examined whether externally added cholesterol could reverse the cytotoxicity induced by syringomycin. Cholesterol (25 µM) was added at various times after the addition of 1 µg syringomycin ml⁻¹ in saline, and cytotoxicity was determined by colony formation on agar plates (Fig. 5). The cells were incubated with the toxin for 30 min. When cholesterol was added to the cell suspension within 10 min of exposure to the toxin, it had a significant reversing effect against the toxin, restoring viability in 70% or more of the treated cells. In contrast, when cholesterol was added to the cell suspension after 20 min of exposure to the toxin, the sterol restored viability in less than 20% of the treated cells. These data are consistent with the theory that the cytotoxicity of syringomycin was established immediately after an

exposure of the cells to the toxin, and cholesterol interferes with an initial process of the cytotoxicity.

Discussion

The present study demonstrated that the toxic effect of syringomycin on yeast is reduced in an environment containing sterols. Among the sterols tested, cholesterol was the most effective. In the presence of 25 µM-cholesterol, syringomycin cytotoxicity was completely abolished. Since cholesterol only gave protection during the early incubation times with syringomycin, it seems to interfere with an initial interaction of syringomycin with the cell, and does not reverse the interaction of previously bound syringomycin. These results are consistent with our hypothesis that membrane ergosterol is critical for syringomycin binding (N. Taguchi and others, unpublished results).

As expected from the rapid stimulation of Ca^{2+} uptake by syringomycin (Takemoto *et al.*, 1991), cytotoxicity was established within 15–20 min of exposure to the toxin. The time required for the cytotoxicity roughly corresponds to the occurrence of Ca^{2+} influx, suggesting that the rapid change in the ion fluxes induced by the toxin is fatal to the cell. Syringomycin was much more toxic to growing cells than to stationary-phase cells. The cytotoxicity was not cell-cycle dependent, since growing cells starved in saline were still highly sensitive to the toxin. The growth-dependent sensitivity could be attributed to differences in wall composition and thickness, as suggested for nystatin which is more toxic to growing cells having thinner cell walls as compared to thick-walled stationary-phase cells (Marini *et al.*, 1961; Lampen *et al.*, 1962).

The primary interaction of the toxin and sensitive cells involving membrane ergosterol may be analogous to that suggested for polyene macrolide antibiotics such as nystatin and amphotericin B. However, the structure of syringomycin is obviously different from that of the polyene antibiotics. Also, the protective effects of different sterols against the cytotoxicity of syringomycin were different from those against the polyene antibiotics where protection was more effective with ergosterol than cholesterol (Lampen, 1966). It has been suggested that membrane sterols are involved in the antifungal activity of the peptidolipidic antibiotic iturin A. The amino acid sequences of iturin A and syringomycin are not related to each other. The binding of iturin A to *S. cerevisiae* was dependent on the nature of sterol present in the membrane. A mutant strain containing cholesterol instead of ergosterol showed the highest affinity for iturin A (Latoud *et al.*, 1990). The effects of various lipids, including sterols and phospholipids, as inhibitors of the antifungal activity of iturin A were examined in *S.*

cerevisiae and cholesterol was found to be the strongest inhibitor (Besson *et al.*, 1979).

Overall, the results suggest that the protective effect of sterols can be attributed to the inhibition of syringomycin interaction with the cell surface of sensitive cells, by preventing access of the toxin to the cell through physical interaction of the toxin with the exogenously added sterols. The protective effect of sterols against the action of syringomycin is consistent with our hypothesis that membrane ergosterol is a critical component for syringomycin binding as suggested by genetic studies (N. Taguchi and others, unpublished). The strong protective effect of cholesterol against the toxicity of syringomycin may offer opportunities to develop preventive agents against the phytopathogens that produce syringomycin or related phytotoxins.

This work was supported in part by a National Science Foundation grant DCB9003398 to J. Y. T.

