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The Immunosuppressant SR 31747 Blocks Cell Proliferation by Inhibiting a Steroid Isomerase in *Saccharomyces cerevisiae*

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SR 31747 is a novel immunosuppressant agent that arrests cell proliferation in the yeast *Saccharomyces cerevisiae*. SR 31747-treated cells accumulate the same aberrant sterols as those found in a mutant impaired in Δ8-Δ7-sterol isomerase. Sterol isomerase activity is also inhibited by SR 31747 in in vitro assays. Overexpression of the sterol isomerase-encoding gene, *ERG2*, confers enhanced SR resistance. Cells growing anaerobically on ergosterol-containing medium are not sensitive to SR. Disruption of the sterol isomerase-encoding gene is lethal in cells growing in the absence of exogenous ergosterol, except in SR-resistant mutants lacking either the *SUR4* or the *FEN1* gene product. These results suggest that sterol isomerase is the target of SR 31747 and that both the *SUR4* and *FEN1* gene products are required to mediate the proliferation arrest induced by ergosterol depletion.

SR 31747 [SR; (*Z*)*N*-cyclohexyl-*N*-ethyl-3-(3-chloro-4-cyclohexylphenyl)propen-2-ylamine hydrochloride] is a chemical agent capable of blocking the proliferation of lymphocytes at a concentration of 10 nM (7). SR does not inhibit the release of interleukin-2 in T cells. Moreover, SR is still capable of inhibiting T-cell proliferation when added as late as 24 h after activation (7). These two properties of SR are shared by rapamycin but not by other immunosuppressants such as cyclosporin A (CsA) and tacrolimus (FK506) (19, 28). The suppressive effect elicited by SR occurs over a concentration range which correlates with the pharmacological profile of the molecule in binding assays, strongly suggesting that SR acts through a receptor-mediated process (7, 29). Rapamycin does not displace [^{3}H]SR binding, and [^{3}H]SR does not bind to the rapamycin acceptor, FKBP12 (7). In contrast, SR is the most potent molecule with respect to capacity to compete with all known sigma ligands, such as haloperidol and pentazocine, although it is reciprocally displaced by none of them (29), which suggests an allosteric relationship between SR and sigma binding sites. The structures and functions of so-called sigma receptors or binding sites are still unknown. They are localized in microsomal fractions and are found in the central nervous system as well as in many peripheral tissues (see reference 36 for a review). The existence of sigma sites in the rat spleen and on human peripheral blood leukocytes (5) and the observation that pentazocine, a typical sigma ligand, suppressed lymphocyte proliferation at micromolar concentrations (6) suggested a physiological role for sigma ligands in modulating immune function. Sigma binding sites have been suggested to ensure the link between the nervous and immune systems and to mediate some aspects of steroid-induced alterations in immune function (36). Although the endogenous ligands of sigma sites have not yet been unequivocally identified, Su (36) has suggested that they belong to the steroid family and possibly include progesterone.

The targets of several immunosuppressants, including CsA, rapamycin, and FK506, are strikingly conserved from humans to yeasts (4, 16, 19). As a preliminary step toward elucidating the nature of the SR target, we have examined the effect of SR in *Saccharomyces cerevisiae*. In this report, we show that SR arrests proliferation in yeast cells. SR-resistant mutants have been obtained. Most mutations map in either two genes, *FEN1* and *SUR4. fen1* mutants are known to be generally resistant to a wide series of post-squalene sterol biosynthesis inhibitors, including azole derivatives, which inhibit the cytochrome P-450 activity that catalyzes lanosterol demethylation, and morpholine derivatives such as fenpropimorph, which inhibit Δ14-sterol reductase and Δ8-Δ7-sterol isomerase (20, 27). Yeast cells are dependent on ergosterol biosynthesis to proliferate aerobically, since they cannot take up exogenous ergosterol in the presence of oxygen (1). Lanosterol demethylase, Δ14-sterol reductase, and Δ8-Δ7-sterol isomerase are encoded by *ERG11*, *ERG24*, and *ERG2*, respectively (see reference 22 for a review). *ERG11* and *ERG24* have been found to be essential for aerobic growth. The *erg11* defect-induced lethality is suppressed by mutations in the *ERG3* gene, which encodes a Δ4-Δ5-desaturase, whereas the *erg24* disruption is not lethal in *fen1* and *fen2* mutants. Surprisingly, the *ERG2* gene has been found to be nonessential for aerobic growth. Therefore, the antifungal properties of fenpropimorph could chiefly be due to Δ14-sterol reductase inhibition. This conclusion is also supported by the observation that overexpressing the Δ14-sterol reductase-encoding *ERG24* gene confers fenpropimorph resistance, whereas overexpressing *ERG2* does not (21). Although SR is not a morpholine derivative, we show in this report that it is a sterol isomerase inhibitor and that *ERG2* is indeed an essential gene in yeast strains except those that harbor *erg2* lethality suppressor genes, such as *fen1* and *sur4* null alleles.

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Source (reference)
C13-ABYS86	*MAT*α *ura3*Δ*5 leu2-3 leu2-112 his3 pra1 prb1 prc1 cps1*	D. Wolf (15)
WA10-3-1D	*MAT***a** *erg2-4*::*LEU2 ura3-52 leu2-3 leu2-112 his3-2 ade5*	M. Bard (2)
EMA1	*MAT***a** *ura1*	F. Lacroute
EMA3	*MAT***a** *ura3*Δ *trp1-4*	F. Lacroute
EMA6	*MAT***a** *ura3*Δ	EMA3 × EMY1
EMA13	*MAT***a** *ura1 sre2-237*	This study
EMA15	*MAT***a** *ura1 sre1-232*	This study
EMA24	*MAT***a** *ura3*Δ *trp1-4 sur4*::*URA3*	This study
EMA40	*MAT***a** *ura3*Δ *trp1-4 leu2 sur4*::*URA3*	EMA24 × EMY30
EMA41	*MAT***a** *ura3*Δ *trp1-4 leu2 fen1*::*LEU2*	EMA39 × EMY30
EMA62	*MAT***a** *leu2 trp1 ura3 sre2-237*	EMY18 × C13-ABYS86
EMY1	*MAT*α *ura1*	F. Lacroute
EMY2	*MAT*α *leu2*	F. Lacroute
EMY4	*MAT*α *ura3*Δ *trp1*	F. Lacroute
EMY17	*MAT*α *ura1 ura3 trp1-4 sre1-232*	EMA15 × EMY4
EMY18	*MAT*α *ura3*Δ *trp1-4 sre2-237*	EMA13 × EMY4
EMY22	*MAT*α *ura3*Δ *sre1-232*	EMA15 × EMY4
EMY27	*MAT*α *ura3*Δ *trp1-4 leu2 fen1*::*LEU2*	This study
EMY30	*MAT*α *ura3*Δ, *trp1-4 leu2*	EMA3 × EMY2
EMY43	*MAT*α *ura3*Δ *trp1-4 erg2*::*TRP1*	This study
EMD7	*MAT***a**/*MAT*α *ura3*Δ/*ura3*Δ *trp1-4*/*trp1-4 leu2*/*LEU2*	EMA3 × EMY30
EMD8	*MAT***a**/*MAT*α *ura3*Δ/*trp1-4*/*trp1-4 leu2*/*LEU2 erg2*::*TRP1*/*ERG2*	This study

