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The *Saccharomyces cerevisiae SGE1* gene product: a novel drug-resistance protein within the major facilitator superfamily

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Abstract Several pleiotropic drug sensitivities have been described in yeast. Some involve the loss of putative drug efflux pumps analogous to mammalian P-glycoproteins, others are caused by defects in sterol synthesis resulting in higher plasma membrane permeability. We have constructed a *Saccharomyces cerevisiae* strain that exhibits a strong crystal violet-sensitive phenotype. By selecting cells of the supersensitive strain for normal sensitivity after transformation with a wild-type yeast genomic library, a complementing 10-kb DNA fragment was isolated, a 3.4-kb subfragment of which was sufficient for complementation. DNA sequence analysis revealed that the complementing fragment comprised the recently sequenced *SGE1* gene, a partial multicopy suppressor of *gal11* mutations. The supersensitive strain was found to be a *sge1* null mutant. Overexpression of *SGE1* on a high-copy-number plasmid increased the resistance of the supersensitive strain. Disruption of *SGE1* in a wild-type strain increased the sensitivity of the strain. These features of the *SGE1* phenotype, as well as sequence homologies of *SGE1* at the amino acid level, confirm that the Sge1 protein is a member of the drug-resistance protein family within the major facilitator superfamily (MFS).

Key words Drug sensitivity · *Saccharomyces cerevisiae* Major facilitator superfamily · Drug expulsion

Introduction

Drug sensitivity in the yeast *Saccharomyces cerevisiae* is influenced by various factors. In order to reach the interior of the cell, molecules have to pass two barriers: the cell wall and the plasmalemma, a limiting membrane between the cell wall and the cytosol. The cell wall has been pictured as an open porous structure (De-Nobel and Barnett 1991), through which compounds can diffuse, depending on their size. For stationary phase yeast cells, the average cell wall pore has been estimated to be 0.89 nm in size, enabling the passage of molecules of up to 760 Da (Scherrer et al. 1974). In an assay with exponentially growing yeast cells, the cell wall permeability threshold was determined to correspond to a globular protein with a molecular weight of 400 kDa (De-Nobel et al. 1990). The majority of drugs do not exceed this size and therefore are thought to be able to pass through the cell wall. A porin protein, such as has been found in bacteria to render the outer plasma membrane permeable for hydrophilic molecules, has so far not been identified in yeast (De-Nobel and Barnett 1991).

In contrast to the cell wall, the plasma membrane represents a permeability barrier for substances present in the growth medium. Data suggest that alterations in the plasma membrane structure may profoundly change the drug sensitivity of yeast. For instance, the mutant pleiotropic drug resistance phenotype of *pdr1* mutants has been associated with reduced plasma membrane permeability (Rank et al. 1975), suggesting exclusion of various test compounds from their intracellular targets. Also, the *ise1* mutation confers increased sensitivity towards a variety of structurally and functionally unrelated inhibitors (Winsor et al. 1987; Nitiss and Wang 1988). *ISE1* has been shown to be allelic to *ERG6* (Graham et al. 1993). Since *erg6* mutants are unable to synthesize ergosterol, a major membrane sterol in yeast (McCammon et al. 1984), they possess an altered membrane structure that leads to a higher permeability of the plasma membrane (Bard et al. 1978).

Expulsion of inhibitors from within the cell may also influence drug sensitivity in yeast cells. In mammalian cell lines, the well-described multiple drug resistance phenomenon has been associated with a more efficient, energy-dependent export of inhibitors (Gottesman and Pastan 1988). In yeast, the *STE6* gene has been found to

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Table 1 *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Source
S288C	α, *gal2*	A. Hinnen
JN127	**a**, *ade1-2, ISE2, ura1, his7-n, lys2, tyr1*	J.C. Wang
YHE1	α, *ade2-40, ilv1-92, trp5-a, ura3Δ5*	This laboratory
YHE4	**a**, *ade2-119, ilv1-92, trp5-b, ura3Δ5*	This laboratory
Ito1A	α, *ade2-40, ilv1-92, trp5-a*	F. Zimmermann
JRY2302	**a/a**, *ura3-52/ura3-52, his3Δ200/his3Δ200, ade2-101/ade2-101, lys2-801/lys2-801*, Met^-/Met^+, Tyr^-/Tyr^+	J. Rine
YAE65	α, *ade2-119, ilv1-92, trp5-b, sge1, ura3Δ5*	This study
YS18	α, *his3-11, his3-15, leu2-3, leu2-112, can*^{r}, *ura3Δ5*	(Sengstag and Hinnen 1987)
JRY254	**a**, *lys1-1*	J. Rine

encode a homologue of the mammalian multidrug resistance P-glycoprotein (McGrath and Varshavsky 1989). Interestingly, the absence of the gene product does not significantly increase the drug sensitivity of the cell (Kuchler et al. 1989). A network of genes has been identified, though, which are involved in cross-resistance to various drugs. As in the mammalian situation, overexpression of the pleiotropic drug resistance gene *PDR5* increases resistance of the yeast cell to cycloheximide and sulphometuron methyl (Leppert et al. 1990). Conversely, disruption of this locus results in higher sensitivity of the strain to a number of drugs (Meyers et al. 1992).

