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SSU1 Encodes a Plasma Membrane Protein with a Central Role in a Network of Proteins Conferring Sulfite Tolerance in *Saccharomyces cerevisiae*†

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The *Saccharomyces cerevisiae SSU1* gene was isolated based on its ability to complement a mutation causing sensitivity to sulfite, a methionine intermediate. *SSU1* encodes a deduced protein of 458 amino acids containing 9 or 10 membrane-spanning domains but has no significant similarity to other proteins in public databases. An Ssu1p-GFP fusion protein was localized to the plasma membrane. Multicopy suppression analysis, undertaken to explore relationships among genes previously implicated in sulfite metabolism, suggests a regulatory pathway in which *SSU1* acts downstream of *FZF1* and *SSU3*, which in turn act downstream of *GRR1*.

The toxicity of sulfite to microorganisms has been widely exploited through its use as a preservative in foods, beverages, and pharmaceuticals (21). The fact that sulfite is also a normal metabolite produced during reductive sulfate assimilation in bacteria, fungi, and plants raises the question of how endogenous toxicity is avoided. Efficient regulation of the reductive sulfate assimilation pathway would minimize pools of intermediates, sulfite among them, and may be the major form of control. In *Saccharomyces cerevisiae*, the formation of acetaldehyde during fermentation appears to be another means of controlling sulfite levels because the two compounds react to form a stable and nontoxic product, 1-hydroxyethane sulfonate (21).

We previously isolated mutants of *S. cerevisiae* in the expectation that sensitive mutants may be impaired in their protective functions and that resistant mutants may have enhanced protection. This analysis identified four genes involved in the sensitive phenotype, *SSU1*, *SSU2*, *SSU3*, and *SSU4*, and a single resistance gene, *RSU1* (24). One of the mutations conferring sensitivity (*ssu2*) was found to be an allele of *GRR1* (1), which others have implicated in glucose signalling (2, 9, 10, 16, 17, 23) and in other functions (3, 4, 7, 15, 22, 23). While the sulfite sensitivity of a *grr1* mutant may be partly due to defective glucose metabolism, sensitivity was observed during growth on nonglucose carbon sources. Further, a suppressor of the glucose repression defect, *rgt1*, failed to completely suppress the sensitivity (1). We also showed that *FZF1* is a multicopy suppressor of the sulfite sensitivity of a *grr1* mutant. *FZF1* encodes a five-zinc-finger putative transcription factor (5), and a particular allele was independently found to confer dominant resistance to sulfite (6). Here we show that the product of the *SSU1* gene, also implicated in sulfite sensitivity, is a plasma membrane protein, and we propose potential relationships among *SSU1* and three other genes involved in protection against sulfite, *FZF1*, *SSU3*, and *GRR1*.

Isolation of *SSU1* and DNA sequence analysis. A genomic clone of *SSU1* was isolated from a centromeric library (19a) by complementation of the sulfite sensitivity of 3088-6d *MAT***a** *ssu1-1 ura3-52 leu2-3,112* (24). A single complementing clone contained a plasmid with an insert of about 9.5 kb, designated pDA17 (Fig. 1). A complementing 3.6-kb *Bam*HI fragment was subcloned in YCplac33 and YIplac211 (11) to yield pDA18 and pDA19, respectively (Fig. 1). The latter construct was integrated in 3088-6d. All 13 tetrads obtained from a cross between the integrant and an *SSU1 ura3* strain produced spores which were resistant to sulfite and which segregated 2+:2− for *URA3*, consistent with pDA19 carrying wild-type *SSU1*. Sequence analysis showed that pDA18 contained two complete open reading frames (ORFs), one encoding 67 and the other encoding 458 amino acids, and part of the glutathione-oxidoreductase ORF, encoding the 146 N-terminal amino acids, which represents about one-third of the protein (Fig. 1). Subcloning established that the centrally located ORF, encoding 458 amino acids (YPL092W), possessed complementing activity and therefore was *SSU1* (GenBank accession no. U20254).