MATERIALS AND METHODS

Strains, media, and growth conditions. All strains used in this study were isogenic derivatives of *S. cerevisiae* wild-type (wt) strain FL100 (ATCC 28383) except for C13-ABYS86 (15), EMA62, and WA10-3-1D (2) (Table 1). Yeast culture media were YPD medium and synthetic minimal (SD) medium containing 2% glucose and adequately supplemented to fulfill the relevant strain's auxotrophic requirement (14). Sporulation media were as described previously (14). Anaerobic conditions were obtained by using an anaerobic glove box (La Calhène, Vélizy, France) under an N_2-H_2-CO_2 (85%-10%-5%) atmosphere. For anaerobic growth, media were supplemented with 0.1% Tween 80 in ethanol and 50 mg of ergosterol per liter in ethanol. Media thus contained ethanol at a final concentration of 1.8%. As cells that display both the *erg2* and *sur4* defects are sensitive to Tween 80 (our unpublished results), such cells were cultivated in the presence of 0.01% Tween 80 and 5 mg of ergosterol per liter when grown anaerobically. Growth was monitored by measuring the optical density at 600 nm with a Spectronic 20D spectrophotometer (Milton Roy Company), 1 optical density unit corresponding to 3×10^7 cells. All media containing SR, fenpropimorph, or fenpropidin were supplemented with 0.1% Tween 20 and 0.9% ethanol to facilitate drug solubilization. SR was prepared as a 50 mM solution in ethanol. Fenpropimorph and fenpropidin were kindly provided by F. Karst, University of Poitiers, Poitiers, France, as 3 mM solutions in ethanol. The final ethanol concentration in these media never exceeded 1% (vol/vol). The *Escherichia coli* strain used for plasmid construction and propagation was RR1 (Bethesda Research Laboratories, Gaithersburg, Md.). TG1 was used for amplification of the phage M13 derivatives.

Recombinant DNA techniques and plasmids. Genetic engineering techniques and yeast DNA extraction were as described previously (14, 34). The DNA probes for Southern blot analyses were labeled by using a nick translation kit from Boehringer (Mannheim, Germany). DNA sequencing was performed by using the dideoxynucleotide chain termination method on recombinant M13 phage single-stranded DNA or directly on denatured double-stranded plasmid DNA or on DNA obtained by PCR amplification. Sequence analyses of *SUR4* and of *FEN1* were performed with the University of Wisconsin Genetics Computer Group package (11). Percent similarity and pairwise homologies were assessed with the GAP software. Database searches were conducted against GenBank and European Molecular Biology Laboratory (EMBL) releases. The yeast genomic library used for the isolation of the *SUR4* and *FEN1* genes was constructed by M. Tuite and kindly provided by M. Crouzet, University of Bordeaux and Centre National de Recherche Scientifique, Bordeaux, France. The library was obtained by ligating partial *Sau*3A digests of SKQ2n genomic DNA into the *Bam*HI site of pUKC200, a pBR322 derivative that contained *TRP1* as the selectable marker as well as an *ARS-CEN3*-containing segment for plasmid replication and maintenance in yeast cells. The mean insert size was estimated at 7 kb. pUKC232/60 and pUKC237/T1 were two clones of the library that had integrated a yeast DNA fragment encompassing *SUR4* (2.9 kb) and *FEN1* (10.5 kb), respectively. The vector pEMR950 is a 2μm-derived *E. coli*-yeast shuttle plasmid and is essentially similar to pEMR515 (23). The *Nhe*I-*Bam*HI expression cassette of pEMR515 was replaced by an *Nhe*I-*Bam*HI segment containing the *URA3* gene as well as the *PHO5* promoter. This promoter was cloned by PCR amplification using a forward primer that contained the *Mlu*I, *Nhe*I, and *Hin*dIII sites and was complementary to a sequence of *PHO5* at positions −541 to −521 and a reverse primer containing the sites *Bgl*II-*Xho*I-*Apa*I-*Xba*I-*Bam*HI and complementary to the sequence at positions −22 to −1 in *PHO5* (accession number V01320). The *SUR4*-encompassing *Hin*dIII-*Sal*I fragment of pUKC232/60 (3.6 kb) was cloned into pEMR950 in place of the *PHO5* promoter to yield pEMR1014; pEMR1055 was obtained by ligating the *FEN1*-encompassing *Hin*dIII-*Sal*I segment of pUKC237/T1 into pEMR950, eliminating the *PHO5* promoter. pEMR1023 was obtained as follows. A 1.4-kb *Pvu*I-*Sph*I fragment of pEMR515 containing the 2μm sequence for plasmid replication in yeast cells was ligated with the *Sph*I-*Cla*I segment of a pUC19 derivative vector, pFL38 (ATCC 77203), to yield pEMR1000. pEMR1023 resulted from ligating this plasmid with the *Nhe*I-*Mlu*I fragment of pEMR950 that encompassed *URA3*, the *Mlu*I-*Eco*RI sequence that contained a *PGK*-derived expression cassette, and finally an *Eco*RI-*Nde*I fragment which encompassed the bacterial origin of transfer required from trans-kingdom conjugation between *E. coli* and *S. cerevisiae* (constructed by E. Liauzun). The *PGK* expression cassette was obtained by separate PCR amplification of the *PGK* promoter and terminator. The oligonucleotides used to amplify the promoter region were A and B; those for the terminator region were C and D. The oligonucleotide sequences corresponded to sequences of *PGK* (accession number J01342) flanked at their external 5′ ends by cloning sites as follows: A, positions −519 to −500 (forward) with an *Mlu*I site; B, positions −20 to −1 (reverse) with *Apa*I, *Pst*I, *Sal*I, and *Bgl*II sites; C, positions +1258 to +1277 (forward) with *Bgl*II, *Ava*I, *Sma*I, and *Bam*HI sites; and D, positions +1510 to +1534 (reverse) with *Eco*RI and *Nde*I sites. The *ERG24* coding sequence (GenBank accession number M99419) was obtained by PCR amplification using two oligonucleotides, E (positions −22 to −2 [forward, *Pst*I] and F (+1314 to +1331 [reverse, *Bam*HI]). The amplified DNA fragment was cloned between the *Pst*I and *Bam*HI sites of the pEMR1023 polylinker to yield pEMR1050. The *Eco*RI-*Nco*I fragment of pEMR1050 that encompassed *ERG24* and a portion of *URA3* was ligated with pFL38 to yield pEMR1205, a centromeric plasmid in which *ERG24* was placed under the control of the *PGK* promoter. The *ERG2* gene (GenBank accession number M74037) was cloned by PCR amplification using two oligonucleotides, G and H. The sequences of G and H corresponded to positions −295 to −276 (forward, *Mlu*I, *Sal*I) and +885 to +904 (reverse, *Cla*I, *Eco*RI), respectively, of the *ERG2* gene. The *ERG2* expression plasmid pEMR1056 was derived from pEMR1023 by replacing the *PGK* promoter and terminator cassette with the cloned *ERG2* gene in an *Mlu*I-*Eco*RI expression cassette and replacing *URA3* with *TRP1* to allow cotransformation by both pEMR1050 and pEMR1056. The *ERG2*-encompassing *Eco*RI-*Sal*I fragment of pEMR1056 was cloned into pFL38 to yield pEMR1200, a centromeric *ERG2* expression vector. All genes cloned throughout this study were entirely sequenced to ensure the absence of mutation.

Genetic methods and transformation procedures. Mating, sporulation, and tetrad analysis techniques were as described previously (14). Crosses involving *ERG2* gene disruptants as matters were done under anaerobic conditions. For random spore analyses, tetrads were incubated in the presence of 50% diethyl ether with vigorous shaking at room temperature for 10 min before plating onto YPD medium. *S. cerevisiae* transformation was performed by the lithium acetate procedure (17). *E. coli* cells were transformed by electroporation (34). SR-resistant mutant strains were obtained as follows. Strains EMA1 (*ura1*) and

EMY2 (*leu2*) were mutagenized with UV light so as to kill about 90% of the cells. UV-induced or spontaneous mutants of strains EMA1 and EMY2 were selected by plating cells onto YPD medium containing 80 μM SR for at least 3 days. Mutant colonies were picked and propagated on rich medium without SR. Complementation tests were performed by crossing a mutant derived from EMA1 with all of the EMY2-derived mutants. Diploids cells were isolated in microtitration plates containing SD medium. SR resistance or sensitivity was determined by growing clones on YPD medium containing 25 μM SR. Two haploid mutants were considered as belonging to the same complementation group when the diploid obtained by mating the two mutants was able to grow in the presence of 25 μM SR. A EMY2-derived mutant of the first complementation group was tested by mating with each of the EMA1-derived mutants so as to identify compatible matters among the population. The same experiment was repeated with a mutant of the second group and so on. The susceptibility to SR of transformants was tested either by replica plating colonies or by plating about 10^5 cells onto SD medium containing different concentrations of SR.

