Identification and analysis of the *Saccharomyces cerevisiae* SYR1 gene reveals that ergosterol is involved in the action of syringomycin

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A 2.5 kb DNA fragment of the *Saccharomyces cerevisiae* SYR1 gene was cloned by complementation of the syr1 mutations that simultaneously lead to resistance to the phytotoxin syringomycin and sensitivity of growth to high Ca2+ concentrations. Sequencing of this fragment revealed a single open reading frame encoding a polypeptide of 365 amino acids. Four hydrophobic regions each separated by hydrophilic regions were present in the protein. SYR1 was identical to ERG3, which is suggested to encode C-5 sterol desaturase required for ergosterol biosynthesis. The protein product of SYR1 was identified by Western blot analysis as a protein of 40 kDa in the particulate fraction. Gene disruption experiments demonstrated that elimination of SYR1/ERG3 is not lethal, but results in membrane C-5 desaturated sterol deficiencies, resistance to syringomycin and sensitivity to high Ca2+. The syr1 mutant cells had significantly decreased ability for syringomycin binding. The results indicated that C-5 desaturated sterols are involved in the binding of syringomycin to the cell, and the lack of the sterols in the mutant membrane results in sensitivity to high Ca2+ and an increased rate of cellular Ca2+ influx.

**Keywords**: *Saccharomyces cerevisiae*, ergosterol, syringomycin, SYR1

**INTRODUCTION**

The phytotoxin syringomycin is a major virulence factor produced by many strains of the bacterial plant pathogen *Pseudomonas syringae* pv. *syringae* (De Vay et al., 1968; Sinden et al., 1971). Syringomycin is a lipodepsipeptide with a molecular mass of 1226 Da (Segre et al., 1989; Fukuchi et al., 1990). In addition to contributing to plant disease, syringomycin inhibits the growth of several fungi including the yeast *Saccharomyces cerevisiae* (Zhang & Takemoto, 1986, 1987; Takemoto et al., 1991). Plants and yeast appear to respond similarly to syringomycin, and the effects suggest action on membrane components. The host plasma membrane appears to be the primary site of syringomycin action. The effects on the membrane include increases in K+ efflux, Ca2+ influx and membrane potential, and protein phosphorylation (Bidwai & Takemoto, 1987; Takemoto et al., 1991).

To elucidate the mechanism of syringomycin action, a molecular genetic approach using syringomycin-resistant mutants of *S. cerevisiae* was undertaken (Takemoto et al., 1991). About 25% of the mutants (comprising at least 12 gene complementation groups) exhibited increased sensitivity of growth to Ca2+ in the medium and higher rates of Ca2+ transport. A single recessive mutation is responsible for both the resistance to syringomycin and for the sensitivity of growth to high Ca2+ concentrations (Takemoto et al., 1991).

In the present study, we cloned the gene SYR1 (syringomycin resistant) that complements the phenotypes of the syringomycin-resistant mutants R4-3G and 3N-H1 (syr1-1 and syr1-2, respectively). The cloned gene was identical with ERG3, which is suggested to be involved in the synthesis of membrane ergosterol (Arthington et al., 1991). The relationship between the phenotypes of syr1 and the alteration in membrane sterol is demonstrated.

**METHODS**

**Strains, media and general recombinant DNA methods.** The Syringomycin-resistant mutant *S. cerevisiae* R4-3G (syr1-1), isolated from the wild-type haploid 8A-1B (*MATα pho3 his3*)
Suitable clones for sequencing were produced using various methods with M13mp18 or M13mp19 (Sanger mutant was crossed with IL1-lD to obtain a mutant R4-3G-12B with desired auxotrophic markers previously (Liu)).

Bacterial strains and yeast plasmid vectors were as described previously (Liu et al.). Disruption of SYRI. Plasmid pNT2 was constructed by replacing the 25 kb XhoI-BamHI fragment of the YEp24-based library with the genomic DNA fragment of the YEp24-based library. The one-step gene disruption method was used (Rothstein, 1983).

Preparation of fusion protein and specific antibody. For the preparation of SYRI fusion protein, the bacterial expression vector pEX3 was used. The Seryl–His3 fragment within the open reading frame (ORF) was inserted at the SmaI site of the expression vector pEX3 to produce the cro–luxZ–SYRI gene fusion. Preparation of fusion protein and specific antibody (Sanger et al., 1977).

