SYR2, a gene necessary for syringomycin growth inhibition of *Saccharomyces cerevisiae*

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The *Pseudomonas syringae* cyclic lipodepsipeptide syringomycin inhibits the growth of *Saccharomyces cerevisiae*. A novel yeast gene, *SYR2*, was found to complement two syringomycin-resistant *S. cerevisiae* mutants. *SYR2* was cloned, sequenced, and shown to encode a 349 amino acid protein located in the endoplasmic reticulum. *SYR2* was identical to *SUR2*, which is involved in survival during nutritional starvation. Gene disruption or overexpression of *SYR2* did not affect cell viability or ergosterol levels, but did influence cellular phospholipid levels. The findings suggest that phospholipids are important for the growth inhibitory action of syringomycin.

Keywords: syringomycin, *SYR2*, *Saccharomyces cerevisiae*, *SUR2*, phospholipid biosynthesis

INTRODUCTION

Syringomycin is a small cyclic lipodepsipeptide produced by stone-fruit and grass isolates of *Pseudomonas syringae* pv. *syringae* (Segre *et al.*, 1989; Fukuchi *et al.*, 1990; Takemoto, 1992). Generally considered a nonhost-specific phytotoxin (Gross & DeVay, 1977), syringomycin also inhibits the growth of fungi, particularly yeasts such as *Saccharomyces cerevisiae* and *Rhodotorula pilimanae* (Zhang & Takemoto, 1986, 1987). Thus, it may play dual roles as a plant virulence factor and an antifungal agent against fungal competitors on plant surfaces (Takemoto, 1992). Several structurally related cyclic lipodepsipeptides produced by *P. syringae* strains have been identified. These include the syringostatins produced by a lilac isolate (Fukuchi *et al.*, 1992), syringotoxin produced by citrus isolates (Ballio *et al.*, 1990), and the pseudomycins produced from a barley isolate mutated with a transposon (Ballio *et al.*, 1994).

The cellular site of action of syringomycin is the plasma membrane. It alters several membrane functions such as membrane potential, protein phosphorylation, H⁺-ATPase activity and cation-transport fluxes (Zhang & Takemoto, 1987, 1989; Bidwai *et al.*, 1987; Bidwai & Takemoto, 1987; Suzuki *et al.*, 1992). The relationships of these activities and their relative importance in the response to syringomycin exposure are not clear. To learn more about the mode of action of syringomycin, recessive

syringomycin-resistant strains of *S. cerevisiae* were isolated and characterized (Takemoto *et al.*, 1991, 1993). One class of mutants (*syrl*) were deficient in membrane ergosterol and sensitive to high external Ca²⁺ levels. *syrl* mutants were complemented by gene *SYR1* (identical to *ERG3*) that encodes the ergosterol biosynthetic enzyme C-5 sterol desaturase (Taguchi *et al.*, 1994). Therefore, ergosterol is necessary for syringomycin action, and the binding of syringomycin to this lipid in the plasma membrane has been proposed (Julmanop *et al.*, 1993). Other classes of syringomycin-resistant mutants with normal ergosterol levels have been identified (Takemoto *et al.*, 1993). Such mutants presumably have defects in other genes whose products are necessary for syringomycin action.

In this paper, we describe the cloning and characterization of yeast gene *SYR2* that complements certain syringomycin-resistant mutants with normal ergosterol levels. *SYR2* is shown to be identical to *SUR2* (Desfarges *et al.*, 1993), which is involved in survival during nutritional starvation. The *SYR2*-encoded protein is shown to associate with the endoplasmic reticulum and to have a role in phospholipid metabolism. The findings suggest that phospholipids are important for syringomycin action.

METHODS

Organisms and general genetic methods. *S. cerevisiae* syringomycin-resistant mutants 13N-F2 and 3N-H1 were isolated from haploid strain KZ1-1C (*MATa his3 leu2 trp1 ura3*), and were previously described by Takemoto *et al.* (1993). Syringomycin-

Abbreviation: GST, glutathione S-transferase.