The predicted protein sequence of *SSU1* did not have significant similarity to proteins from public databases. A search at TMpred (14) suggested 9 or 10 transmembrane domains which form a central hydrophobic core flanked by hydrophilic domains which presumably face the cytoplasm. Although Ssu1p does not have significant similarity to proteins from transporter families, this structure suggests a facilitator-transporter protein. The carboxy terminus of 49 amino acids is hydrophilic and has 10 (20%) serine residues, some being putative phosphorylation sites. Ssu1p lacks the nucleotide binding sequence typical of ATP binding cassette transporters (13) and the conserved amino acid stretches which form the catalytic site of P-type ATPases (8).

An SSU1p-GFP fusion protein is localized to the plasma membrane. To obtain an Ssu1p-GFP fusion protein, the *GFP* ORF containing the S65T mutation (12) from pCM153 (15b) was cloned in YCplac33 as a *Bam*HI/*Sac*I fragment to yield pDA8 (data not shown). The *SSU1* ORF and 150-bp upstream region were generated by PCR using appropriate primers, and the remaining *SSU1* upstream region was subcloned from

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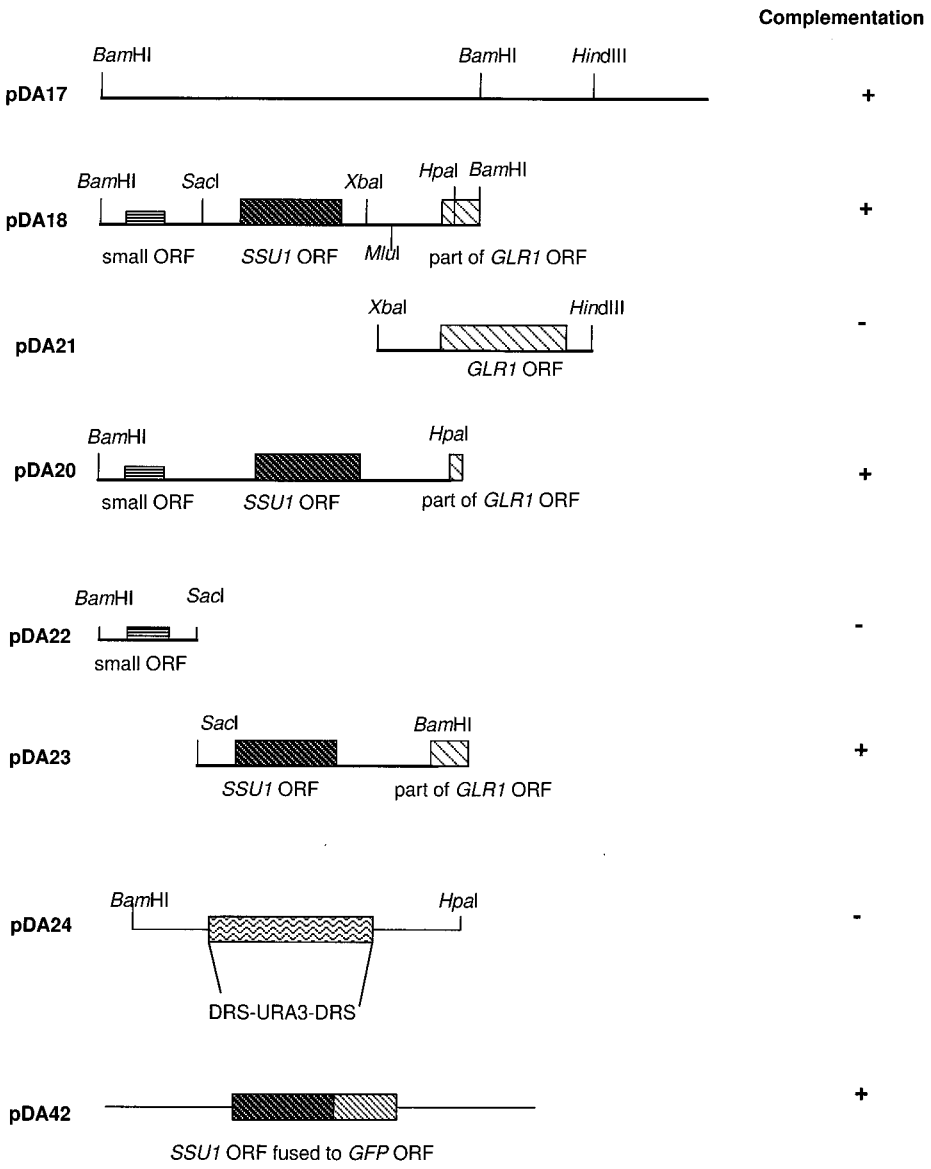