Protein electrophoresis and Western blot analysis. Yeast cells were grown to late-exponential-phase in 10 ml YPD medium. The cells were harvested and washed twice with 1 ml buffer 1 (50 mM imidazole/HCl; pH 7.0, 1 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM PMSF, 10 μg ml⁻¹ each of leupeptin and pepstatin). Cell extracts were prepared by vortexing the cell pellet with glass beads (0.45 mm; Braun) in a microcentrifuge tube. After removing the glass beads, the lysate was centrifuged at 12000 r.p.m. for 15 min at 4 °C. Equal amounts (40 μg) of protein samples (supernatant and particulate fractions) were analysed by SDS-PAGE. After electrophoresis, the proteins were transferred onto a Biodyne membrane, and the protein was identified by Western blotting using antisera against Syrl. Protein samples were not heat treated in sample buffer for solubilization, as the treatment caused loss of Syrl protein due to aggregation.

Extraction of nonsaponifiable lipids. The procedure for sterol extraction from yeast was as described by Woods (1971), using cells grown for 48 h at 28 °C in YPD medium.

Binding assay for syringomycin. Syringomycin (40 μg) was added to S. cerevisiae cells (2 × 10⁷) suspended in 200 μl YPD medium in a microcentrifuge tube. After incubation at 28 °C for various periods of time (0–60 min), the suspension was clarified by centrifugation for 1 min at 10000 g. A 50 μl sample was taken from the supernatant fluid, and the amount of syringomycin was determined semi-quantitatively by measuring the growth inhibitory effect of the toxin using wild-type S. cerevisiae as assay cells.

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

RESULTS
SYRI complements Ca²⁺ sensitivity of syringomycin-resistant mutants

Syringomycin-resistant mutants R4-3G and 3N-H1 were previously selected from the wild-type strains 8A-1B and KZ1-1C, respectively, on the basis of their ability to grow on YPD agar containing 2 μg syringomycin ml⁻¹. The growth of R4-3G and 3N-H1 was inhibited by 0.4 M and 0.2 M CaCl₂, respectively. The respective wild-type parental strains were able to grow with these levels of Ca²⁺. Both mutants showed increased rates of ⁴⁰Ca²⁺ uptake from the medium (Takemoto et al., 1991). Genetic analyses of these mutants showed single recessive mutations that simultaneously led to Ca²⁺-sensitive growth and syringomycin resistance (Takemoto et al., 1991). The two mutations, designated syr-1 and syr-2, belonged to the same complementation group. For cloning of the genes that complement the syr-1 phenotypes, we used genomic libraries of S. cerevisiae made with a centromere vector (YCPN1 or YCP50) and the multi-copy vector YEp24. Since R4-3G did not have appropriate auxotrophic markers for selection of these plasmids, strain R4-3G-12B (MATA tyr1-1 ura3 trp1 his3 leu2) was obtained by crossing R4-3G with KZ1-1D. After transformation of R4-3G-12B and 3N-H1 with the genomic libraries, transformants were tested for the phenotypes to verify that the increased resistance to Ca²⁺ was due to the presence of the plasmid. The plasmids were then purified, amplified in E. coli, and used to transform the mutants to verify that the plasmids complemented Ca²⁺-sensitive growth.

About 5000 and 9000 transformants of R4-3G-12B were obtained using the YCPN1- and YEp24-based libraries, respectively. From these transformants, one (clone 22) YCPN1- and four (clones 1, 3, 273 and 225) YEp24-based syr-1-complementing clones were obtained. From about 3000 YCP50-library transformants of 3N-H1, five complementing clones were isolated (clones 28, 29, 32, 37 and 35). Restriction enzyme analysis of these clones suggested that they all contained a common DNA fragment found in the shortest clone, clone 1. The restriction maps of some of these clones are shown in Fig. 1. Southern blot analyses of genomic DNA digested with various restriction enzymes,
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Clone 22