The GenBank accession number for the sequence reported in this paper is SCU10427.

resistant mutant 28N-D6 was also derived from KZ1-1C and was shown by diploid analysis to be in the same genetic complementation group as 13N-F2. Resistant mutant 3N-H1 was defective in *SYR1* (also designated *ERG3*), which encodes the sterol biosynthetic enzyme C-5 sterol desaturase (Taguchi *et al.*, 1994). For gene cloning, *Escherichia coli* strains DH5 and HB101 were used (Sambrook *et al.*, 1989). Yeast plasmid shuttle vectors YCpN1 (Liu *et al.*, 1991), YCp50 (Rose *et al.*, 1987), YE24 (Rose & Broach, 1991) and pYES2 (Invitrogen) were used. Genomic libraries of KZ1-1C were prepared in YCp50 and YCpN1 as described by Rose & Broach (1991). Yeast extract-peptone-dextrose (YPD), minimal salts-dextrose minus uracil (SD-URA) and minimal salts-galactose minus uracil (SG-URA) growth media were prepared as described by Rose *et al.* (1990). Syringomycin was added to growth media at concentrations ranging from 0.5 to 2.0 $\mu\text{g ml}^{-1}$. Yeast transformations were done using the lithium acetate method (Ito *et al.*, 1983) or by electroporation (Becker & Guarente, 1991). After transformation, yeast cells from single colonies growing on selective agar media were suspended in 100 μl water in microtitre plate wells. Cells were replica-plated onto YPD agar medium and YPD agar medium containing syringomycin. After 48 h the plates were visually inspected for clones showing little or no growth on syringomycin-containing plates but normal growth on plates without syringomycin. Genetic complementation tests of syringomycin-resistant strains were performed as previously described by Takemoto *et al.* (1993).

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

Nested deletion subcloning of *SYR2*. Nested deletions were performed using the Erase-a-Base system (Promega). A 4.8 kb *SYR2* clone (*SphI-SphI*) contained in pBluescript II (Stratagene) was used. The plasmid was linearized at *KpnI* and *SalI* restriction sites, and then subjected to exonuclease III digestion for varying lengths of time to unidirectionally excise portions of insert DNA. After exonuclease III excision, the DNA was treated in succession with S1 nuclease, Klenow DNA polymerase and T4 DNA ligase according to the manufacturer's protocol. The resulting *SYR2* subclones were transferred to YCpN1 to test for functional complementation of *syf2* strains.

***SYR2* nucleotide sequence determination.** DNA sequencing was performed using the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977). Sequences were determined on both strands from clones prepared by nested deletion or from clones prepared with suitable restriction enzymes in pGEM-3Z (Promega).

Northern blot analysis. Yeast total RNA was isolated using TRI-Reagent (Molecular Research Center). RNA was treated with glyoxal and electrophoresed on 1% (w/v) agarose gels as previously described (Franke *et al.*, 1987). RNA fragments were transferred to nylon membranes as described by Sambrook *et al.* (1989). DNA probes were labelled with the ECL direct labelling and detection system (Amersham) and used for hybridization to the RNA fragments. A 1.2 kb *PstI-SphI SYR2* fragment created using PCR was used as the probe.

Cell fractionation and Western blot analysis. Two cell fractionation methods were employed. For small-scale preparations, the glass-bead lysis method of Harlow & Lane (1988) was used. For large-scale preparations, a procedure similar to one described by Serrano (1983) was used. Yeast cells were grown to late exponential stage in 500 ml YPD medium. Cells were centrifuged in a Beckman JA10 rotor at 5000 r.p.m. for 10 min, and the cell pellets were washed with 1.5 M sorbitol. The cells were resuspended in 40 ml homogenizing buffer

(0.1 M Tris/HCl, pH 7.5, 5 mM EDTA, 2 mM DTT) and 6% (w/v) sorbitol. Zymolyase 100T (Seikagaku America; 200 U) was added. Cells were incubated at 30 °C for 60–90 min. Spheroplasts were collected, resuspended in 40 ml ice-cold homogenizing buffer, and lysed using 15–20 strokes in a chilled Dounce glass homogenizer. Lysates from both preparations were centrifuged at 100 000 *g* for 30 min at 4 °C to isolate the pelleted crude membranes. Crude membranes from the large-scale isolation were fractionated using a sucrose step gradient containing 3 ml 53% (w/v) sucrose, 3.5 ml 43% (w/v) sucrose and 3.5 ml 26% (w/v) sucrose. The gradient was centrifuged overnight at 68 000 *g* in a Beckman SW41 rotor at 4 °C. Plasma and endoplasmic reticulum membrane fractions were collected from the lower and upper interfaces, respectively. Vanadate-sensitive H^+ -ATPase and NADPH-cytochrome-*c* oxidoreductase activities were used to verify the identification of the plasma and endoplasmic reticulum membrane fractions, respectively (Marriot & Tanner, 1979). Samples (30–50 μg protein per lane) were analysed by SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membranes and Western-blotted as described by Harlow & Lane (1988).

