Sit4p Protein Phosphatase Is Required for Sensitivity of *Saccharomyces cerevisiae* to *Kluyveromyces lactis* Zymocin

Daniel Jablonowski,* Andrew R. Butler,†,[1] Lars Fichtner,* Donald Gardiner,† Raffael Schaffrath* and Michael J. R. Stark†

**Institut für Genetik, Martin-Luther Universität Halle-Wittenberg, D-06120 Halle (Salle), Germany and* †*Division of Gene Regulation & Expression, School of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom*

Manuscript received June 25, 2001
Accepted for publication September 18, 2001

ABSTRACT

We have identified two *Saccharomyces cerevisiae* genes that, in high copy, confer resistance to *Kluyveromyces lactis* zymocin, an inhibitor that blocks cells in the G_1 phase of the cell cycle prior to budding and DNA replication. One gene (*GRX3*) encodes a glutaredoxin and is likely to act at the level of zymocin entry into sensitive cells, while the other encodes Sap155p, one of a family of four related proteins that function positively and interdependently with the Sit4p protein phosphatase. Increased *SAP155* dosage protects cells by influencing the sensitivity of the intracellular target and is unique among the four *SAP* genes in conferring zymocin resistance in high copy, but is antagonized by high-copy *SAP185* or *SAP190*. Since cells lacking *SIT4* or deleted for both *SAP185* and *SAP190* are also zymocin resistant, our data support a model whereby high-copy *SAP155* promotes resistance by competition with the endogenous levels of *SAP185* and *SAP190* expression. Zymocin sensitivity therefore requires a Sap185p/Sap190p-dependent function of Sit4p protein phosphatase. Mutations affecting the RNA polymerase II Elongator complex also confer *K. lactis* zymocin resistance. Since *sit4*Δ and *SAP*-deficient strains share in common several other phenotypes associated with Elongator mutants, Elongator function may be a Sit4p-dependent process.

KILLER strains of the yeast *Kluyveromyces lactis* secrete a protein toxin or zymocin that inhibits the proliferation of several different yeasts including *Saccharomyces cerevisiae* (Stark *et al.* 1990; Schaffrath and Breunig 2000). In the presence of *K. lactis* zymocin, sensitive *S. cerevisiae* cells become blocked in the G_1 phase of the cell division cycle prior to budding and with unreplicated DNA (White *et al.* 1989; Butler *et al.* 1991c), suggesting that the zymocin acts to inhibit one or more of the events normally triggered at Start and that are required for G_1 exit. Although the zymocin is a heterotrimeric protein, intracellular expression of just the γ-subunit is sufficient to promote the G_1 arrest phenotype (Tokunaga *et al.* 1989; Butler *et al.* 1991b). The two larger subunits are probably involved in entry of the zymocin into the cell, a process that involves an interaction between the α-subunit and cell wall chitin. Thus chitin-deficient mutants are zymocin resistant (Takita and Castilho-Valavicius 1993; Jablonowski *et al.* 2001) and the α-subunit has a domain that shows *in vitro* chitinase activity and has sequence similarity to other chitinases and chitin-binding proteins (Butler *et al.* 1991a). Recent work has demonstrated that mutations affecting at least five genes encoding components of the RNA polymerase II Elongator complex lead to zymocin resistance (Frohloff *et al.* 2001). Elongator is a multisubunit complex that binds to the elongating form of RNA polymerase II (RNAPII: Otero *et al.* 1999; Wittschieben *et al.* 1999; Fellows *et al.* 2000) and loss of Elongator function causes a range of phenotypes including delayed activation of genes under changing growth conditions, hypersensitivity to 6-azauracil and caffeine, slow growth, and temperature sensitivity (Otero *et al.* 1999; Frohloff *et al.* 2001). However, Elongator itself is dispensable; so although zymocin inhibition is an Elongator-dependent process, the zymocin cannot simply be acting to block Elongator function.