Clone 1

Clone 3

Clone 273

The \textit{SYR1} gene encodes C-5 sterol desaturase

The 1.2 kb insert of clone 1 was sequenced. A sequence presumed to be part of an ORF was found, but a stop codon was missing at the C-terminus. Thus, the nucleotide sequence of the 2.5 kb \textit{XhoI-BamHI} fragment of clone 273 was determined (Fig. 1). In this fragment, an ORF of 1095 nucleotides that could encode a 365-amino-acid protein (427 kDa) was found (data not shown). The sequence of clone 1 corresponded to amino acid residues 1–322 of the ORF, indicating that the \textit{SYR1} protein is not essential for restoring \textit{Ca}^{2+} sensitivity of \textit{syrl}. The hydrophobicity profile of the predicted \textit{SYR1} gene product revealed several hydrophobic domains sufficient in length to span a membrane, suggesting that the \textit{SYR1} gene product is a membrane protein. Comparison of the \textit{SYR1} sequence with those in the sequence databases showed that \textit{SYR1} is identical to yeast \textit{ERG3}, a gene suggested to encode C-5 sterol desaturase required for ergosterol biosynthesis (Arthington \textit{et al.}, 1991).

The \textit{SYR1} gene is not essential for growth, and its disruption causes syringomycin resistance and \textit{Ca}^{2+} sensitivity

To investigate the relationship between the \textit{Syrl} protein and syringomycin resistance, \textit{SYR1} was inactivated by the one-step gene disruption technique (Rothstein, 1983). The \textit{XhoI-BamHI} fragment of plasmid pNT2 was constructed by replacing a 300 bp \textit{SalI-SalI} region within the ORF with the \textit{SalI-SmaI} fragment of \textit{HIS3} (Fig. 3). The diploid \textit{S. cerevisiae} strain RAY-3A-D was transformed with pNT2 linearized with \textit{PstI}. Gene disruption was confirmed by Southern hybridization (Fig. 3). Tetrad analysis of the diploid was carried out upon sporulation. Viable spores were scored for genotype according to their auxotrophic requirements, and 1:1 segregation patterns of \textit{syrl-M1} and \textit{SYR1} alleles were observed. Consistent with the results of \textit{ERG3} disruption (Arthington \textit{et al.}, 1991), all four spores were able to grow on YPD, showing that \textit{SYR1} is not essential for mitotic growth. However, \textit{SYR1} disruptants (\textit{His} \textsuperscript{+} colonies) were all resistant to syringomycin, and sensitive to \textit{Ca}^{2+} (Fig. 4). Cells with
Fig. 3. (a) Disruption of SYRI. This was done as described in Methods. (b) Confirmation of gene disruption by Southern hybridization. Genomic yeast DNA (10 μg) was digested with EcoRV and prepared for hybridization. The probe was the same as that used in experiments shown in Fig. 2. Lanes: WT, DNA from RAY-3D-A; D, DNA from His' transformed diploid (YT1).

3.5 kb

5.0 kb

Fig. 4. Phenotypes of the syr1::HIS3 (syr1-Δ1) disrupted meiotic segregant. Tetrad analysis of the syr1-Δ1/SYRI diploid (YT1) was performed. Growth of selected tetrads were tested on (a) YPD medium, (b) SD without histidine, (c) YPD medium containing 2 μg syringomycin ml⁻¹ and (d) YPD containing 400 mM Ca²⁺.

Fig. 5. Western blot analysis of the Syr1 protein. Equal amounts of proteins (30 μg) were separated by electrophoresis in a 10% (w/v) polyacrylamide gel in the presence of SDS and analysed by Western blotting as described above. Total cell lysates prepared from wild-type cells of YT1-1a (lane 1) and YT1-1a harbouring SYRI on the YEpl4 plasmid (lane 2) were immunoblotted with affinity-purified antibody against Syr1. Total cell extract (lane 3) prepared from YT1-1a harbouring SYRI was fractionated to soluble (lane 4) and particulate (lane 5) fractions, as described in Methods, and analysed by Western blotting. No bands were formed when total cell lysates were immunoblotted with control serum.

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Identification of Syr1 protein

For immunological detection of the SYR1 product, Syr1, antiserum against LacZ–Syr1 fusion protein was prepared. Western blot analysis of whole-cell extracts prepared from a strain overexpressing SYR1 from a multi-copy-number plasmid vector showed an immunoreactive polypeptide with a molecular mass of 40 kDa, corresponding in size to that expected from the deduced amino acid sequence (42.7 kDa). An increase in the intensity of this band was observed when SYRI was overexpressed from a YEp24 multi-copy-number plasmid, but only a very weak band was seen in wild-type cells (Fig. 5), implying that Syr1 is a minor protein of the cell. Syr1 was found in the particulate fraction, suggesting that it is bound to the membrane.