Other multiple drug resistances have also been described in yeast. Mutations in the plasma membrane ATPase *PMA1* gene give rise to multiply drug resistant strains (Ulaszewski et al. 1987); however, they exhibit a phenotype quite different from the *pdr* resistances. In a different approach, the genes *SNQ1*, *SNQ2* and *SNQ3* have been identified, which, on multicopy plasmids, confer a hyper-resistance phenotype. Disruption of these loci results in sensitivity to various mutagens (Haase et al. 1992).

The availability of drug-sensitive yeast mutants is of major importance in the field of genotoxicity testing. Although yeast has been proven as a promising test organism for the detection of a large range of genetic events (Zimmermann et al. 1975), wide use of *S. cerevisiae* as a standard genotoxicity test system has so far been hampered by its poor sensitivity compared to other test organisms, presumably due to low general permeabiliy of the cells (Morita and Yanagihara 1985). Various chemical, physical and enzymatic methods have been described to permeabilize yeast cells (Felix 1982); however these methods are not appropriate for use in tests, as special treatment of the cells is required and decreases the viability of the cells. Therefore, the use of sensitive mutants is more favourable. Here we report the construction of a drug-sensitive yeast strain and cloning and sequencing of the gene which complements the sensitivity. It is shown to be identical to the recently sequenced *SGE1* gene, a partial multicopy suppressor of *gal11* mutations (Amakasu et al. 1993).

Materials and methods

Yeast and bacterial strains

The genotypes of the *S. cerevisiae* strains used in this study are shown in Table 1. For strain constructions, standard yeast genetic methods were used (Guthrie and Fink 1991). Yeast transformations were performed as described (Klebe et al. 1983). Bacterial strains DH5αF' and XL1-blue™ (Stratagene) were used for propagation of recombinant plasmids.

Media and culture conditions

Strains were grown in standard media. Complete medium (YPD) contained 1% yeast extract, 2% bactopeptone and 2% glucose. Minimal medium YM [0.67% yeast nitrogen base without amino acids (Difco), 2% glucose] was appropriately supplemented with amino acids as described (Sherman 1991). Sporulation medium contained 0.3% potassium acetate and 0.02% raffinose. *Escherichia coli* was grown in LB medium supplemented with 150 μg/ml ampicillin when appropriate.

Drug sensitivity assay

Strains were pregrown in liquid medium to late log phase. Cultures were diluted to about 3×10^6 cells/ml (absorbance at 600 nm of 0.3). Five microlitres of these cell suspensions and of 1:10, 1:100 and 1:1000 dilutions were placed on YPD or supplemented YM agar either lacking or containing various concentrations of crystal violet (Merck). Plates were incubated for 2 to 3 days at 30° C.

Plasmid constructions

pAE9b was obtained from a yeast genomic library of S288C constructed in pCS19 (Sengstag and Hinnen 1987). pAE281 and pAE292 were constructed by ligating a 3-kb *Xho*I and a 3.4-kb *Cla*I fragment of pAE9b in the *Xho*I and *Cla*I sites of pRS316 (Sikorski and Hieter 1989), respectively. pAE440 is a pRS316 derivative and contains a 7-kb *Sma*I-*Xho*I fragment of pAE9b. pAE260 was obtained by deleting a 2.6-kb *Sac*I fragment from a YEp352 (Hill et al. 1986) derivative containing a 9-kb *Pst*I fragment of pAE9b. To obtain pAE321, a 3.4-kb *Sal*I-*Sma*I fragment of pAE292 was inserted into the pBR322 (Peden 1983) *Sal*I/*Eco*RV sites; subsequently, a 1.6-kb *Xba*I-*Nco*I fragment was replaced by a 1.1-kb blunt-ended *Hin*dIII fragment containing the *URA3* gene. For pAE378, the 2.9-kb *Cla*I fragment of pAE321 was inserted in the *Cla*I site of pRS313 (Sikorski and Hieter 1989).

DNA sequence analysis

Two *Cla*I-*Eco*RI segments and one *Eco*RI fragment of plasmid pAE292 were subcloned in pBluescript KS and pBluescript SK

(Stratagene). With the *Cla*I/*Eco*RI subclones, a series of deletions were created using exonuclease III and S1 nuclease (Ausubel et al. 1990). DNA sequence analysis was performed on single-stranded DNA (Sambrook et al. 1989) by the dideoxy chain-termination method (Sanger et al. 1977) using the Sequenase version 2.0 DNA Sequencing kit (U.S. Biochemical Corporation) according to the manufacturer's instructions. [α-^{35}S]dATP was purchased from DuPont.