FIG. 1. Restriction map of the primary *SSU1*-containing fragment and derived subclones (not drawn to scale). The primary subclone was designated pDA17. pDA18 contains a 3.6-kb *Bam*HI fragment from pDA17 cloned in YCplac33 which includes the small ORF, the entire *SSU1* ORF, and one-third of the *GLR1* ORF. pDA21 contains the entire *GLR1* ORF. pDA20 contains the small ORF and the *SSU1* ORF, and pDA22 contains only the small ORF. pDA23 contains the *SSU1* ORF, and pDA24 contains the disrupted *SSU1* ORF (consisting of the *URA3* gene flanked by direct repeats [DRS]). pDA42 contains the *SSU1* ORF fused to the *GFP* ORF.

pDA19, generating pDA42 (Fig. 1). pDA42 complemented the sulfite sensitivity of 3090-9d-T4-L1 *MAT*α *ssu1*Δ *leu2-3,112 ura3-52*. The construct was then cut with *Bst*XI to delete *CEN* and *ARS* sequences and was integrated at the *SSU1* locus in 3090-9d-T4-L1. Examination of a wild-type strain expressing the Ssu1p-GFP fusion protein by laser scanning confocal microscopy revealed peripheral fluorescence (Fig. 2). Protoplasts expressing the same construct also showed peripheral fluorescence (data not shown), confirming a plasma membrane location.

An *SSU1* null mutant is viable and sensitive to sulfite. *SSU1* was disrupted (18) using the disruption cassette of pDIS4 (15a) to generate pDA24, which was linearized with *Dra*I and *Aat*II and used to transform 3090-9d *SSU1 ura3* to yield 3090-9d-T4 *SSU1*Δ*::URA3*. Integration of the disruption construct at the *SSU1* locus was confirmed by PCR (19) with two *SSU1* primers and a *URA3* primer (1). The null mutant was found to be viable, prototrophic, and sensitive to sulfite but was neither UV sensitive nor temperature sensitive for growth at 37°C. Sporulation of a homozygous *ssu1*Δ/*ssu1*Δ diploid was qualitatively indistinguishable from that of a wild-type diploid.

Multicopy suppression analysis. Multicopy suppression analysis was undertaken to uncover possible functional relationships among four genes implicated in the sulfite-sensitive phenotype, based on the premise that defects upstream in a regulatory pathway can be suppressed by overexpressed genes downstream, but not vice versa. Overexpression of *GRR1*, *FZF1*, and *SSU1* was performed reciprocally in each of the single null mutants. Because *SSU3* has not yet been isolated, its overexpression could not be evaluated. However, multicopy *GRR1*, *FZF1*, and *SSU1* were tested individually in an *ssu3* background.

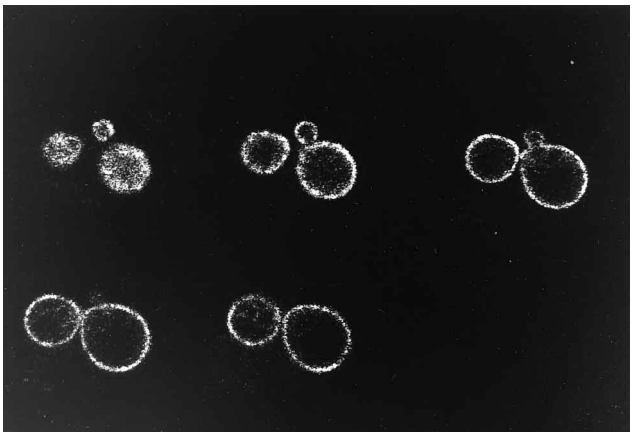

FIG. 2. Localization of the Ssu1p-GFP fusion protein. GFP fluorescence was observed in living cells visualized with a Leica TCS 4D confocal microscope with images processed with Adobe Photoshop. The five frames represent serial sections of the same cells.