***SUR4*, *FEN1*, and *ERG2* disruptions.** The *SUR4* deletion was made by replacing a *Hin*dIII-*Hin*dIII internal fragment (1.4 kb) of *SUR4* by *URA3* in pUKC232/60. A *Bcl*I fragment (2.4 kb) containing the resulting *SUR4* deletion was purified and used to transform EMA3 (*ura3*) or EMY22 (*ura3 sre1-232*) cells to Ura^+. The *FEN1* gene disruption was created as follows. A *fen1*::*LEU2* hybrid gene was synthesized by PCR amplification using two hybrid oligonucleotides as primers. The sequence of the first primer corresponded to the 59 nucleotides that preceded the *FEN1* initiation codon (positions −67 to −9) followed by the 15 nucleotides located at the 5′ end of the *LEU2* promoter (positions −647 to −632; accession number J01333), whereas that of the second primer corresponded to the region that encompassed the stop codon of *FEN1* (positions 1044 to 1101) followed by 15 nucleotides at the 3′ end of *LEU2* (positions 1340 to 1355) in the noncoding DNA strand. The resulting 2.4-kb DNA segment was purified and used to transform EMY30 (*leu2*) or EMA62 (*leu2 sre2-237*) cells to Leu^+. Both disruptions were performed by the one-step gene replacement method (14) and verified by PCR and Southern blot analyses. The *ERG2* gene was disrupted as follows. The promoter and terminator regions of the *ERG2* gene were separately isolated by PCR amplification with oligonucleotides complementary to sequences from positions −295 to −276 (forward, *Sal*I) and −3 to +18 (reverse, *Nhe*I, *Hin*dIII) for the promoter and from positions +632 to +651 (forward, *Hin*dIII, *Mlu*I) and +885 to +904 (reverse, *Cla*I, *Eco*RI) for the terminator. Both fragments were associated in the correct direction and cloned into pFL38. The *TRP1* gene isolated from pEMR1056 was inserted between the two sequences at the *Nhe*I and *Mlu*I sites. The resulting *Nco*I-*Cla*I fragment (1.6 kb) that contained the *erg2*::*TRP1* deletion was purified and used to transform EMD7 (*trp1-4*/*trp1-4*) cells directly to Trp^+ (Table 1). The *erg2* disruption was ascertained on one transformant by PCR and Southern blot analyses of genomic DNA. Transformed cells were sporulated. Ten tetrads were dissected. Spores were germinated anaerobically on rich medium supplemented with 0.2% Tween 80 and 50 mg of ergosterol per liter. The tryptophan and uracil requirements of each spore were determined under anaerobic conditions. An *erg2*::*TRP1* gene disruptant, EMY43 (Table 1), was kept for further studies.

Sterol analyses. Cells were grown for 24 h in YPD medium containing SR at different concentrations. Sterols were extracted from lyophilized cells as previously described (25) in the presence of a constant amount of cholesterol (130 μg/50 mg [dry weight]) when specified. Sterols were analyzed on a VG ZAB-2E instrument for gas chromatography (GC)-mass spectrometry (MS) (electronic impact [EI] mode). Experimental mass spectra of the different peaks were automatically compared with reference spectra included in the NIST spectral bank. Sterols were separated by GC with a Fisons chromatograph, using a 30-m OV1 column (0.32-mm inside diameter), and quantified by the area method in comparison with cholesterol (25).

Δ8-Δ7-Sterol isomerase assay. EMA3[pEMR1056] cells were disrupted by glass bead homogenization (0.45-mm diameter) in 0.1 M phosphate buffer (pH 7.5) in the presence of 1.5 mM reduced glutathione and 30 mM nicotinamide for 10 min (4°C). Cell debris, mitochondria, and nuclei were removed by centrifugation at 10,000 × *g* for 20 min. Microsomes were isolated by centrifuging the supernatant at 100,000 × *g* for 20 min and resuspending the pellet in an Elvehjem-Potter homogenizer in 0.1 M phosphate buffer (pH 7.5) containing 3 mM reduced glutathione and 20% (vol/vol) glycerol. Four hundred microliters of microsome suspension, containing 1.3 mg of protein, was incubated in the presence of 75 μM exogenous cholest-8-en-3β-ol and 0.1% (wt/vol) Tween 80 at 30°C for 3 h. The reaction was stopped by the addition of 1 ml of 6% KOH-ethanol. Sterols were extracted three times with a total volume of 15 ml of *n*-hexane. The extracts were further analyzed by thin-layer chromatography on silica gel, using dichloromethane as the eluant (two migrations). The 4-desmethylsterols (R_f = 0.30) were separated from 4α-methylsterols (R_f = 0.40) and from 4,4-dimethylsterols (R_f = 0.45). After elution from the silica gel, an aliquot of the 4-desmethyl fraction was analyzed by either gas-liquid chromatography (GLC) or reverse-phase high-pressure liquid chromatography (RP-HPLC). For GLC analysis, a fused-silica capillary column (WCOT; 30 m by 0.32 mm [inside diameter]) with 0.25-μm film coated with DB17 (240 to 280°C, 2°C/min) and hydrogen as the carrier gas were used. The residual substrate cholest-8-en-3β-ol (retention time [t_R] relative to cholesterol = 1.026) and the product formed, cholest-7-en-3β-ol (t_R = 1.079), were readily separated from each other and from the bulk of endogenous sterols, including ergosterol (t_R = 1.155). In the case of RP-HPLC analysis, two analytical C_{18} Ultrasphere 5-μm columns (250 by 4.6 mm) connected in series were used, with a mobile phase of methanol-water (99.7:0.3 [vol/vol]) at a flow rate of 1 ml/min at room temperature. RP-HPLC analysis was carried out with a Waters 510 instrument equipped with a single pump and linked to a UV spectrophotometric detector at 210 nm. Cholest-8-en-3β-ol (t_R relative to ergosterol = 1.152) and cholest-7-en-3β-ol (t_R = 1.201) were readily separated from each other and from the bulk of endogenous sterols. The Δ7 metabolite produced by the reaction was unequivoqually identified as cholest-7-en-3β-ol by coincidental retention times in GLC and RP-HPLC and by an electron impact spectrum identical to that of an authentic standard obtained from Sigma. In both analytical procedures, the conversion ratio was calculated from the areas of the peaks of cholest-7-en-3β-ol and cholest-8-en-3β-ol and corrected from endogenous components of same t_R (if present) with the values obtained in the corresponding assay using boiled microsomes. The rate of substrate isomerization was calculated from the conversion ratio and the concentration of substrate used in the reaction. Cholest-8-en-3β-ol was synthesized as previously described (3).

Nucleotide sequence accession number. The *SUR4* sequence data have been assigned EMBL accession number X82033 (under the designation SRE1).

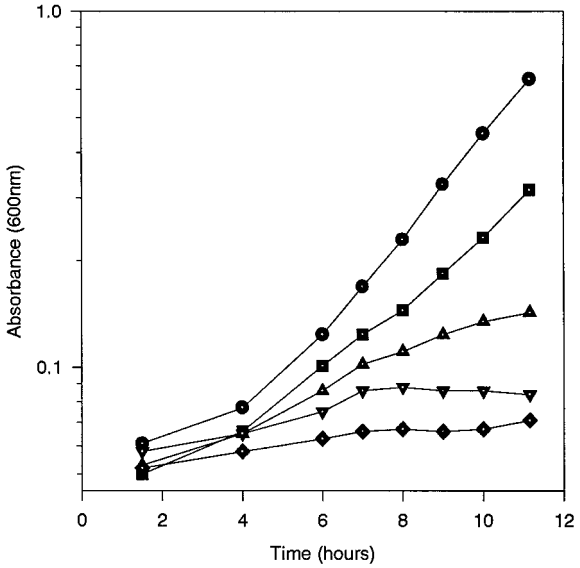


FIG. 1. Growth of EMA6 cells in YPD in the absence (circle) or presence of SR at various concentrations: squares, 3 μM; upward-pointing triangles, 5 μM; downward-pointing triangles, 7.5 μM; diamonds, 12 μM.