Protein determination. Protein concentrations were determined using Coomassie Protein Assay Reagent (Pierce) and BSA as standard.

Alkaline phosphatase treatment of crude membranes. Crude membranes (50 μg protein) in 200 μl phosphatase buffer (10 mM Tris/HCl, pH 9, 1 mM ZnCl_2 , 1 mM MgCl_2) were treated with 17 U alkaline phosphatase (Boehringer Mannheim) at 37 °C for 30 min.

Preparation of *Syr2* fusion protein and antiserum. The bacterial vector pGEX-KG (Guan & Dixon, 1991) was used to prepare a *Syr2*-glutathione-S-transferase (GST) fusion protein. Initially, an *EcoRI-SalI* fragment containing intact *SYR2* was cloned into the vector. Expression of this GST-*Syr2* fusion protein severely restricted the growth of *E. coli* and the fusion protein was not isolated in sufficient quantities. Subsequently, the *HpaI-SphI* fragment from *SYR2* (encoding the C-terminal 124 amino acids) (Fig. 2b) was inserted in-frame with the GST sequences to produce a GST-*Syr2* fusion protein. The 5'-end of the fragment (*HpaI*) was ligated with blunt ends to the *SmaI* site of the pGEX-KG vector. A *HindIII* site was borrowed from pBluescript II phagemid (Stratagene) and used to clone the 3'-end of *SYR2* into pGEX-KG at the corresponding site. *E. coli* HB101 was transformed with the construct. Upon IPTG induction, a 40 kDa polypeptide presumed to be the fusion product was produced. The fusion protein was purified and the *Syr2* peptide was cleaved with thrombin as described by Smith & Johnson (1988). Peptide *Syr2* was purified by SDS-PAGE using a 8–15% (w/v) gradient polyacrylamide gel. After staining with Coomassie Blue, the *Syr2* band was cut from the gel. The gel slice was fragmented and prepared for injection as described by Harlow & Lane (1988). Rabbit antisera were prepared by immunizing rabbits with 75 μg protein in Freund's complete adjuvant. Booster injections of 40 μg protein in Freund's incomplete adjuvant were given 10–14 d before collection of sera. The sera were used directly for Western blot analysis.

To confirm the *Syr2* peptide sequence, the peptide was transferred to Immobilon-P membrane (Millipore) using the method of LeGendre & Matsudaira (1989). The membrane was stained with Coomassie Brilliant Blue, the *Syr2* fragment was excised, and the N-terminus (31 amino acids) was sequenced at the Utah State University Biotechnology Center (Logan, UT, USA). The sequence included two residues (Gly-Ser) encoded by vector sequences remaining after thrombin cleavage.

SYR2 disruption. A 2.3 kb pBluescript clone of *SYR2* created by nested deletion was used to construct the *SYR2*-disruptant strain. Plasmid C1 was constructed by replacing the *HpaI*–*EcoRV* segment of *SYR2* with a 1.2 kb *HindIII* fragment containing *URA3* from plasmid YEp24. The C1 plasmid was digested with *SacI* and *SphI*, and the resulting 3.14 kb linear fragment was purified and used to transform diploid strain W303-1A (*MATa/MATα his3 leu2 trp1 ura3*). The one-step gene disruption method was used (Rothstein, 1983). Transformants were induced to sporulate, and the spores were dissected for tetrad analysis (Rose *et al.*, 1990). A typical *SYR2*-disrupted segregant was selected and designated strain WΔ*SYR2* and a corresponding nondisrupted strain was selected and designated W303C.