Southern analysis

Total DNA was isolated from yeast cells (Hoffman and Winston 1987); 500 ng DNA was digested with *Sac*I and *Cla*I, and the DNA fragments were separated on a 0.8% agarose gel. Transfer to Zeta-Probe GT charged nylon membrane (Biorad) and hybridization were performed according to the supplier's recommendations. Radioactive probes were obtained by use of the Random Primed DNA labelling kit from Boehringer, Mannheim. [α-^{32}P]dGTP was obtained from DuPont.

Results

Construction of a crystal violet supersensitive, transformable yeast strain

In order to obtain a hypersensitive yeast tester strain for genotoxicity assays, we have attempted to make use of the previously described *ISE2* mutation. An *ISE2* strain of *S. cerevisiae*, JN127 (Nitiss and Wang 1988), has been shown to exhibit a pleiotropic drug-sensitive phenotype. The strain displays increased sensitivity to 4'-(9-acridinylamino)-methanesulphon-*m*-anisidide (mAMSA), aphidicolin, camptothecin and cycloheximide, presumably due to enhanced drug permeability. We have tested the effect of various crystal violet concentrations on the *ISE2* mutant. A stationary culture of the strain was diluted to 3×10^6 cells/ml, and aliquots of serial dilutions were placed on complete medium either lacking or containing the drug. Plates were incubated for 2 days at 30° C (Fig. 1). In contrast to the normally sensitive strain YHE4, JN127 showed a severe growth defect on crystal violet concentrations as low as 0.3 μg/ml. Therefore, the crystal violet sensitivity of JN127 can be regarded as a further characteristic of the *ISE2* mutation and was used as a phenotypic marker during the following strain constructions.

With the aim of introducing the supersensitivity-conferring gene *ISE2* into the background of our tester strains, JN127 was crossed to haploid strain YHE1. However, the resulting JN127 × YHE1 diploid failed to sporulate. Since such lack of sporulation may be related to an abnormal ploidy level, we hypothesized that JN127 might have become homodiploid as a consequence of its previous exposure to ethyl methanesulphonate (Nitiss and Wang 1988). In order to test this possibility, JN127 was crossed to a homodiploid strain (JRY2302) of the opposite mating type, and presumptive tetraploids were selected phenotypically. Subjecting the resulting strain to sporulation conditions indeed resulted in asci containing predominantly four viable

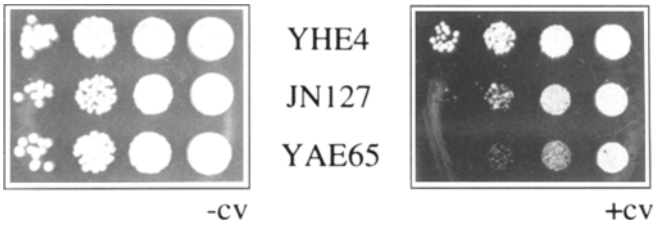

Fig. 1 Crystal violet sensitivity of *Saccharomyces cerevisiae* strains YHE4, JN127 and YAE65. Cell density of the strains pregrown in liquid complete medium was adjusted to 3×10^6 cells/ml; 5 μl of the cell suspensions and of 1:10, 1:100 and 1:1000 dilutions were placed on complete medium (−cv) and on complete medium containing 0.3 μg/ml crystal violet (+cv). Plates were incubated at 30° C for 2 days

spores. This result strongly argues for our assumption regarding the ploidy of JN127. Presumptive diploid spores were then dissected by micromanipulation, and a clone was chosen that exhibited the crystal violet supersensitive phenotype and which did not mate with either an **a** or and α tester strain. This putative diploid was subjected to another round of sporulation, and a supersensitive haploid of mating type α was selected. The strain was then crossed to YHE4, and the resulting diploid was tested for crystal violet sensitivity. Unexpectedly, it showed no enhanced crystal violet sensitivity, which suggested that increased sensitivity is a recessive rather than a dominant trait (data not shown). A crystal violet- supersensitive haploid from this cross was chosen, and two backcrosses to strain YHE4 were performed to obtain strain YAE65. Apart from containing the *ura3Δ5* marker and the genotoxicity markers *ade2-119*, *ilv1-92* and *trp5-b*, this strain exhibits a severe growth defect on plates containing 0.3 μg/ml crystal violet (Fig. 1). Since transformation of YAE65 with a *URA3*-containing plasmid was successful, this strain was chosen for cloning of the wild-type counterpart of the crystal violet supersensitivity-conferring gene.