RESULTS

Isolation and identification of the *SUR4* and *FEN1* genes. Yeast vegetative growth was inhibited by SR in a dose-dependent manner (Fig. 1). At drug concentrations exceeding 8 μM, proliferation was completely arrested. We isolated about 300 spontaneous or UV-induced mutants that were able to form colonies in the presence of SR at concentrations exceeding 56 μM. The SR resistance phenotype was found to be recessive in all mutants. None displayed enhanced levels of resistance to rapamycin compared with the wt control, suggesting that FKBP12 was not involved in mediating the SR effect on yeast cells (16, 19). The majority of the mutants fell into only two major complementation groups represented by 123 and 106 mutants. One spontaneous mutant of each group was mated with an SR-sensitive strain. As expected, segregation analyses showed a regular 2 sensitive:2 resistant pattern, indicating that the resistance phenotype in each of these mutants was due to a single mutated gene, *sre1-232* or *sre2-237*, respectively.

The wt alleles corresponding to *sre1-232* and *sre2-237* were isolated from a library of yeast genomic DNA constructed in the low-copy-number centromeric vector pUKC200. The clones were selected by their ability to correct the recessive SR resistance phenotype in strain EMY17 and EMA62, two Trp^-

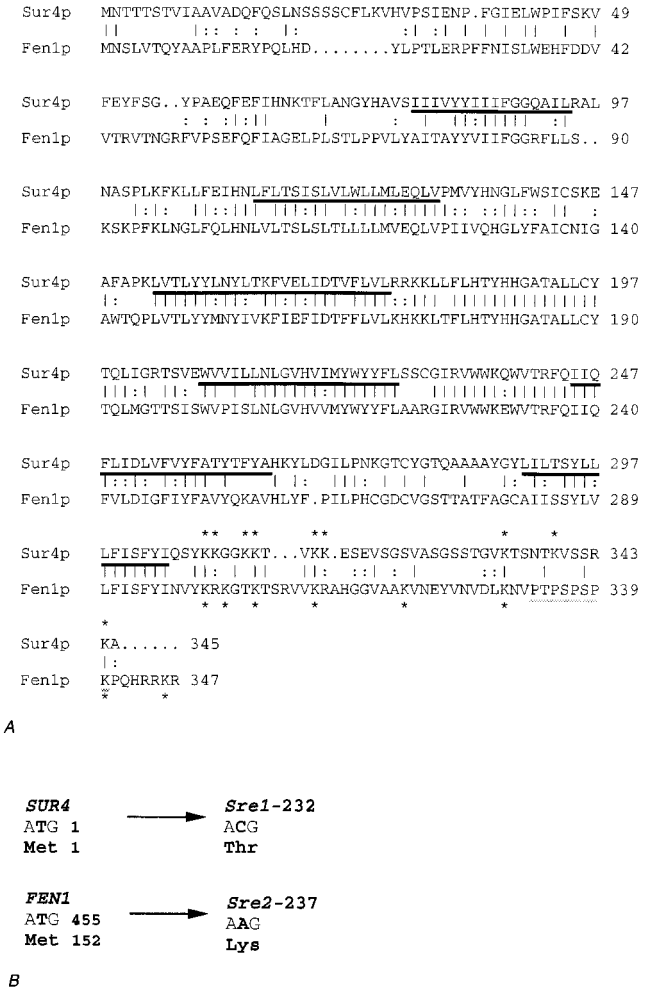
strains that harbored the *sre1-232* and *sre2-237* alleles, respectively (Table 1). About 5,000 colonies of EMY17 transformants were screened by replicating onto medium containing 50 μM SR, and five SR-sensitive colonies were obtained. Subsequent transformation of EMY17 (*sre1-232*) with DNA extracted from two of these colonies revealed a complete coincidence of inheritance of both Trp^+ and SR sensitivity, confirming that the SR sensitivity displayed by these clones was a plasmid-linked phenotype. The two plasmids contained the same 2.9-kb insert. Sequencing of this insert revealed only one long open reading frame with a coding capacity for a 345-amino-acid polypeptide.

In a similar experiment, two genomic clones were found to complement the *sre2-237*-linked phenotype of EMA62. We verified that the SR sensitivity phenotype was a plasmid-linked trait in both cases. Restriction analysis of these two clones revealed the presence of two overlapping inserts of about 7 and 10.5 kb. The region needed to restore SR sensitivity in the *sre2-237* mutant could be assigned to one gene encoding a 347-amino-acid peptide (Fig. 2A).

The gene that complemented the *sre1-232*-linked phenotype was identical to *SUR4* located on chromosome XII (12, 33). This gene was first described under the name *APA1* by Garcia-Arranz et al. (13). However, since another team had already chosen *APA1* to name a different gene in *S. cerevisiae* 5 years before Garcia-Arranz's paper was published (30), the name *SUR4* will be used in this study. Garcia-Arranz's *apa1* mutants were isolated on the basis of their reduced levels of the plasma membrane ATPase synthesis (13), whereas *sur4* mutants were identified by their ability to suppress the reduced viability upon starvation phenotype of *rvs* mutants (8, 10).

The cloned gene that complemented the SR resistance of EMA62 was identical with YCR521 (39, 41), a nonessential open reading frame located on chromosome III. Although the function of YCR521 is unknown, an allele of this gene, *fen1-1*, has recently been identified in Karst's laboratory as conferring resistance to various ergosterol biosynthesis inhibitors (18, 20). The *SUR4* and *FEN1* gene products (Sur4p and Fen1p) are 50% identical (67% similar) and display a very hydrophobic and conserved core with four to six potential transmembrane segments. The N and the C termini are less conserved, hydrophilic, and lysine rich (Fig. 2A).

Sur4p and Fen1p share an essential function and mediate the sensitivity to SR. *SUR4* and *FEN1* gene disruptions were obtained by replacing the entire coding sequences of *SUR4* and *FEN1* with *URA3* and *LEU2*, respectively. Wild-type strains were transformed with a linearized fragment containing either the *sur4*::*URA3* or the *fen1*::*LEU2* gene disruption to produce strains EMA24 and EMY27, respectively (Table 1). Southern blot and PCR analysis of genomic DNA confirmed that the gene replacement event had occurred at the correct locus in both cases (data not shown). The growth of EMA24 and EMY27 in rich medium was delayed by a factor of about 2 compared with the wt control. *SUR4* and *FEN1* gene disruptants were found to display the same level of SR resistance as the mutant strains do (Fig. 3A). The diploid cells obtained by crossing EMA24 (*sur4*::*URA3*) and EMY27 (*fen1*::*LEU2*) with the mutant strains EMY22 (*sre1-232*) and EMA62 (*sre2-237*), respectively, were SR resistant, as expected. These diploid strains yielded only rare spores and germination was very poor, impeding a clear-cut interpretation of allelism. The issue of the allelism of the cloned genes with *sre1-232* and *sre2-237*, respectively, was therefore examined in two different ways. First, the *sur4*::*URA3* and *fen1*::*LEU2* gene disruptions were introduced into the genomes of EMY22 (*sre1-232*) and EMA62 (*sre2-237*), respectively, using the gene replacement technique. The resulting disruptants were mated with a wt strain, and the progeny from each cross was analyzed. A dozen tetrads were obtained in each case, and they all displayed a regular 2 resistant:2 sensitive segregation pattern, as expected. Second, the *SUR4* and *FEN1* loci in EMA15 (*sre1-232*) and in EMA13 (*sre2-237*), respectively, were compared with the same loci in EMY30 (*SUR4 FEN1*) (see Table 1) by gene sequencing experiments. The *SUR4* or *FEN1* gene in the mutants differed from the respective wt counterpart by a single nucleotide