syr1 mutants have altered membrane sterols

Since SYR1 encodes C-5 sterol desaturase, we measured the UV spectra of the nonsaponifiable lipid extracts to see if the levels of membrane ergosterol are altered in the syr1 mutants (Fig. 6). Two of the sterols most abundant in S. cerevisiae, ergosterol and 24(28)-dehydroergosterol, are readily identified by their UV absorption spectra, but the other sterols present in the yeast membranes are not detectable from the absorption spectra (Breivik & Owades, 1957). The lipid extract prepared from YT1-1a, a SYRI segregant of the diploid YT1 heterozygous at the

the disrupted allele had an elevated rate of Ca²⁺ uptake, as previously observed with syr1-1 mutants (Takemoto et al., 1991). When the syr1-Δ1 segregant was transformed with the YEpl4 plasmid bearing SYRI, both Ca²⁺-sensitive and syringomycin-resistant phenotypes were restored to wild-type levels. These results indicate that the function

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SYR1 locus, showed that this strain contains ergosterol and possibly 24(28)-dehydroergosterol as the major UV-absorbing materials, as described by Bard (1972). In contrast, a syr1Δ mutant did not contain detectable amounts of UV-absorbing materials in the corresponding fraction, suggesting that ergosterol is not essential for the growth of yeast cells (Fig. 6). This result is consistent with the data of Arthington et al. (1991), which demonstrated the lack of C-5-desaturated sterols in the mutant strains by GC–MS analysis. When SYR1 was introduced into syr1Δ mutant cells using YCp or YEp vectors, the level of ergosterol was restored close to the wild-type level. The cells of syr-1Δ1 had a very low level (less than 10% of that in wild-type cells) of the UV-absorbing substances (Fig. 6).

SYR1 was cloned on the basis of its ability to complement the Ca2+-sensitive phenotype of syr1 mutants. The cloned gene also suppressed syringomycin resistance when introduced into the mutant by a centromere-based vector. The gene had interesting phenotypic and molecular features. The mutant with the syr1 allele was resistant to syringomycin, showed Ca2+-sensitive growth, and had a greatly increased Ca2+-uptake rate compared to wild-type cells (Takekomo et al., 1991). Since the syringomycin-resistant and Ca2+-sensitive phenotypes caused by the null mutation in SYR1 were very similar to those of syr1 mutants, the mutations possibly resulted in loss of function of the gene product. The sequence of SYR1 was identical with the recently reported S. cerevisiae gene ERG3, which is suggested to code for C-5 sterol desaturase, the third terminal enzyme of ergosterol synthesis (Arthington et al., 1991). The ERG3 gene was cloned by complementation of the cycloheximide sensitivity of an erg3 mutant, which was originally identified as a mutation conferring nystatin resistance.

Western blot analyses using antibody directed against a LacZ-Syr1 fusion protein revealed a protein with an apparent size of 40 kDa in the particulate fraction. The size of the protein detected agrees with that expected from the gene. The microsome is expected to be the location of terminal sterol biosynthetic enzymes such as C-5 sterol.

**DISCUSSION**

Syringomycin presumably binds to the surface of sensitive cells as the first step of its action. To measure the syringomycin-binding ability of the cells, cell samples were incubated with syringomycin in YPD medium, and the amount of syringomycin remaining in the medium was measured. Cells were pelleted by centrifugation at various periods of incubation, and toxin concentration in the clarified supernatant fluid was determined semi-quantitatively by bioassay using sensitive S. cerevisiae as indicator (Fig. 7). With wild-type cells, the amount of toxin in the supernatant rapidly decreased. In contrast, the toxin level did not appreciably decrease with syr1Δ cells, suggesting that the syringomycin-binding activity of the mutant is much lower than that of wild-type cells (Fig. 7). The decreased binding ability of syr1Δ was restored when SYR1 was introduced by transformation with YCpN1 (SYR1) or YEp24 (SYR1) (Fig. 7). Supporting the possibility that membrane sterols are involved in the binding of syringomycin to the cell, the toxic effect of syringomycin for sensitive cells was relieved by the presence of low concentrations of sterols, such as cholesterol and ergosterol, added to the medium (data not shown).