SYR2 overexpression. The *PstI*–*SphI* fragment of *SYR2* was cloned into the *GAL1* promoter-containing vector pYES2 to yield pY*SYR2*. pY*SYR2* allowed overexpression of *SYR2* in SG-URA growth medium. The *PstI*–*SphI* fragment was missing the first ATG start codon and the following 24 codons, yielding an expressed protein that was shorter than intact *Syr2*.

Chromosomal localization. Yeast chromosomes were separated by PFGE using a Bio-Rad CHEF DR-II system. Chromosomal DNA was electrophoresed in 1% (w/v) agarose at 200 V. Pulse time was 60 s for 15 h followed by 90 s for 9 h. DNA for Southern blotting was digested with appropriate restriction enzymes and separated on 0.7% (w/v) agarose gels. DNA was transferred to nylon membranes as described by Sambrook *et al.* (1989). The *SYR2* probe was the same as described above for Northern blot analysis. A *Dictyostelium* actin-8 probe was also used, prepared by digesting an actin-8 clone contained in pMB9 (Romans & Firtel, 1985) (supplied by Dr Dennis L. Welker, Utah State University, UT, USA) with *HindIII* and labelling with the ECL system as described above.

Phospholipid and ergosterol analyses. Cells were extracted with chloroform/methanol (3:1, v/v) and the phospholipids were purified as described by Radin (1969). Total lipid masses were determined after drying the chloroform/methanol lipid extracts. Total phospholipid phosphate amounts were determined using a modified Fiske & Subbarow method described by Cooper (1977). Individual phospholipids were resolved by one-dimensional TLC (silica gel G-60, Merck; 20 × 20 cm) using chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) as the developing solvent. For phospholipid detection, plates were sprayed with molybdenum blue reagent (Dittmer & Lester, 1964). Authentic samples of phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and cardiolipin (Sigma) were used as standards. For ergosterol analysis, nonsaponifiable lipid extracts (Radin, 1969) were chromatographed on silica gel G-60 (Merck; 20 × 20 cm) thin-layer plates using *n*-hexane/diethyl ether/acetic acid (70:30:1, by vol.). After chromatography, the plates were sprayed with phosphomolybdic acid spray reagent (Sigma) for visualization of ergosterol.

RESULTS

SYR2 cloning

Genetic complementation analyses showed that syringomycin-resistant strains 13N-F2 (Takemoto *et al.*, 1993) and 28N-D6 had closely linked mutant alleles designated *sy2*. In contrast to other mutations that conferred syringomycin resistance, *sy2* mutations did not affect ergosterol biosynthesis (Takemoto *et al.*, 1993; Taguchi *et al.*, 1994). Strains 13N-F2 and 28N-D6 were

Fig. 1. Partial restriction maps of *SYR2* clones. (a) Clone 246 (10.5 kb) isolated by complementation of the *sy2* mutant 28N-D6. The *SYR2* gene is located within a 2.5 kb *BstBI* fragment (bold line). (b) Restriction map of 2.5 kb *BstBI* clone S4 showing the sequenced region (bold) and the position of the *SYR2* ORF. Restriction sites: H, *HindIII*; R, *EcoRI*; P, *PstI*; E, *EcoRV*; B, *BstBI*; S, *SphI*; Sm, *SmaI*; Hp, *HpaI*.

transformed with genomic libraries prepared in yeast centromeric vectors YCpN1 and YCp50. Inserts for the two libraries averaged 12 kb. Approximately 5000 transformants of each strain were screened for phenotypic complementation of *sy2*. Transformants were replica-plated onto YPD medium containing 1–2 µg syringomycin ml⁻¹ and inspected for growth after 48 h. Plasmids were isolated from transformants that showed little or no growth with syringomycin. Five clones were isolated with inserts ranging from 10.5 to 14 kb. Restriction fragment analysis revealed that the clones were allelic, containing large overlap regions (data not shown). The smallest clone, 246-D6 (derived from a YCpN1 library), was selected for isolation of the *SYR2* gene (Fig. 1). The gene was subcloned on a 2.5 kb *BstBI* fragment with restriction endonucleases and then on a 1.68 kb fragment by nested deletion. Smaller subclones generated by S1 nuclease treatment were not expressed in yeast, suggesting that coding or upstream regulatory sequences had been excised.