Cloning of a crystal violet supersensitivity-complementing gene

Supersensitive strain YAE65 was transformed to uracil prototrophy with a genomic library of wild-type strain S288C constructed in the centromere-based *E. coli*/*S. cerevisiae* shuttle vector pCS19 (Sengstag and Hinnen 1987; Sengstag 1993). Transformants were pooled and subsequently spread on selective medium supplemented with 0.3 μg/ml crystal violet. Six out of a total of 13000 transformants grew that showed the normal level of sensitivity to the dye. The normal sensitive phenotype of four of the transformants was reconfirmed in a drug sensitivity assay (see the Materials and methods). Upon amplification in *E. coli* of the plasmids rescued from these transformants, and retransformation into YAE65, isolate pAE9b was found to reconstitute normal levels of sensitivity on the crystal violet-supersensitive strain.

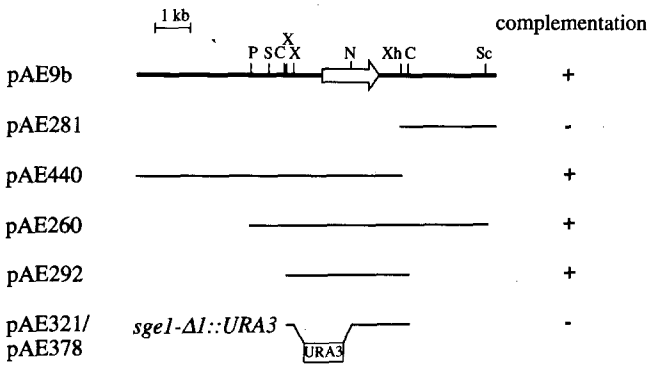

Fig. 2 Maps of plasmids complementing the crystal violet sensitivity. The *upper line* represents the yeast DNA insert of the original pCS19 gene library plasmid that complements crystal violet supersensitivity of strain YAE65. Only selected restriction sites are shown above the line. The *arrow* indicates length and direction of the *SGE1* open reading frame. The lines *below* represent fragments contained in constructed subclones. Plasmids pAE281, 292 and 440 are pRS316 derivatives. Plasmids pAE378, 260 and 321 are derived from pRS313, YEp352 and pBR322, respectively. *Plus* or *minus* symbols to the right identify those plasmids which were able or unable to complement the crystal violet supersensitivity of YAE65, respectively. Restriction site abbreviations: C, *Cla*I; N, *Nco*I; P, *Pst*I; S, *Sal*I; Sc, *Sac*I; X, *Xba*I; Xh, *Xho*I

Analysis of various subclones of pAE9b (Fig. 2) revealed that the complementing activity was confined to a 3.4-kb *Cla*I fragment.

The complementing 3.4-kb *Cla*I fragment comprises the *SGE1* gene

Figure 3 depicts the extent of DNA sequence determination on the 3407-bp *Cla*I fragment. The complete sequence of both DNA strands was determined. For sequencing purposes, subfragments were cloned into pBluescript KS and pBluescript SK. For large subfragments, exonuclease III was used to generate unidirectional nested deletions enabling sequencing of internal DNA stretches. Single-stranded DNA of the subclones was prepared (Sambrook et al. 1989), which was sequenced according to the dideoxy chain-termination method (Sanger et al. 1977). An open reading frame extending over 1629 nucleotides was identified. In the putative gene, many codons occur which are rarely used in *S. cerevisiae*, suggesting it to be a gene that is expressed at a low level in yeast. The codon bias index (CBI; Bennetzen and Hall 1982), which has a value of 1 if only the 22 preferred codons are used, and a value of 0 if all codons are used randomly, was calculated to be − 0.041. The nucleic acid sequence codes for a predicted protein of 543 amino acids (Fig. 4) with a calculated molecular weight of 59425. Considering Ala, Leu, Ile, Val, Pro, Phe, Tyr and Met as hydrophobic residues (Lehninger 1977), the protein consists of 52% hydrophobic amino acids. It has one putative N-glycosylation site at amino acid position 183, which lies within a hydrophilic stretch.

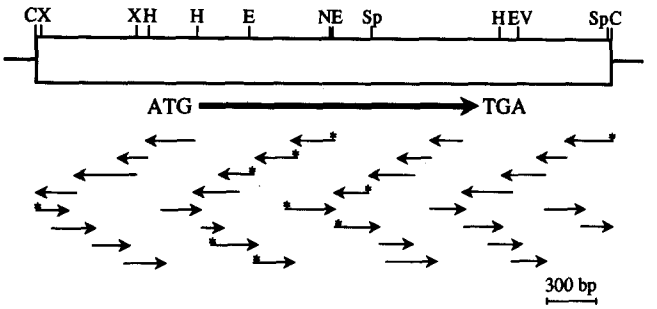

Fig. 3 Restriction map and sequencing strategy of the supersensitivity complementing *Cla*I fragment of pAE292. Selected restriction sites are indicated. The position and length of the *SGE1* open reading frame is given by the *bold arrow*. *Thin arrows* show the extent of DNA sequence determination: *starred arrows* show subclone-derived DNA sequences; all others show DNA sequences from exonuclease III-derived clones. Restriction site abbreviations: C, *Cla*I; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; N, *Nco*I; Sp, *Spe*I; X, *Xba*I