The execution of Start requires the Sit4p protein phosphatase and temperature-sensitive *sit4* mutants to arrest in G_1 prior to bud emergence and DNA replication (Sutton *et al.* 1991; Fernandez-Sarabia *et al.* 1992). This requirement is at least in part because Sit4p is needed for proper expression of G_1 cyclins such as *CLN1*, *CLN2*, and *PCL1*. However, while ectopic expression of *CLN2* from a Sit4p-independent promoter can relieve the block to DNA replication in *sit4-102* Ts^- cells, these cells still remain largely unbudded and thus blocked for bud emergence (Fernandez-Sarabia *et al.* 1992). Thus, independently of its effect on *CLN2* expression, Sit4p is also needed for other events required for

Corresponding author: Michael J. R. Stark, Division of Gene Regulation & Expression, School of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom.
E-mail: m.j.r.stark@dundee.ac.uk

[1] *Present address:* Department of Biochemistry, School of Biological Sciences, University of Leicester, University Road, Leicester LE1 7RH, United Kingdom.

G_1 exit. The phenotype of *sit4* mutants is complicated by its dependence on a second polymorphic gene, *SSD1* (Sutton *et al.* 1991). In *ssd1-d* strains deletion of *SIT4* is lethal, but in *SSD1-v* strains *sit4* deletion is tolerated, although it leads to a G_1 delay and a slow growth rate that is not improved by ectopic *CLN2* expression (Fernandez-Sarabia *et al.* 1992). Sit4p associates in a cell-cycle-dependent manner with the members of a protein family termed the SAPs (Sutton *et al.* 1991) and deletion of all four *SAP* genes (*SAP4*, *SAP155*, *SAP185*, and *SAP190*) confers the same phenotype as loss of *SIT4* (Luke *et al.* 1996). A variety of evidence shows that the SAPs and Sit4p function in an interdependent manner, leading to models whereby the SAPs are either positive activators of Sit4p phosphatase or Sit4p effectors (Luke *et al.* 1996). Sit4p also binds Tap42p, an element in the TOR signaling pathway; on nutrient starvation or inhibition of the TOR kinases by rapamycin, Tap42p dissociates from Sit4p (Di Como and Arndt 1996). Nutrient starvation or treatment with rapamycin leads to a variety of responses including a G_1 arrest, reduced translational initiation, reduced ribosome biosynthesis, and changes to the amino acid transporters expressed on the cell surface (see Schmidt *et al.* 1998; Cardenas *et al.* 1999). Dissociation of Sit4p from Tap42p has been proposed to trigger dephosphorylation of at least some of the proteins that act as effectors of the TOR pathway and that mediate some of these responses (Schmidt *et al.* 1998; Beck and Hall 1999).

We previously isolated two genes that confer *K. lactis* zymocin resistance when present specifically on high-copy plasmids (Butler *et al.* 1994). One of these (*KTI12*) had previously been defined by recessive mutations that also lead to zymocin resistance. Since either loss of *KTI12* function or high-copy *KTI12* conferred zymocin resistance, we hypothesized that Kti12p could be part of a protein complex required for zymocin action that was perturbed by excess Kti12p (Butler *et al.* 1994). Consistent with this idea, *KTI12* has recently been shown to be allelic with *TOT4*, a component of the Elongator complex (Frohloff *et al.* 2001). Here we describe the isolation of *GRX3* and *SAP155* (encoding one of the Sit4p-associated SAPs) as additional genes that promote zymocin resistance when present in high copy. In addition, we show that cells lacking *SIT4* are both refractory to *K. lactis* zymocin and share many of the other phenotypes of Elongator mutants, placing Sit4p within a pathway required for zymocin action and linking Sit4p to Elongator function.