A

B

FIG. 2. Amino acid sequence alignment of the *SUR4* and the *FEN1* gene products (A) and mutational variation of *sre1-232* and *sre2-237* alleles (B). (A) Identical residues (|) and conservative replacements (:), as determined with the GAP software of the Genetics Computer Group package, are indicated. Seven gaps are represented by dots. The putative transmembrane domains of Sur4p are underlined by solid lines. The lysines in the C-terminal part of Sur4p and Fen1p are indicated by asterisks. A proline-rich domain in Fen1p is marked by dotted lines. (B) Differences in DNA and deduced amino acid sequences between *SUR4* and *sre1-232* and between *FEN1* and *sre2-237*. The EMBL accession numbers for the nucleotide sequences of *SUR4* (*SRE1*) (this work), *SUR4*, *APA1*, and *FEN1* (YCR521) are X82033, L28723, X78326, and S19446, respectively. Four differences were found between our *SUR4* sequence and that of *APA1* isolated from a different strain (13). These variations include two nucleotide changes, one of which, G for T at position 104 with respect to the 5′ end of the initiation codon, changes Glu-35 to Asp. Another variation, T for C at position 621, replaces Trp-208 with Arg. The two other variations are an insertion of T at 986 and a deletion of T at position 992, respectively, thus changing Ser-329–Thr-330 to Phe-Tyr. No differences were found between our sequence and that determined by Revardel et al. (33) on another *SUR4* clone obtained independently from the same library as ours.

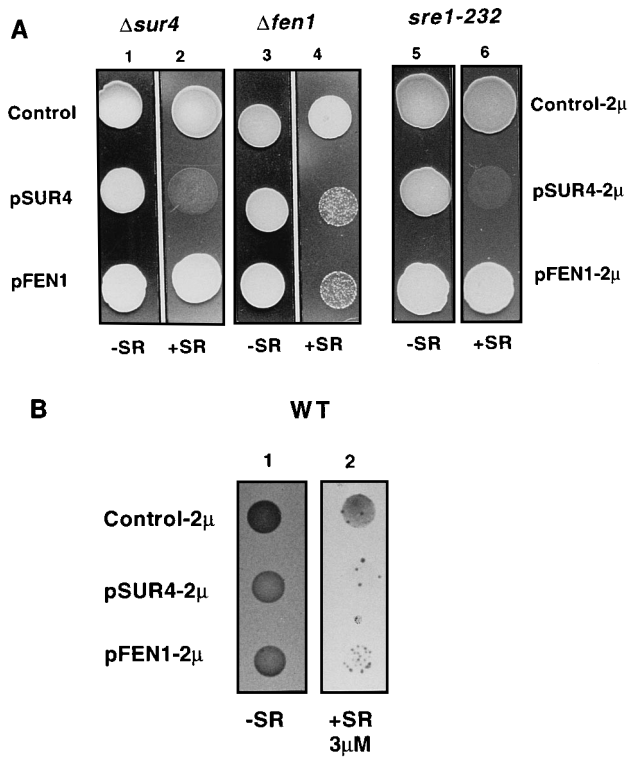


FIG. 3. SR resistance of various yeast strains, including *sur4* and *fen1* mutants (A) and a wt strain (B), transformed by *SUR4* and *FEN1* expression plasmids. (A) Plasmid DNAs used for transformation were as follows. Lanes 1 to 4, pUKC200 (Control) is the centromeric vector used as a negative control; pUKC232/60 (pSUR4) and pUKC237/T1 (pFEN1) are pUKC200 derivatives containing a wild-type yeast genomic insert that encompasses *SUR4* and *FEN1*, respectively. Lanes 5 and 6, pEMR950 (Control-2μ), pEMR1014 (pSUR4-2μ), and pEMR1055 (pFEN1-2μ). Recipient strains were EMA24 (Δ*sur4*), EMY27 (Δ*fen1*), and EMY22 (*sre1-232*). About 10^5 transformant cells were incubated for 48 h either in the absence (−SR) or in the presence of 50 μM SR (+SR) on solid minimal medium. (B) Plasmids used to transform EMY30 cells (Table 1) were pEMR950 (Control-2μ), pEMR1014 (pSUR4-2μ), and pEMR1055 (pFEN1-2μ). pEMR1014 and pEMR1055 were derived from pEMR950 by the insertion of *SUR4* and *FEN1*, respectively. Transformed cells were grown for 48 h in the presence or in the absence of 3 μM SR.

change, which abolished the *SUR4* start codon or resulted in a Met-152→Lys substitution in *FEN1*, respectively (Fig. 2B).

Analysis of the progeny from cross EMA24 × EMY27 confirmed that the double disruption of *SUR4* and *FEN1* was lethal (33). However, 4 of 96 colonies derived from this cross by random spore selection harbored both the *fen1* and the *sur4* disruptions. Further phenotypical analyses indicated that these colonies had retained a mating type and could not sporulate. PCR analysis of genomic DNA revealed that these double disruptants harbored an additional wt allele of either *SUR4* or *FEN1* together with the mutant alleles, suggesting that these strains were aneuploids. The exact roles of *fen1* and *sur4* disruptions in generating these aberrations remain to be clarified further. Multicopy vectors containing a wt allele of either *SUR4* or *FEN1* conferred SR hypersensitivity to transformed cells (Fig. 3B). SR sensitivity was restored in a *fen1* disruptant strain by the presence of a centromeric plasmid containing either *SUR4* or *FEN1* (Fig. 3A). In contrast, *FEN1* did not confer SR sensitivity to a *sur4* gene disruptant, even when cloned at a high copy number. Therefore, *FEN1* and *SUR4* are not completely interchangeable.

SR inhibits ergosterol biosynthesis at the Δ8-sterol isomerization step. The *sre2-237* gene is allelic to *fen1-1*, a mutation that specifically confers resistance to a large series of structurally unrelated sterol biosynthesis inhibitors. Azoles, such as ketoconazole, flusilazol, or LAB 170250, inhibit the C-14 lanosterol demethylase step, whereas the N-substituted morpholines, such as fenpropimorph, block Δ8-Δ7-sterol isomerase and Δ14-sterol reductase (25, 27). To determine whether the sterol biosynthesis pathway was impaired in SR-treated cells, membrane sterols were extracted from EMY30 (wt) cells grown in the presence of 3 μM SR and analyzed by GC-MS (Fig. 4A). Unusual sterols were detected instead of ergosterol, as expected. They were identified as ergosta-5,8,22-trien-3-β-ol (peak 2) and ergost-8(14)-en-3β-ol (peak 3). The same unusual sterols were found in an *erg2* mutant impaired in Δ8-Δ7-sterol isomerase (Fig. 4A). In the presence of increasing concentrations of SR, the ergosterol content of cells grown aerobically decreased in a dose-dependent manner (Fig. 4B and C). At 1.2×10^{-8} M SR, the ergosterol level was reduced approximately threefold (Fig. 4C). The level of the sterol corresponding to peak 3 increased concomitantly to reach a plateau at 3 μM SR, a concentration at which ergosterol was no longer detected. These results strongly suggested that SR inhibited sterol isomerase activity in *S. cerevisiae*. This conclusion was ascertained by assaying sterol isomerase in vitro in the presence and in the absence of SR. As expected, enzyme activity was inhibited in the presence of SR (Fig. 5).