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  1  MKSTLSLTLCVISLLLTLFLAALDIVIVVTLYDTIGIKFH
 41  DFGNIGWLVTGYALSNAVFMLLWGRLAEILGTKECLMISV
 81  IVFEIGSLISALSNSMATLISGRVVAGFGGSGIESLAFVV
121  GTSIVRENHRGIMITALAISYVIAEGVGPFIGGAFNEHLS
161  WRWCFYINLPIGAFAFIILAFCNTSGEPHQKMWLPSKIKK
201  IMNYDYGELLKASFWKNTFEVLVFKLDMVGIILSSAGFTL
241  LMLGLSFGGNNFPWNSGIIICFFTVGPILLLLFCAYDFHF
281  LSLSGLHYDNKRIKPLLTWNIASNCGIFTSSITGFLSCFA
321  YELQSAYLVQLYQLVFKKKPTLASIHLWELSIPAMIATMA
361  IAYLNSKYGIIKPAIVFGVLCGIVGSGLFTLINGELSQSI
401  GYSILPGIAFGSIFQATLLSSQVQITSDDPDFQNKFIEVT
441  AFNSFAKSLGFAFGGNMGAMIFTASLKNQMRSSQLNIPQF
481  TSVETLLAYSTEHYDGPQSSLSKFINTAIHDVFYCALGCY
521  ALSFFFGIFTSSKKTTISAKKQQ  543
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Fig. 4 The deduced amino acid sequence of *SGE1*. Single-letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp and Y, Tyr

Shortly after completion of the sequence determination, an internal 1954 bp stretch was found to be identical to the recently published *S. cerevisiae SGE1* gene sequence (Amakasu et al. 1993). Minor discrepancies between this sequence and the one determined by us were found in the 5' and 3' untranslated regions of the gene. At positions 779 and 780 of the sequence were two thymidine residues compared to two cytidines of Amakasu et al. (1993). At positions 855–858, we report the sequence GCGC instead of GGCC. Furthermore, we found three instead of four adenosine residues at positions 2618–2620. The nucleotide sequence 1–3407

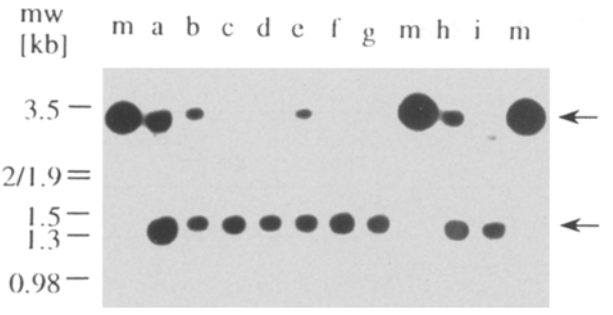

Fig. 5 Detection of chromosomal homologues of the crystal violet complementing *Cla*I fragment of pAE292 in various *S. cerevisiae* strains. Total DNA was double-digested with *Cla*I and *Sal*I to liberate a 3.4-kb *SGE1* containing fragment (*upper arrow*) and a 1.4-kb *Sal*I/*Cla*I *LEU2* fragment (*lower arrow*) as a control. Southern analysis was performed according to the Materials and methods. The 3.4-kb *Cla*I fragment of pAE292 and a 1.4-kb *Ssp*I *LEU2* fragment were used as probes. Lanes a, S288C; b, JN127; c, YAE65; d, YHE4; e, JRY2302; f, YHE1; g, Ito1A; h, YS18; i, YS18 *sge1-Δ1::URA3*; m, 0.3 ng *Cla*I digested pAE292; mw, molecular weight standard (sizes are given in kb). In strains YAE65, YHE4, YHE1 and Ito1A, the 3.4-kb *Cla*I fragment is absent. YS18 *sge1-Δ1::URA3* DNA contains a hybridizing 2.9-kb *Cla*I band which corresponds to the size of the *Cla*I fragment in the disruption construct pAE321 and confirms correct disruption of *SGE1*

has been deposited in the GenBank data bank (Bilofsky and Burks 1988; accession no. U02077).