References

- BESSON, F., PEYPOUX, F., MICHEL, G. & DELCAMBE, L. (1979). Antifungal activity upon *Saccharomyces cerevisiae* of iturin A, mycosubtilin, bacillomycin and of their derivatives; inhibition of this antifungal activity by lipid antagonists. *Journal of Antibiotics* **32**, 828–833.
- BIDWAI, A. P. & TAKEMOTO, J. Y. (1987). Bacterial phytotoxin syringomycin, induces a protein kinase-mediated phosphorylation of red beet plasma membrane polypeptides. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 6755–6759.
- DEVAY, J. E., LUKEZIC, F. L., SINDEN, S. L., ENGLISH, H. & COPLIN, D. L. (1968). A biocide produced by pathogenic isolates of *Pseudomonas syringae* and its possible role in the bacterial canker disease of peach trees. *Phytopathology* **58**, 95–101.
- GROSS, D. C. & DEVAY, J. E. (1977). Role of syringomycin in holcus spot of maize and systemic necrosis of cowpea caused by *Pseudomonas syringae*. *Physiological Plant Pathology* **11**, 1–11.
- GROSS, D. C., DEVAY, J. E. & STADMAN, F. (1977). Chemical properties of syringomycin and syringotoxin; toxigenic peptides produced by *Pseudomonas syringae*. *Journal of Applied Bacteriology* **43**, 453–463.
- LAMPDEN, J. O. (1966). Interference by polyenic antifungal antibiotics (especially nystatin and filipin) with specific membrane functions. *Symposium of the Society for General Microbiology* **16**, 111–130.
- LAMPEN, J. O., ARNOW, P. M., BOROWSKA, A. & LASKIN, A. I. (1962). Location and role of sterol at nystatin-binding sites. *Journal of Bacteriology* **84**, 1152–1160.
- LATOUD, C., PEYPOUX, F. & MICHEL, G. (1990). Interaction of iturin A, a lipopeptide antibiotic, with *Saccharomyces cerevisiae* cells: influence of the sterol membrane composition. *Canadian Journal of Microbiology* **36**, 384–389.
- LONGLEY, R. P., ROSE, A. H. & KNIGHTS, B. A. (1968). Composition of the protoplast membrane from *Saccharomyces cerevisiae*. *Biochemical Journal* **108**, 401–412.
- MARINI, F., ARNOW, P. & LAMPEN, J. O. (1961). The effect of monovalent cations on the inhibition of yeast metabolism by nystatin. *Journal of General Microbiology* **24**, 51–62.
- REIDL, H. H. & TAKEMOTO, J. Y. (1987). Mechanism of action of bacterial phytotoxin, syringomycin. Simultaneous measurement of early responses in yeast and maize. *Biochimica et Biophysica Acta* **898**, 59–69.
- SINDEN, S. L., DEVAY, J. E. & 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. *Physiological Plant Pathology* **1**, 199–213.

- TAKEMOTO, J. Y., ZHANG, L., TAGUCHI, N., TACHIKAWA, T. & MIYAKAWA, T. (1991). Mechanism of action of the phytotoxin syringomycin: a resistant mutant of *Saccharomyces cerevisiae* reveals an involvement of Ca^{2+} transport. *Journal of General Microbiology* **137**, 653–659.
- TANAKA, K., MATSUMOTO, K. & TOH-E, A. (1988). Dual regulation of the expression of the polyubiquitin gene by cyclic AMP and heat shock in yeast. *EMBO Journal* **7**, 495–502.
- ZHANG, L. & TAKEMOTO, J. Y. (1986). Mechanism of action of *Pseudomonas syringae* phytotoxin, syringomycin. Interaction with the plasma membrane of wild-type and respiratory-deficient strains of *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta* **861**, 201–204.
- ZHANG, L. & TAKEMOTO, J. Y. (1987). Effect of *Pseudomonas syringae* phytotoxin, syringomycin, on plasma membrane fraction of *Rhodotorula pilimanae*. *Phytopathology* **77**, 297–303.