If the proliferation arrest is the consequence of this inhibition, this effect should be relieved by ergosterol supplementation. Yeast wt cells do not take up ergosterol when grown aerobically (1). In contrast, when placed under strictly anaerobic conditions, as several steps of the post-squalene sterol biosynthesis require molecular oxygen, yeast cells need ergosterol supplementation for growth. As expected, SR exerted no inhibitory effect on cells that were growing anaerobically on ergosterol-containing medium (Fig. 6).

***ERG2* gene overexpression confers SR resistance.** To ascertain the role of sterol isomerase in SR sensitivity, we tested whether increasing the number of copies of the *ERG2* gene would alter the SR susceptibility phenotype in corresponding transformed cells. Since most drugs that inhibit Δ8-Δ7-sterol isomerase are known to inhibit sterol Δ14-sterol reductase too (27), we also checked the effect of increasing the gene number of the Δ14-sterol reductase-encoding gene, *ERG24* (26, 21). Multicopy plasmids containing either *ERG2* or *ERG24* were used to transform EMY30 cells, and the growth of each transformants was examined in the presence of either SR, fenpropimorph, or fenpropidin. Interestingly, multiple copies of *ERG2*, but not of *ERG24*, increased the level of SR resistance up to 21 μM SR (Fig. 7). Lai et al. (21) showed that overexpressing *ERG24*, but not *ERG2*, conferred fenpropimorph resistance in strain Y294. In our hands, overexpressing *ERG24* alone was sufficient to confer resistance to fenpropidin, as already observed by Marcireau et al. (26), but not to fenpropimorph. In contrast, overexpressing *ERG24* and *ERG2* concomitantly in the same cell conferred a slight fenpropimorph resistance. The functionality of our *ERG* genes was ascertained both by gene sequencing and by testing the ability of each gene to restore the corresponding defect of a mutant when cloned into a low-copy-number centromeric vector. No difference was found with sequences from databases except for silent variations (data not shown), and complementation was achieved in each case. Using an *ERG24* clone isolated by complementation from a conventional library in Karst's laboratory (26), we obtained the same results, confirming that overexpressing *ERG24* alone was not sufficient to confer any significant fenpropimorph resis-

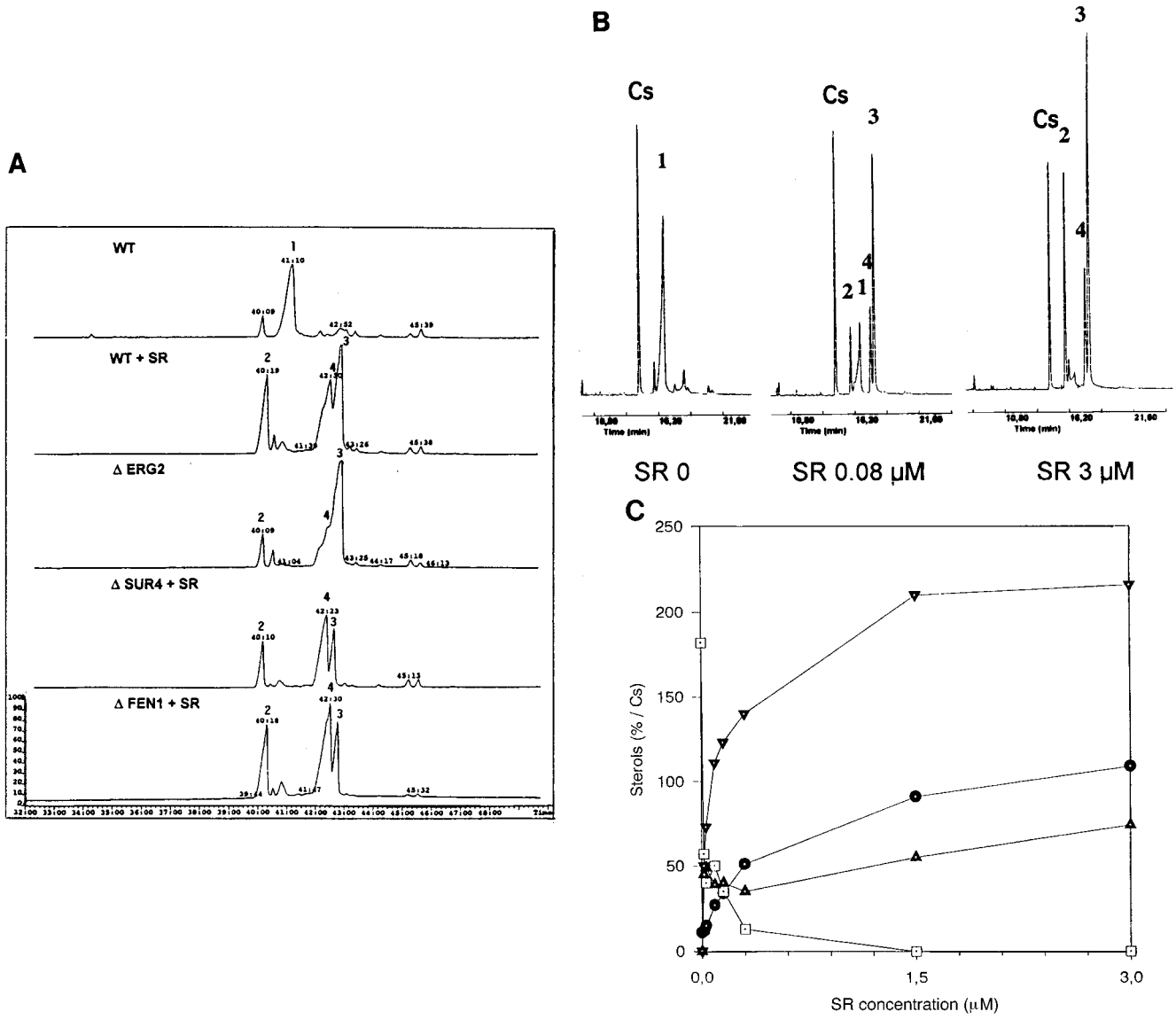


FIG. 4. GC-MS and GC analyses of membrane sterol extracted from various yeast strains. (A) Comparative GC-MS profiles of membrane sterols extracted from various yeast strains, including wild-type EMA3 cells grown in the absence (WT) or in the presence of 3 μM SR (WT + SR), an anaerobically grown *erg2* disruptant arrested by oxygen (ΔERG2), and *sur4* and *fen1* disruptants grown in the presence of 3 μM SR (ΔSUR4 + SR and ΔFEN1 + SR, respectively). The t_R and mass spectrum of peak 1 were identical with those of a commercial sample of ergosterol. Peaks 2, 3, and 4 in each assay were recognized by their mass spectra as ergosta-5,8,22-trien-3-ol (3β,22E), ergost-8(14)-enol, and ignosterol, respectively (not shown). (B and C) Sterols were extracted from EMA3 cells grown for 24 h in the presence of various concentrations of SR as indicated, separated by GC (B), and quantified (C). (B) Examples of GC profiles corresponding to three SR concentrations (0, 0.08, and 3 μM). Cs, cholesterol added as an internal standard; sterol 1, ergosterol; sterols 2 to 4, Δ8-sterols corresponding to peaks 2 to 4 in panel A. (C) Quantification of ergosterol (squares), sterol 2 (circles), sterol 3 (downward-pointing triangles), and sterol 4 (upward-pointing triangles). Amounts are expressed in arbitrary units in comparison with added cholesterol. One unit is equivalent to 1% of the cholesterol level.

tance. As fenpropimorph is known to inhibit both sterol isomerase and sterol reductase activities, a simple hypothesis for these apparently contradictory results was that sterol isomerase and sterol reductase were both essential in some strains, such as FL100, whereas other strains did not require sterol isomerase to proliferate in the absence of ergosterol.