Gene disruption of *SGE1* in a wild-type strain confers crystal violet sensitivity

To determine the effect of the loss of *SGE1*, the gene was disrupted in *S. cerevisiae* strain YS18. For this purpose, plasmid pAE321 was constructed, in which an internal 1.6-kb *Xba*I-*Nco*I fragment of *SGE1* is replaced by *URA3* (Fig. 2). The *Cla*I fragment containing the disrupted allele was cloned into the centromere-based vector pRS313 to give pAE378. Strain YAE65 transformed with pAE378 was unable to grow on crystal violet-containing medium (data not shown), demonstrating that the disruption abolished *SGE1* gene function. The wild-type strain YS18 was then transformed with the isolated 2.9-kb *Cla*I fragment of pAE321. A subsequent Southern analysis of the Ura^+ transformants was performed to ensure correct disruption of the *SGE1* locus (Fig. 5, lanes h and i). The *sge1-Δ1::URA3* mutant exhibited a slight growth disadvantage on drug-free medium compared to the *SGE1* parental strain YS18 (Fig. 6A). However, at a crystal violet concentration of 0.8 μg/ml, the disrupted strain clearly exhibited a crystal violet-sensitive phenotype, whereas growth of strain YS18 was not impaired. This result demonstrates that inactivation of the *SGE1* function in a wild-type strain increases its crystal violet sensitivity. The sensitivity of the *sge1-Δ1::URA3* disruptant, however, was not as pronounced as the sensitivity of YAE65, indicating that (an) other mutation(s) in YAE65 might contribute to its sensitivity phenotype.

YAE65 is a *sge1* null mutant

In an attempt to characterize the *sge1* mutation in YAE65, Southern analysis of YAE65 DNA was performed. No band hybridizing to the 3.4-kb complementing *Cla*I fragment from pAE9b was detected. In addition, the *Cla*I fragment was shown to be missing in

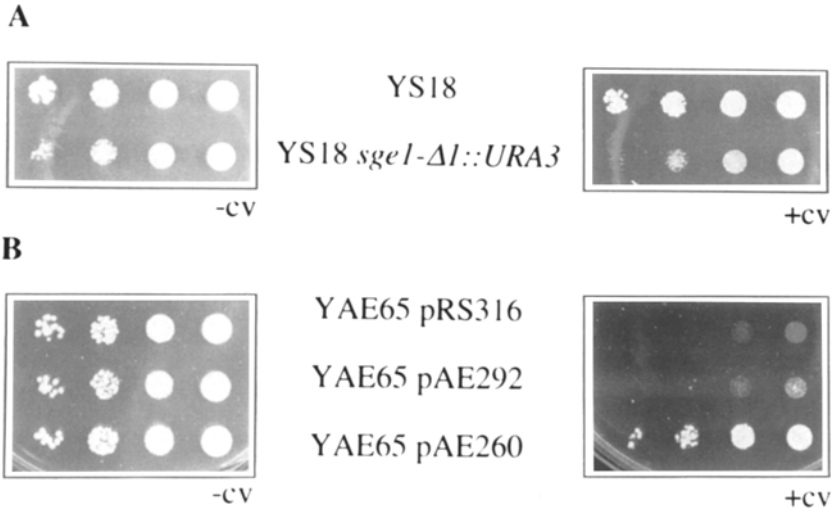

Fig. 6 **A** Effect of *sge1* disruption on crystal violet sensitivity. YS18 and YS18 *sge1-Δ1::URA3* were pregrown in supplemented minimal medium. Serial dilutions (see the Materials and methods) were placed on supplemented minimal agar with (+cv) or without (−cv) 0.8 μg/ml crystal violet. Plates were incubated for 3 days at 30° C. **B** *SGE1* overexpression increases the crystal violet resistance of YAE65. YAE65 transformed with a control plasmid (pRS316), a single copy (pAE292) and a high copy plasmid (pAE260) containing the *SGE1* gene was pregrown in supplemented minimal medium. The crystal violet sensitivity was assayed (see the Materials and methods) on supplemented minimal agar with (+cv) or without (−cv) 1 μg/ml crystal violet. Plates were incubated for 3 days at 30° C

YHE1, YHE4 and one of their progenitors, Ito1A, while being present in S288C, JN127, JRY2302 and YS18 (Fig. 5). The finding that JN127 contained the *SGE1* fragment indicated that the *sge1* deletion in YAE65 did not stem from JN127, and therefore, that *SGE1* was not allelic to *ISE2*. This view is supported by the fact that the *SGE1* homologue cloned from JN127 was capable of complementing the supersensitivity of YAE65 (data not shown) and thus was not mutated nor responsible for the sensitivity phenotype of JN127.

Further investigation revealed that YAE65 and YHE4 were deleted for a DNA region of approximately 7 kb encompassing the *SGE1* gene (data not shown). Therefore, YAE65 as well as YHE4 represent *sge1* null mutants. In fact, YHE4 showed increased crystal violet sensitivity compared to the wild-type strain YS18, which is not deleted in *SGE1*. Moreover, the fact that expression of *SGE1* from a single-copy plasmid in YHE4 increased the resistance of the strain to crystal violet up to the maximum tested concentration of 1 μg/ml crystal violet (data not shown) confirms the observation that the *SGE1* gene function is missing in YHE4. Nevertheless, YAE65 displays increased crystal violet sensitivity compared to YHE4, although both strains are *sge1* null mutants. This observation strongly suggests that one or more additional mutations are present in YAE65, which discriminate it from YHE4 and which contribute, in addition to the *sge1* deletion, to its strong crystal violet-supersensitive phenotype.