A portion of the 1.68 kb fragment was used as a probe for chromosomal mapping of *SYR2*. Yeast chromosomes of strain KZ1-1C were separated by CHEF gel electrophoresis and blotted onto nylon membranes. Hybridization of the *SYR2* probe to chromosomal blots revealed that *SYR2* is located on chromosome IV (Fig. 2). To look for genes having similar or overlapping function with *SYR2*, the strain KZ1-1C genome was hybridized to the *SYR2* probe using low stringency conditions. No similar genes were found under hybridization conditions that allowed *Dictyostelium* actin-8 (Romans & Firtel, 1985) to bind yeast actin genes (approximately 78% identity) (data not shown).

Sequence analyses

A 2 kb fragment that included the 1.68 kb fragment was sequenced. An ORF sequence was located, beginning at ATG position 270, containing 1047 nucleotides and

Fig. 2. Mapping of *SYR2* to chromosome IV by hybridization to a CHEF gel blot. An ethidium-bromide-stained CHEF gel is shown in lane 1. Hybridization of the *SYR2* probe to a blot of the CHEF gel is shown in lane 2.

predicted to encode a protein of 349 amino acids (Fig. 3). The ORF was preceded by regulatory sequences (CAAT) and (TATAA) beginning at positions -259 and -128, respectively. Northern blot analysis showed a transcript of approximately 1.4 kb (data not shown), consistent in size with a polyA-modified transcript encoded by the ORF. The predicted amino acid sequence was confirmed by sequencing the N-terminal 31 amino acids of a thrombin-cleaved GST-Syr2 (derived from a *HpaI*-*SphI* DNA fragment of *SYR2*) fusion protein. The determined sequence (Thr227-Ala253) corresponded to the predicted sequence.

SYR2 (GenBank accession no. SCU10427) was identical to *SUR2* (GenBank accession no. SCU07171), identified in *S. cerevisiae* and related to survival during nutrient starvation (Desfarges *et al.*, 1993). No other homologues of the encoded Syr2/Sur2 protein were found in the standard protein computer databases.

Syr2 possessed the C-terminal dilysyl motif for retrieval to the endoplasmic reticulum (Letourneur *et al.*, 1994). Syr2 hydropathy profiles suggested six potential membrane-spanning regions with hydrophobicity exceeding 1.25 using the parameters of Kyte & Doolittle (1982) (Fig. 4). The C-terminus contained 26% lysine residues within a stretch of 50 residues, giving Syr2 an overall basic character. A pI of 9.32 was calculated. Potential protein phosphorylation and *N*-glycosylation sites were revealed