The essentiality of the *ERG2* gene in aerobically growing cells is suppressed by deleting *FEN1* or *SUR4*. *erg2* gene disruptants were reported to be viable, even when placed under ergosterol starvation conditions in aerobiosis, suggesting that sterol isomerase was not essential in yeast cells (2). Since this conclusion was not supported by our results, the issue of the essentiality of *ERG2* in our strains was addressed by replacing most of the *ERG2* coding sequence by the *TRP1* selectable marker in the *trp1-4*/*trp1-4* diploid strain EMD7 (Table 1). Southern blot and PCR analyses confirmed that one copy of the *ERG2* gene was deleted in the resulting Trp^+ strain, EMD8 (data not shown). Tetrad analyses of anaerobically obtained EMD8 progeny revealed that all the single-spore colonies that required tryptophan for growth were ergosterol producers and viable in ergosterol-free medium under aerobic conditions, whereas the other single-spore colonies that were prototrophic for tryptophan produced no ergosterol and were nonviable in ergosterol-free medium (Fig. 8A). PCR analysis confirmed that the Trp^+ and Trp^- spores contained a disrupted allele and a wt allele, respectively, of the *ERG2* gene

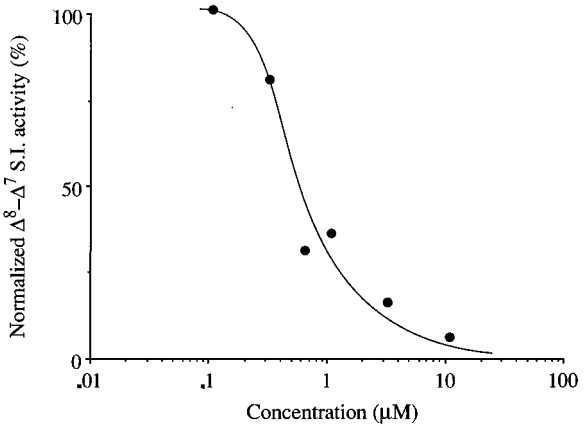


FIG. 5. Inhibition of Δ8-Δ7-sterol isomerase (Δ^8-Δ^7 S.I.) by SR. Inhibition is expressed in percent residual sterol isomerase activity as a function of SR concentration. Under these experimental conditions (see Materials and Methods), the SR concentration required to obtain 50% inhibition is 0.6 μM.

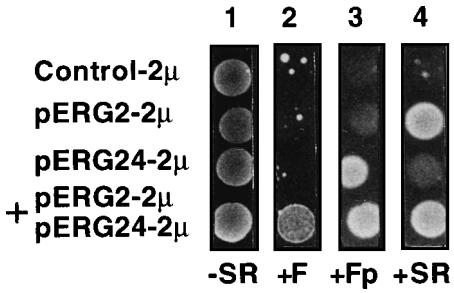


FIG. 7. Aerobic growth of transformants overexpressing *ERG2*, *ERG24*, or both genes in the presence of various drugs. Plasmids used to transform EMY30 cells were as follows. pEMR1023 (Control-2μ) is a 2μm-derived multicopy plasmid containing *URA3* as the selectable marker; pEMR1050 (pERG24-2μ) and pEMR1056 (pERG2-2μ) were derived from pEMR1023 by the insertion of the *ERG24* coding sequence and of the *ERG2* gene, respectively. In pEMR1050, the *ERG24* coding sequence was placed under the control of the *PGK* promoter. In pEMR1056, the cloned *ERG2* sequence included the promoter. *URA3* was replaced by *TRP1* in pEMR1056 so as to enable cell cotransformation with pEMR1050 and pEMR1056 DNA. Transformant cells were grown in the absence of drug (−SR) or in the presence of 21 μM SR (+SR), 3 μM fenpropimorph (+F), or 3 μM fenpropidin (+Fp).

(data not shown). Thus, our experiments indicated that *ERG2* was an essential gene at least in the FL100 genetic background. Haploid strains that harbored disrupted alleles of both *erg2* and *fen1* or *sur4* genes were constructed. Such strains were found to be able to proliferate under ergosterol starvation conditions, indicating that in the absence of Fen1p or Sur4p, the *ERG2* gene was no longer essential (Fig. 8B). Ergosterol contents of *fen1* gene and *sur4* gene disruptants were found to be similar to that of the wt strain (i.e., about 50 μg/100 mg [dry weight]). Furthermore, the aberrant sterols found in the *erg2* mutant were also produced by SR-treated *sur4* and *fen1* disruptants (Fig. 4A). These results indicated that no drastic change in the sterol metabolism could account for the SR resistance phenotype of these disruptants.

Ashman et al. (2) showed that the disruption of the *ERG2* gene was not lethal in strains derived from WA10 by sporulation, even under ergosterol starvation conditions. To determine whether such *erg2* gene disruptants might harbor a lethality suppressor gene, we analyzed six tetrads derived from a cross between one of our *erg2*::*TRP1* gene disruptants and WA10-3-1D, an *erg2*::*LEU2* gene disruptant derived from WA10 (2). Of the 12 *erg2*::*LEU2* gene disruptants obtained in the progeny, 6 were viable and 6 were not, suggesting that viability was due to a single suppressor allele unlinked to the *ERG2* locus. Moreover, four tetrads displayed a regular 2 viable:2 nonviable segregation pattern, which corroborated that the difference in viability phenotype could be due to a single pair of alleles. However, the two remaining tetrads were made of three viable *erg2* strains, one of which grew very slowly, and the fourth was nonviable. This kind of result, which could be expected since the two parent strains were not genetically related one to the other, might simply indicate that the *ERG2* lethality phenotype could be corrected by certain genetic combinations, even in the absence of any specific suppressor gene.

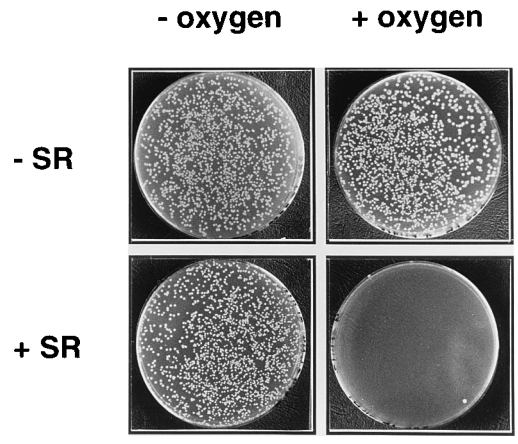


FIG. 6. Growth of EMY30 cells in the presence or in the absence of 50 μM SR (+ SR or − SR, respectively) and in the absence or in the presence of oxygen (− oxygen or + oxygen, respectively). Five hundred to 1,000 aerobically grown EMY30 cells (Table 1) were plated onto rich medium supplemented with ergosterol and Tween 80. Plates were incubated at 30°C for 72 h.

DISCUSSION

In contrast to CsA or FK506 but like rapamycin, SR interferes with a late event in the interleukin-2-dependent activation of lymphocytes (7). However, the targets of both SR and rapamycin are clearly distinct. Our results strongly suggest that SR blocks cell proliferation by inhibiting sterol isomerase. This hypothesis is supported by three points: (i) sterol isomerase activity is inhibited by SR both in vivo and in vitro, and sterol isomerase is essential for aerobic growth; (ii) overexpressing *ERG2*, the sterol isomerase-encoding gene, confers SR resistance; and (iii) cells are insensitive to SR when grown anaerobically in the presence of ergosterol, conditions under which sterol isomerase is not required for proliferation. It is worth noting that although SR is not structurally related to the N-substituted morpholine fungicides, it nevertheless shows some electrochemical and steric similarities with fenpropimorph. The mechanism of isomerization is likely to involve protonation at C-9 on the α face of the sterol nucleus, resulting in a high energy intermediate with a carbonium at C-8. This carbonium ion is then stabilized by the loss of a proton at C-7 (37). It has been proposed that inhibitors of the morpholine family are protonated at physiological pH and thus can mimic this high-energy reaction intermediate (37, 38). Similarly, the protonated form of the tertiary amine function of SR could interact with the active-site domain that stabilizes this putative C-8 carbonium intermediate.