Genetic analysis of the crystal violet sensitivity in YAE65

Several findings indicate that the crystal violet-supersensitive phenotype of YAE65 is not caused by the deletion of *SGE1* alone, but that (a) further, so far unidentified mutation(s) contribute(s) to its strong sensitivity phenotype. Therefore, we performed a genetic analysis of the crystal violet sensitivity of YAE65 to test this notion. For this purpose, YAE65 was mated to wild-type strain JRY254; a diploid was selected phenotypically and was subjected to sporulation and tetrad analysis. In a subsequent crystal violet sensitivity assay with the segregants, three distinct phenotypes were found. Haploids insensitive to 1 μg/ml crystal violet corresponded to the wild-type parental strain JRY254. Haploids displaying strong growth inhibition at 0.3 μg/ml crystal violet resembled the parental strain YAE65 and were designated supersensitive. Thirdly, haploids exhibiting an intermediate phenotype of sensitivity to a crystal violet concentration of 0.5 to 0.8 μg/ml were observed. Accordingly, we were able to distinguish between three classes of asci: parental ditype (PD), where wild-type sensitivity and supersensitivity segregated 2:2, non-parental ditype (NPD), where all four segregants exhibited an intermediate sensitivity, and tetratype (T), where the tetrad contained two haploids of intermediate sensitivity, one supersensitive and one wild-type haploid. The results of this genetic analysis are presented in Table 2. These results indicate that the diploid YAE65 × JRY254 is heterozygous at more than one genetic locus bearing on sensitivity. Moreover, the observation that PD ~ NPD ≪ T argues for the presence of two sensitivity-conferring mutations that segregate independently of each other. Presumably, the *sge1* null mutation constitutes one of these mutations. We cannot exclude the possibility that the other mutation is allelic to *ISE2*.

Table 2 Genetic analysis of the crystal violet sensitivity in strain YAE65

Cross	Tetrad types		
	Parental ditype	Non-parental ditype	Tetratype
YAE65 × JRY254	4	2	9

Effect of *SGE1* overexpression on crystal violet sensitivity

To test the effect of *SGE1* gene dosage, supersensitive strain YAE65 was transformed (Klebe et al. 1983) with plasmids pAE292 and pAE260 (cf. Fig. 2), which are centromere- and 2 μm-based plasmids, respectively, and contain the *SGE1* gene. While the *SGE1*-containing centromere plasmid could relieve the supersensitivity of YAE65 at 0.3 μg/ml crystal violet, the high-copy-number plasmid could complement the sensitivity up to a concentration of 1 μg/ml (Fig. 6B). Therefore, *SGE1* overexpression further increases crystal violet resistance.

Discussion

We have constructed a supersensitive yeast strain, which shows growth inhibition at a crystal violet concentration of 0.3 μg/ml and contains the markers *ade2-119*, *ilv1-92* and *trp5-b* suitable for genotoxicity testing (Zimmermann et al. 1975). We have further isolated an approximately 10-kb genomic DNA fragment from a wild-type strain, which complements the dye supersensitivity on a single-copy plasmid. A 3.4-kb subfragment is sufficient to restore normal sensitivity in the supersensitive strain.

The complementing 3.4-kb fragment, which has been sequenced on both strands, contains a 1629 bp open reading frame recently identified as *SGE1* (Amakasu et al. 1993) which encodes a putative protein of 543 amino acids. Because of its high content of hydrophobic amino acids and its hydrophobicity profile as calculated by the Kyte-Doolittle algorithm (Kyte and Doolittle 1982), we predict that the protein is embedded in the membrane. Other yeast proteins involved in sensitivity/resistance such as Snq2p (Servos et al. 1993), Pdr5p (Wang et al. 1992) and Ygl022p (Chen et al. 1991) have previously been proposed to be integral membrane proteins. Interestingly, SNQ2 and PDR5 both confer distinct resistance phenotypes when present on multi-

copy plasmids and their products show extended homology with the products of the *Drosophila melanogaster* genes *white* and *brown*; the latter are thought to function as pigment transport proteins in the eye and other tissues (Riordan et al. 1989). Therefore, *SNQ2* has been suggested to encode an export permease (Servos et al. 1993). Snq2p and Pdr5p also share common features with the yeast *STE6* gene product, a yeast homologue of P-glycoproteins (McGrath and Varshavsky 1989), which mediate multidrug resistance in mammalian cells, due to the energy-driven extrusion of a wide variety of drugs (Gottesman and Pastan 1988). Likewise, the Ste6 protein exports the yeast **a**-factor mating pheromone. Unlike Snq2p, Pdr5p and Ste6p, Ygl022p does not contain any putative ATP-binding domains that might imply an energy-dependent manner of substrate export (Chen et al. 1991). Nevertheless, disruption of *YGL022* affects sensitivity to various drugs (Sauer 1992).