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-270 TCTTTCGACCAATTTTCACAGAAAATTTCTTTTGGGTACTAAAAATTTCTTGT
-210 GCCTATTGCTTGTATTTTACTCTCTTCTCATCCTGCTGCGCTGTGTTGCCGCTT
-150 TTTGCTTGTGTTTGGGATTATAAAGAAAGTAAAGATTACATATCTTCTATTCCCTT
-90 GCATTACTCGGGTCTTCTCTTTACTGTTTGGTCTGGGTATTCTAACATCTAGTCGG
-30 AAGAGGGTGTATACGAAAGAAATATACGATGAACGTAACATCGAATGCAACTGCAGCC
      M N V T S N A T A A
30 GGTTCCTTCCACTAGCATTGGTCTCAAGACCTCATTGGGTTTATGCATTATGCCAAG
   G S F P L A F G L K T S F G F M H Y A K 30
90 GCCCTGCCATTAAATTACGCCCAAGGAATCCTTGCTGCCGAAATGAGTGTGTTG
   A P A I N L R P K E S L L P E M S D G V 50
150 CTGGCCTTGGTTGCGCCGGTTGTTGCTACTGGGCGTTGCTGGTATATTCATGTAATA
   L A L V A P V V A Y W A L S G I F H V I 70
210 GACATTTCCATCTGGCTGAGAAGTACAGAATTCATCCGAGCGAAGAGGTTGCCAAGAGG
   D T F H L A E K Y R I H P S E E V A K R 90
270 AACAGGCGTCGAGAATGCATGTTTCTTGAAGTGATTCTACACATATCATACAGACC
   N K A S R M H V F L E V I L Q H I I Q T 110
330 ATTTGTTGGCTTATCTTTATGCACCTCGAGCCGATCTACATGACTGGGTTTGAAGAAAT
   I V G L I F M H F E P I Y M T G F E E N 130
390 GCCATTTGGAAGCTTCGTGCAGACCTTCTCGGATTATTCAGATGCCGCTATTTTATAC
   A M W K L R A D L P R I I P D A A I Y Y 150
450 GGCTATATGTACGGAATGTCGCTTTGAAGATCTTTCAGGCTTTTATTCGTTGATACA
   G Y M Y G M S A L K I F A G F L F V D T 170
510 TGGCAATCTTTTGCATAGATTGATGCATATGAATAAGACCTTATACAAATGGTTCAC
   W Q Y F L H R L M H M N K T L Y K W F H 190
570 TCTGTTTCATCATGAATATACGTGCCATATGCTTACGGTGTCTTTTCAACAATCCTGTT
   S V H H E L Y V P Y A Y G A L F N N P V 210
630 GAGGGCTTCTGTTAGATCTTTGGGAACCGGTATTGCCATGACGTTAACTCATTGTACT
   E G F L L D T L G T G I A M T L T H L T 230
690 CACAGAGAGCAAAATCATTCTTTTACCTTTGCCACCATGAAGCTGTCGATGACCATGT
   H R E Q I I L F T F A T M K T V D D H C 250
750 GGGTATGCTTTGCCACTTGACCATTCGAATGGCTTTTCCCTAATAACGCTGTCTACAC
   G Y A L P L D P F Q W L F P N N A V Y H 270
810 GATATCCACCACCAATTTGGTATCAAGACGAACCTTGTCAACCATTTTTCACCTTTC
   D I H H Q Q F G I K T N F A Q P F F T F 290
870 TGGGACAAATTTGTCAAACTAATTTAAGGGTTTGAAGAATATCAAGAAGAGCAAGA
   W D N L F Q T N F K G F E E Y Q K K Q R 310
930 CGTGTCCATCGACAAGTACAAGAGTTTTCGAAGAGAGAGATTGGAAAAAGAGAG
   R V T I D K Y K E F L Q E R E L E K K E 330
990 AAACCTCAAAACTTCAAGCTATGAATGCTGCTGAATGAAGTAAGAAAGAGAAATAA
   K L K N F K A M N A A E N E V K R E K * 349
1050 CCCTTTTGCATACCTCCGTTCAATGCTGGGTAAAGCAATGCTCCGACTTCCACGTT
1110 TGTATTATTATCCATTATATACATCAAGACATATTAATAGTGTATAGCGGTGACTTT
1170 GACTATATTATGTTTGAACGAAAGATACATCAAAATCAACTATGAATGGTTATT
1230 ATTGTGATGCAAGCTT

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Fig. 3. Nucleotide and predicted amino acid sequences of *SYR2*. Upstream transcriptional regulatory sequences (CAAT) and (TATAAA) are underlined. The C-terminal endoplasmic reticulum retrieval signal is highlighted in bold. The asterisk indicates the position of translational termination.

Fig. 4. Hydropathy plot of Syr2. Hydropathy was analysed as described by Kyte & Doolittle (1982). Calculations were normalized on a scale from 0 to 2.

using computer programs PROFILESCAN and MOTIF (GCG Corporation, Madison, WI, USA) (Altschul *et al.*, 1990).

SYR2 disruption

All four meiotic segregants recovered after *SYR2* disruption in diploid strain W303-1A were viable in YPD growth medium. A 2:2 cosegregation of syringomycin

Fig. 5. Disruption of *SYR2*. (a) Structure of the DNA fragment used for the construction of the *SYR2* null allele. (b) Tetrad analysis of the disrupted diploid showing cosegregation of syringomycin resistance and the *URA3* marker. (c) Southern blot analysis of *Pst*I-digested genomic DNA from diploid strain W303-1A (lane 1) and *SYR2*-disruptant WΔ*SYR2* (lane 2). The *URA3* marker contains a *Pst*I site 1.6 kb downstream of the *SYR2* *Pst*I and creates two bands in the null allele.

resistance and *ura*⁺ phenotypes occurred, indicating *URA3* replacement in the wild-type *SYR2* allele (Fig. 5). Disruption was confirmed by Southern blot hybridization (Fig. 5c). No *SYR2* transcripts were detected by Northern blot analyses in the disruptant strains. The WΔ*SYR2* segregants were viable in SD media and insensitive to ethanol, heat shock and high concentrations of sorbitol (1.25 M) and Na⁺ (1 M). They grew with maltose, galactose, glycerol and acetate as carbon sources.