Analysis of multicopy suppression of *GRR1*, *FZF1*, and *SSU1* gave internally consistent results, suggesting that *GRR1* acts upstream of *FZF1*, which in turn acts upstream of *SSU1* (Table 1; Fig. 3). Overexpression of the three genes in an *ssu3* background placed *SSU3* downstream of *GRR1* but upstream of *SSU1*. These results are in agreement with previous observations of multicopy *FZF1* suppressing the sulfite sensitivity of a *grr1* mutant and partially suppressing that of an *ssu3* mutant (1), independent isolation of *SSU1* from a high-copy DNA library as a multicopy suppressor of the sensitivity of an *fzf1* mutant (data not shown), and activation of a *lacZ* ORF-*SSU1* promoter fusion by multicopy *FZF1* (1a).

We speculate that if *SSU1* encodes a transporter, it may be involved in sulfite efflux rather than uptake because (i) an *SSU1* null mutant is sulfite sensitive rather than resistant; (ii) deletions or mutations in genes upstream in the pathway that appear to regulate or are required for *SSU1* function (*GRR1*, *FZF1*, and *SSU3*) also result in sulfite sensitivity rather than resistance, except for a particular allele of *FZF1* which confers resistance, perhaps due to hyperactivation of *SSU1* (6); and (iii) biochemical studies of sulfite uptake support passive diffusion (20). The latter point is consistent with sulfite being a normal yeast metabolite but an unusual source of exogenous sulfur. The present study has uncovered a regulatory pathway involved in sulfite metabolism, and its relationship to reductive sulfate assimilation and acetaldehyde production merits further investigation.

TABLE 1. Multicopy suppression of sulfite sensitivity of various mutants

Mutation	Multicopy gene	Suppressing activity[a]
*grr1*Δ	*FZF1*	+
*grr1*Δ	*SSU1*	+
*fzf1*Δ	*GRR1*	−
*fzf1*Δ	*SSU1*	+
*ssu1*Δ	*GRR1*	−
*ssu1*Δ	*FZF1*	−
ssu3-7	*GRR1*	−
ssu3-7	*FZF1*	±
ssu3-7	*SSU1*	+

[a] Suppressing activity was tested as growth on plates containing 1.5 mM sulfite. +, −, and ±, wild-type growth, no growth, and slow growth, respectively. On this medium, mutants carrying the vector alone did not grow. In a wild-type background, multicopy *SSU1* conferred resistance to 3 mM sulfite while single-copy *SSU1* permitted growth on no more than 1.5 mM sulfite.

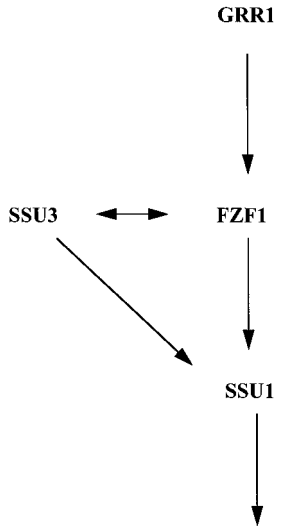

FIG. 3. Regulatory model for sulfite detoxification in *S. cerevisiae*. The sulfite-proximal member of this pathway is Ssu1p, a putative transporter which when mutated causes sulfite sensitivity. Expression of *SSU1* is controlled by Fzf1p, independently shown to be involved in sulfite tolerance. Based on *lacZ* ORF-*SSU1* promoter fusion analysis, activation by Fzf1p requires a functional *SSU3* gene, which when mutated also causes sensitivity to sulfite. Expression of the *SSU1* promoter also requires a functional *GRR1* gene (1a). The sulfite sensitivity of *grr1* is suppressed by multicopy *FZF1* and *SSU1*, and thus, Grr1p is presumed to regulate both.

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