In fact, a whole family of proteins lacking an ATP-binding cassette has been identified, which catalyse the efflux of various drugs such as quinolone, tetracycline and methylenomycin A in several bacterial species (Marger and Saier 1993). Designation as a member of the family is not only based on the common phenotype suggesting an efflux function, but also on mutual amino acid comparisons indicating high homology scores. These drug-resistance proteins form a family within the major facilitator superfamily (MFS; Marger and Saier 1993). The MFS proteins characteristically consist of "two-times six" α-helices separated by a dispensable central cytoplasmic domain, and do not contain an ATP-binding consensus sequence. So far, only three yeast genes, the aminotriazole resistance *ATR1* gene (Marger and Saier 1993) and two yeast chromosome III open reading frames *YCL069W* and *YCR023C* have been reported to belong to the drug-resistance protein family (Goffeau et al. 1993). The Ycl069w protein, whose function in yeast so far remains elusive, has been proposed to consist of 10 membrane-spanning domains, and its best homology has been reported to be to the *E. coli* tetracycline transporter (Goffeau et al. 1993). It shows strong homology to the Sge1 protein (38% identity, 62% similarity). The Sge1 protein furthermore shares extended homologies with the *Streptomyces rimosus* tetracycline-resistance determinant *tetA* gene product (19.7% identity, 47% similarity), the *Staphylococcus aureus* fluoroquinolone-resistance *norA* gene product (24% identity, 57% similarity) and the *Bacillus subtilis* methylenomycin A-resistance *mmr* gene product (25.5% identity, 52% similarity). All three proteins are members of the drug-resistance protein family (Marger and Saier 1993). Also, Sge1p is 24.5% identical and 52% similar to the *Nocardia lactamdurans cmc*T gene product, which has been proposed to function as an export protein for cephamycin C and is similar to the yeast *ATR1* gene product (Coque et al. 1993).

From these findings we conclude that the *SGE1* gene product constitutes a novel member of the family of drug-resistance proteins within the major facilitator superfamily. We furthermore suggest that Sge1p functions as an export permease by analogy to the other members of the family. This view is supported by the features of the *SGE1* phenotype: the supersensitivity of the *sge1* null mutant YAE65 is a recessive rather than a dominant trait, and disruption of the chromosomal *SGE1* locus in a normally sensitive strain results in increased sensitivity. Alternatively, Sge1p might increase the expression of yet another export gene or repress expression of a drug import gene. Conversely, overexpression of the *SGE1* gene increases resistance. This characteristic resembles the multidrug hyper-resistance phenotype of strains that overexpress *SNQ2* and *PDR5*. Therefore, we would expect that *SGE1* also affects sensitivity towards other substances apart from crystal violet. Since crystal violet sensitivity in some cases is representative of high plasma membrane permeability (Bard et al. 1978), one might consider a possible involvement of *SGE1* in the synthesis of membrane components, as was found for the *ise1* mutation (Graham et al. 1993). This does not seem likely, though, because of the hydrophobic nature of Sge1p and the observed homologies to the MFS proteins.

The *SGE1* gene was originally identified as a partial suppressor of *gal11* mutations (Amakasu et al. 1993). *GAL11* is required for maximizing transcription of some, but not all, galactose-inducible genes (Suzuki et al. 1988). Surprisingly, one additional copy of *SGE1* has been reported to be sufficient to suppress the Gal^- phenotype of *gal11* mutants. Other *gal11* phenotypes (Fassler and Winston 1989; Nishizawa et al. 1990) such as α-specific sterility and slow growth on non-fermentable carbon sources were not suppressed by *SGE1*. *SGE1* has therefore been suggested specifically to play a role in transcription of *GAL4*-dependent genes. In the view of our results, *SGE1* might also be involved in the transcription of other, *GAL4*-independent genes that affect the drug sensitivity or the plasma membrane permeability of the cell.

Direct transcriptional activation through the Sge1 protein via protein-protein interaction as proposed for the Gal11 protein seems unlikely, once again because of the hydrophobicity and putative membrane location of the Sge1 protein. Interestingly, the "MF" superfamily, to which the Sge1 protein is proposed to belong, also comprises a large group of transporters specific for sugars such as glucose and galactose. One might therefore speculate on a possible role of Sge1p in galactose import. Overexpression of *SGE1* might thereby increase the intracellular galactose level, which might indirectly increase the expression of *GAL4*-dependent genes, for instance, by a post-transcriptional regulatory mechanism. In this context, examination of the effect of *SGE1* overexpression in a mutant deficient in the galactose import protein Gal2 might help elucidate this possible function of the Sge1 protein.

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