Ashman et al. (2) showed that the disruption of the *ERG2*

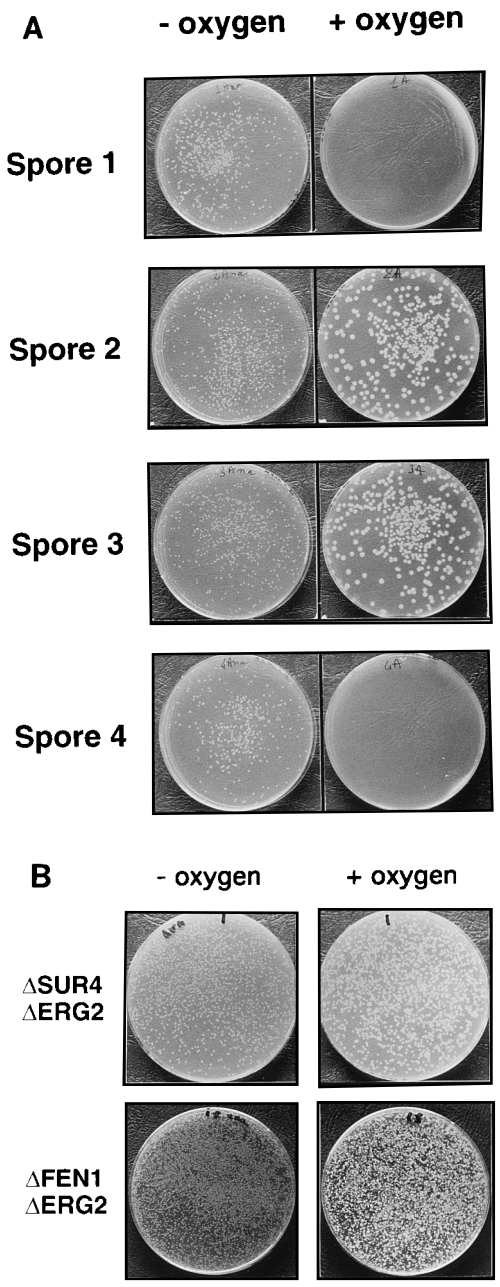


FIG. 8. Growth of an *erg2* gene disruptant (A) and *erg2 sur4 or erg2 fen1* double disruptants (B) under anaerobic or aerobic conditions. (A) EMD8 cells (Table 1) were sporulated, tetrads were dissected, and spores were germinated under anaerobic conditions. Each one-spore colony was maintained and characterized under anaerobic conditions. About 200 anaerobically grown cells from each one-spore colony were plated onto rich medium supplemented with ergosterol and Tween 80. Plates were incubated at 30°C. The tryptophan-requiring Erg^+ cells (spores 2 and 3) form colonies in the presence of oxygen, whereas the *erg2* gene disruptants do not (spores 1 and 4). Similar results were obtained with the nine other tetrads analyzed, confirming that the *erg2* gene disruptants were not viable under aerobic conditions. (B) Diploid cells derived from EMY43 (*erg2*::*TRP1*) mated either with EMA40 (*sur4*::*URA3*) or with EMA41 (*fen1*::*LEU2*) were sporulated, and spores were germinated and phenotypically characterized under aerobic and anaerobic conditions. About 1,000 anaerobically grown cells of the *erg2 sur4* or *erg2 fen1* double disruptants were plated onto rich medium supplemented with ergosterol and Tween 80 and incubated in the presence or the absence of oxygen.

gene was not lethal in WA10-derived strains, even under ergosterol starvation conditions. We have found that deleting the *ERG2* coding sequence confers ergosterol auxotrophy in FL100 congenic strains. By crossing the *ERG2* gene disruptant of Ashman et al. with ours, we have confirmed that even their *ERG2* gene disruption could be lethal under different genetic backgrounds in aerobically growing cells. In these strains that require the *ERG2* gene function to proliferate, increasing the *ERG24* gene copy number is not sufficient to confer fenpropimorph resistance. In contrast, overexpressing *ERG2* is sufficient to confer SR resistance to these cells, which confirms that sterol isomerase inhibition is the main cause for the proliferation arrest. The reasons why sterol isomerase could be essential for proliferation are not clear. In an *erg2* mutant, as well as in SR-treated wt cells, unusual sterols displaying a Δ8 unsaturation in the B ring are accumulated instead of ergosterol. These sterols might exert a toxic effect by altering the membrane fluidity and physiology (32, 40). Ergosterol at a nanomolar level has been suggested to play a role in controlling exit from the G_1 phase, also known as the sterol sparking effect (9, 24, 31). However, microscopic observation of SR-arrested cells did not reveal any accumulation of unbudded cells, which indicated that SR did not specifically impede exit from the G_1 phase of the cell cycle. This observation was confirmed by FACScan analyses of the DNA content, which indicated no accumulation of cells harboring only 1*n* content of DNA. Therefore, the sterol sparking theory cannot fully explain the SR effect. It is nevertheless worth noting that although Δ8-sterols may be accumulated, proliferation is maintained as long as SR-treated cells are producing detectable amounts of ergosterol. Thus, the proliferation arrest could be due to a specific ergosterol requirement that is not fulfilled by Δ8-sterols.

Mutations in *SUR4* or *FEN1* suppress the lethality of the *erg2* gene disruption. Similarly, Ladevèze et al. (20) have shown that the *fen1-1* mutation suppresses the lethal effect of the *erg24* gene disruption. In the absence of either Fen1p or Sur4p, ergosterol depletion does not arrest proliferation; consequently, *sur4* and *fen1* mutants resist fenpropimorph, SR, and other structurally unrelated sterol biosynthesis inhibitors as well as the lethal effect of the *erg2* or *erg24* gene disruption. Mutations in *SUR4* or *FEN1* induce pleiotropic phenotypes, including abnormal phospholipid composition (10, 33), aberrant budding behavior (12), the suppression of the reduced viability upon starvation phenotype of mutants impaired in an actin-binding protein (12, 35), and a transcriptional downregulation of the gene that encodes plasma membrane H^+-ATPase (13). *FEN1* and *SUR4* gene products thus appear to play a key role in controlling membrane and cytoskeletal functions. It is tempting to speculate that ergosterol, or a metabolic derivative of this sterol, is required as a signal to promote cytoskeleton rearrangements and related membrane movements. In the absence of ergosterol, both gene products would impose a cell proliferation arrest. In contrast, when any of these proteins is lacking, the ergosterol signal would no longer be required. Overproducing any of these proteins results in SR hypersensitivity. If our model is correct, one explanation for this higher SR susceptibility could be that Fen1p and Sur4p, when they are overproduced, require higher concentrations of ergosterol to transduce a signal essential for proliferation. This model is currently under investigation.

CsA, FK506, and rapamycin are inhibitors of peptidylprolyl *cis-trans* isomerases. Although ubiquitous, these isomerases nevertheless mediate the immunosuppressive properties of these drugs by forming complexes that result in the inhibition of other enzymes specifically required for T-cell proliferation, e.g., calcineurin and phosphatidylinositol kinases (19). We

have found that SR is an inhibitor of sterol isomerase in yeast cells. As such, SR provokes a proliferation arrest signal in strains such as FL100 in which *ERG2* is an essential gene for aerobic proliferation. Experiments are in progress to determine whether sterol isomerase is indeed inhibited by SR in mammalian cells. If so, it will be of interest to determine whether the inhibition of sterol isomerase by SR is the cause of immunosuppression or whether this enzyme is an immunophilin of a new kind. Su (36) pointed to a nonidentified steroid as an endogenous ligand of the sigma receptors. It is worth noting that this steroid ligand could also belong to the sterol family. Especially if SR, an atypical sigma ligand, appears to inhibit sterol isomerase in mammalian cells as it does in yeast cells, the issue of a possible relationship between the sigma receptors and the post-squalene sterol biosynthesis enzymes will have to be addressed.

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