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**Fig. 7.** Syringomycin binding activity of the syr1 mutant. The decrease in the amount of syringomycin in the fluid during incubation of cells in suspensions (2 × 10⁷ cells in 200 μl YPD medium) for 0, 10, 30 and 60 min were measured. A 50 μl sample of clarified fluid was taken, and the amount of toxin was determined semi-quantitatively by bioassay using syringomycin-sensitive S. cerevisiae as indicator. (a) YT1-1a (SYR1); (b) null mutant YT1-1b (syr1Δ); (c) YT1-1b harbouring YCpN1 (SYR1) plasmid. 1. Incubated without cells for 60 min; 2-5, incubated with cells for 0 (2), 10 (3), 30 (4) and 60 min (5).
desaturase (Osumi et al., 1979). The predicted SYR1 C-terminal four amino acids, Lys-Lys-X-X, are a retention signal for transmembrane endoplasmic reticulum proteins in mammalian cells (Jackson et al., 1990; Shin et al., 1991). However, clone 1, which contains an ORF lacking the C-terminal 43 amino acids of the SYR1 protein, complemented gyr1, suggesting that the C-terminal portion is not essential when introduced on a multi-copy plasmid.

The availability of the gene responsible for susceptibility to syringomycin and its identification as the gene coding for an enzyme of ergosterol biosynthesis afforded an opportunity to correlate syringomycin action with sterol composition of the membrane. The gyr1 mutant cells lacked ergosterol and possibly C-5 desaturated sterols, as determined by the UV absorption spectrum of the non-saponifiable lipid extract (Fig. 6). A similar relationship between membrane sterols and the resistance of mutants to polycene antibiotics, such as nystatin and amphoterin B, has been demonstrated (Lampen et al., 1962; Woods, 1971; Bard, 1972). Ergosterol has been identified as the cellular binding site for nystatin (Lampen et al., 1962). Binding experiments showed that the rate of syringomycin binding to gyr1 cells is lower than to wild-type cells (Fig. 7). These results suggest that membrane ergosterol facilitates the binding of syringomycin. The absence or severe reduction of ergosterol in the membrane results in resistance to the toxin. It is not clear, however, whether syringomycin binds directly to ergosterol, as does nystatin, or to another surface component dependent upon ergosterol biosynthesis. It was recently reported that sterol membrane composition affects the interaction of S. cerevisiae cells with iturin A, a lipopeptide antibiotic structurally unrelated to syringomycin (Latoud et al., 1990). The involvement of ergosterol could be the common mechanism for antifungal action of lipopeptides.

The sensitivity of the gyr1 strain to high Ca2+ concentrations, and the increased Ca2+ influx of the mutant, may be ascribed to lowered membrane ergosterol levels. Some sterol mutants of yeast have altered sensitivities to pulses (e.g. 10 min) of hypertonic salt solutions (2 M), including NaCl and CaCl2, suggesting a change in ion permeability as a result of the alteration of the membrane sterol composition (Bard et al., 1978). Also, some sterol mutants become more sensitive to cycloheximide (Gaber et al., 1989; Arthington et al., 1991; Ashman et al., 1991). The gyr1 mutant did not exhibit increased sensitivity to other ions tested, such as KCl and MgCl2, but the mutant was more sensitive to NaCl than wild-type cells (data not shown). Studies of Ca2+-sensitive mutants by several groups have shown that yeast has various mechanisms which allow it to grow with high Ca2+ concentrations. For example, vacuoles possess a potent Ca2+-uptake system driven by a H+-ATPase and serve as an intracellular store for Ca2+. Mutants of the subunit of the vacuolar H+-ATPase which are defective in vacuolar acidification have sixfold higher cytosolic Ca2+ levels and were Pet− (type IV els mutants) (Ohya et al., 1991). These mutants exhibit increased Ca2+ uptake activity and their growth is Ca2+ sensitive. Although the gyr1 mutants exhibited similar phenotypes with respect to Ca2+ sensitivity and 45Ca2+ uptake, the gyr1 mutants have normal levels of vacuolar ATPase activity and are not Pet− (data not shown).

Mutants ( gyr2 and gyr4) that exhibited increased resistance to syringomycin without changes in Ca2+ sensitivity and had the wild-type sterol composition have been identified. Syringomycin bound to these mutants normally, but was unable to kill the cell. We are examining the defects of these mutants by cloning the genes that complement the phenotype due to the gyr mutations. The structure of these genes may further provide information on the mechanism of syringomycin action in yeast.

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