Syr2 detection and cell localization

Western blot analysis using antisera directed against Syr2 identified an approximately 43 kDa immunoreactive protein, which was slightly larger than the predicted mass of Syr2. The protein was detected in whole-cell extracts, microsomal fractions and endoplasmic reticulum mem-

Fig. 6. Western blot analysis of Syr2. (a) Immunoreaction with the GST-Syr2 fusion protein and the Syr2 peptide that was used for antibody production (lane 1). Pre-immune sera did not react with either polypeptide (lane 2). (b) Immunoreaction to a 43 kDa protein in yeast microsomal extracts (lane 1) and yeast extracts enriched for endoplasmic reticulum enzymes (lane 2). Syr2 was not detected in plasma membrane fractions (lane 3) or microsomal fractions prepared from *SYR2*-disrupted mutants (lane 4). Pre-immune sera did not react with microsomal extracts (lane 5).

branes, but not the plasma membrane (Fig. 6). The strongest immunoreaction occurred with the cellular fractions enriched for endoplasmic reticulum markers. Syr2 was not detected in extracts of the WΔ*SYR2* disruptants nor in extracts of strain 28N-D6. It was, however, detected in strain 13N-F2 membrane fractions.

In extracts from *SYR2*-overexpression strains, two equally intense immunoreactive bands on Western blots were often observed. This observation raised the possibility that Syr2 exists in two forms. To test the possibility that one form may be phosphorylated, crude membrane preparations were treated with alkaline phosphatase before SDS-PAGE and Western blotting. Both forms were still detected in the phosphatase-treated samples (data not shown). Likewise, no glycosylated form of Syr2 was evident after staining equivalent gels and blots with periodic acid/Schiff base reagent (data not shown).

Fig. 7. Ergosterol levels in *SYR2*-overexpressing and disruption strains. Equal amounts (1 mg) of total cell nonsaponifiable lipid extracts were chromatographed on thin-layer silica gel plates and stained with phosphomolybdic acid reagent. Authentic ergosterol (1 mg) was applied for comparison. The chromatographic origin (o) is indicated.

Table 1. Total cell phospholipid levels of *SYR2*-overexpression and disruption strains

Strain	Growth medium	Lipid phosphate [μg (mg dry wt cells) $^{-1}$]*
KZ1-1C(p Δ SYR2)	SD-URA	1.35 (0.31)
KZ1-1C(p Δ SYR2)	SG-URA	2.47 (1.19)
W Δ SYR2	YPD	1.71 (0.73)
W303C	YPD	3.88 (1.03)

* Values are means from three separate experiments. Standard deviations (*n*-weighted) are indicated in parentheses.

SYR2 overexpression

To further analyse *SYR2* function, overexpression vector p Δ SYR2, containing the *Pst*I-*Sph*I portion of *SYR2* under the regulation of the *GAL1* promoter, was constructed. When *sy*r2 mutant strains 13N-F2 and 28N-D6 were transformed with p Δ SYR2 and grown in SG-URA medium, sensitivity to syringomycin was restored. Transformants W303C(p Δ SYR2) and KZ1-1C(p Δ SYR2) displayed syringomycin sensitivities comparable to the corresponding nontransformed strains, W303C and KZ1-1C, when grown in galactose-containing media. Also, p Δ SYR2 transformants of *sy*r1 mutant 3N-H1 maintained levels of resistance to syringomycin in SG-URA medium similar to those of non-transformants. *SYR2* overexpression was confirmed by Northern blot and Western blot analyses.