MATERIALS AND METHODS

Strains: All yeast strains used in this study are listed in Table 1. LL20-2 was generated from LL20-1 by transformation with pHO1.5 (a YEp vector carrying the *HO* and *URA3* genes; M. Pocklington) to promote mating-type switching, followed by 5-fluoroorotic acid-induced plasmid loss and verification of mating type by crossing with tester strains (Herskowitz and Jensen 1991; Sikorski and Boeke 1991). CY4029α was generated from CY4029 in the same manner. LFY5 and LFY6 were constructed from AY925 by one-step disruption using the PCR primers described previously (Frohloff *et al.* 2001).

General methods: All yeast growth media and general yeast methods were as described by Kaiser *et al.* (1994). Yeast transformation was carried out according to Gietz *et al.* (1992). When direct selection for the *leu2^d* marker gene was employed, the selective plates were supplemented with 0.0075% (w/v) Bacto-yeast extract. To test multiple strains for growth phenotypes on agar plates, strains were first grown on plates with selection for all markers and then colonies of similar size were resuspended in fresh medium at the same cell density ($\sim 10^6$ cells/ml). Culture samples ($\sim$5 μl) together with three 10-fold serial dilutions were spotted onto plates using a multipronged inoculating manifold (Dan-Kar). General recombinant DNA procedures were carried out as described by Sambrook *et al.* (1989). DNA sequencing was performed manually using the Sequenase version 2.0 kit (United States Biochemical, Cleveland) and double-stranded plasmid DNA templates. A combination of specific subclones and synthetic oligonucleotide primers allowed determination of the sequence of *SAP155* on both strands. To identify pMA3a clones encoding $tRNA_3^{glu}$ sequences, plasmid DNA was digested with *Eco*RI and *Sal*I and Southern blot analysis was performed using the 435-bp *Bsa*AI-*Hin*dIII fragment from pYF1 (Butler *et al.* 1994). Similar analysis was performed using a 550-bp fragment of *KTI12* excised from pJHW27 to identify *KTI12*-encoding clones. In each case, radiolabeled probes were generated using random hexamer priming (Feinberg and Vogelstein 1983). RNA isolation and RT-PCR were both carried out essentially as described by Frohloff *et al.* (2001) using the following primer pairs: *TOT1* (5′-CTTGGTGTATGAAACTCGCG-3′ and 5′-TTCTTACCTCTGCCAGTACC-3′), *TOT2* (5′-AACCTGATGAGACTTCAGGC-3′ and 5′-CAAACCTAACACAGGAACGG-3′), *TOT3* (5′-TCAGTCCTTGTACGAAGACG-3′ and 5′-ATAAGCTCGACCTGATCTGG-3′), *TOT4* (5′-TCCGGTATCAACTTCACTGC-3′ and 5′-CTTGTTCCGTTACTTACCCC-3′), and *HHT1* (5′-AGCAAGAAAGTCCACTGGTG-3′ and 5′-GAATGGCAGCCAAGTTGGTA-3′).

Plasmids: Table 2 summarizes plasmids used in this study. The *TAP42* fragment in pDJ14 was obtained by PCR amplification followed by *in vivo* gap repair (to recover the coding region from the genome) and complemented the lethality of a *tap42* deletion strain (not shown). The high-copy library used in this study comprised partial *Sau*3A fragments from yeast genomic DNA (6–10 kb) inserted into the vector pMA3a (*leu2^d* 2μ) and has also been described previously (Crouzet and Tuite 1987). pARB19 was constructed in two stages: the large *Eco*RI fragment from the pARB106 insert was first ligated into the *Eco*RI site of YEplac181 and then the *Sal*I fragment from this construct was replaced with the *Sal*I fragment of pARB15 (Table 2) such that the bulk of the original insert of pARB106 was reconstructed. pDG8 was made by first cloning the 1.96-kb *Xba*I fragment of pARB100 (encoding the promoter and 5′ half of *SAP155*) into the *Xba*I site of YEplac181 such that the *SAP155* open reading frame (ORF) read in the opposite direction to the *lacZ* α-fragment. The *Sal*I-*Pst*I interval from the insert was then removed and replaced with the *SAP155* *Sal*I-*Pst*I fragment from pDG6, reinstating a complete *SAP155* gene. To make a *sap155::HIS3* deletion allele, pDG8 was digested with *Bcl*I to excise the bulk of the *SAP155* coding region, which was replaced by the 1.3-kb *HIS3* *Bam*HI fragment of YDpH (Berben *et al.* 1991). This resulted in the deletion of codons 85–999 in *SAP155* and yielded pDG11 and pDG12, which differ in the orientation of the *HIS3* insert.