Sterol and phospholipid compositions

The cellular ergosterol levels of disruptant strain W Δ SYR2 and strain W303C were similar (Fig. 7). Also, the ergosterol levels of KZ1-1C(p Δ SYR2) cells grown in minimal medium with either galactose or glucose were similar (Fig. 7). In contrast, W Δ SYR2 had lower levels of total cellular phospholipid (Table 1) and of phosphatidyl-

Fig. 8. Phospholipid profiles of *SYR2*-overexpressing and disruption strains. Equal amounts (10 mg) of total cell lipid extracts of strains KZ1-1C(pYES2) grown in SD-URA medium (lane 1) and SG-URA medium (lane 2), KZ1-1C(p Δ SYR2) grown in SD-URA (lane 3) and SG-URA medium (lane 4), W Δ SYR2 (lane 5) and W303C (lane 6) were chromatographed on thin-layer silica gel G-60 plates and stained with molybdenum blue reagent. Positions of authentic lysophosphatidylcholine (LP), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) are indicated. The chromatographic origin (o) is indicated.

ethanolamine, phosphatidylserine and phosphatidylinositol (Fig. 8) than W303C. On the other hand, KZ1-1C(p Δ SYR2) cells grown with galactose had higher total cellular levels of phospholipids (Table 1) and of phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol when compared to cells grown with glucose (Fig. 8).

DISCUSSION

It was determined in the present study that a novel gene, *SYR2*, is required for yeast growth inhibition by syringomycin. *SYR2* differs from other identified syringomycin response genes since it does not involve ergosterol biosynthesis (Takemoto *et al.*, 1993). Although its precise biochemical function is not known, *SYR2* appears to have a role in cellular phospholipid biosynthesis. *SYR2* is not essential for viability and, therefore, it is unlikely that it catalyses a critical step in the phospholipid biosynthetic pathways. Mutant *sy*r2 alleles, however, lead to syringomycin resistance, indicating that normal phospholipid

levels are required for syringomycin action. Previous work showed that the plasma membrane is the site of action of this lipopeptide (Takemoto, 1992), and we suggest that the two major lipid classes of the yeast plasma membrane, ergosterol and phospholipids, are important for its action.

SYR2 is identical to the yeast gene *SUR2*. Mutant *sur2/sur2* alleles, as well as *sur1*, *sur3* and *sur4* mutations, are suppressors of *rus161* (Desfarges *et al.*, 1993), which impairs survival of yeast during nutrient starvation. Like *sur2/sur2*, *sur1* and *sur4* lead to alterations in the relative levels of various phospholipids. Desfarges *et al.* (1993) have hypothesized that all four identified *SUR* genes function in the same biochemical pathway.

Sequence analysis of Syr2 revealed several potential sites for phosphorylation and *N*-glycosylation. However, evidence for covalent modification of Syr2 was not observed. Syr2 was often seen as a doublet on Western blots of crude extracts obtained from *SYR2*-overexpression strains. The doublet may reflect a modified or degraded form of Syr2. The overexpressed Syr2 did not possess an N-terminal signal sequence, eliminating the possibility that one form resulted from cleavage of a signal peptide.

Hydropathy profiles suggested that Syr2 is a membrane protein. As expected for an enzyme involved in phospholipid biosynthesis, an association with the endoplasmic reticulum was demonstrated. This location is consistent with the dilysyl motif at the C-terminus, which is an endoplasmic reticulum retrieval and coatamer interacting signal (Letourneur *et al.*, 1994). Yeast proteins Wbp1p and C-5 sterol desaturase (encoded by *ERG3/SYR1*) also contain this signal (Taguchi *et al.*, 1994; Arthington *et al.*, 1991; Te Heesen *et al.*, 1992; Letourneur *et al.*, 1994), and in the former, the dilysyl motif occurs in the cytoplasmic domain (Townsend & Pelham, 1994; Gaynor *et al.*, 1994). By analogy, we suggest that the Syr2 hydrophilic C-terminus is exposed on the cytoplasmic side of the endoplasmic reticulum.

ACKNOWLEDGEMENTS

This work was supported by the National Science Foundation (grant DCB 9003398). We thank S. Stock for technical assistance.

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Received 4 July 1995; revised 25 October 1995; accepted 6 November 1995.