***K. lactis* zymocin methods:** Killer eclipse assays for zymocin sensitivity were performed as described previously (Kishida *et al.* 1996), using *K. lactis* strains AWJ137 (zymocin producer) and NK40 (nonproducer). For most other assays, serial dilu-

TABLE 1

Yeast strains

Strain	Genotype[a]	Source
K. lactis		
AWJ137	*MAT***a** *leu2 trp1* [pGKl1$^+$ pGKl2$^+$]	Kämper *et al.* (1991)
NK40	*MAT*α *ade1 ade2 leu2* [pGKl1° pGKl2$^+$]	Gunge *et al.* (1981)
S. cerevisiae		
AY925	*MAT***a** W303 *ssd1-d2*	Kim Arndt
CY3938	*MAT***a** W303 *sit4*Δ*::HIS3 SSD1-v1*	Luke *et al.* (1996)
CY4029	*MAT***a** W303 *SIT4 SSD1-v1*	Luke *et al.* (1996)
CY4029α	*MAT*α W303 *SIT4 SSD1-v1*	This study
CY5220	*MAT***a** W303 *sap4::LEU2 sap155::HIS3 SSD1-v1*	Luke *et al.* (1996)
CY5224	*MAT***a** W303 *sap185::ADE2 sap190::TRP1 SSD1-v1*	Luke *et al.* (1996)
CY5236	*MAT***a** W303 *sap4::LEU2 sap155::HIS3 sap185::ADE2 sap190::TRP1 SSD1-v1*	Luke *et al.* (1996)
DJY8	*MAT*α W303 *sap4::LEU2 SSD1-v1*	CY4029α × CY5236
DJY9	*MAT*α W303 *sap155::HIS3 SSD1-v1*	CY4029α × CY5236
CY4917	*MAT***a** W303 *sap185::ADE2 SSD1-v1*	Kim Arndt
CY4380	*MAT***a** W303 *sap190::TRP1 SSD1-v1*	Kim Arndt
DJY10	*MAT*α W303 *sap4::LEU2 sap185::ADE2 SSD1-v1*	CY4029α × CY5236
DJY11	*MAT*α W303 *sap4::LEU2 sap190::TRP1 SSD1-v1*	CY4029α × CY5236
DJY12	*MAT*α W303 *sap155::HIS3 sap185::ADE2 SSD1-v1*	CY4029α × CY5236
DJY13	*MAT*α W303 *sap155::HIS3 sap190::TRP SSD1-v1*	CY4029α × CY5236
DJY14	*MAT*α W303 *sap155::HIS3 sap185::ADE2 sap190::TRP1 SSD1-v1*	CY4029α × CY5236
DJY15	*MAT*α W303 *sap4::LEU2 sap185::ADE2 sap190::TRP1 SSD1-v1*	CY4029α × CY5236
DJY16	*MAT*α W303 *sap4::LEU2 sap155::HIS3 sap190::TRP1 SSD1-v1*	CY4029α × CY5236
DJY17	*MAT*α W303 *sap4::LEU2 sap155::HIS3 sap185::ADE2 SSD1-v1*	CY4029α × CY5236
LA	*MAT*α *leu2-3 leu2-112 his3-11 his3-15 ura3::pARB1* Gal$^+$	From LL20-1
LFY5	*MAT***a** W303 *tot3::TRP1 SSD1-v1*	This study
LFY6	*MAT***a** W303 *kti12::TRP1 SSD1-v1*	This study
LL20	*MAT*α *leu2-3 leu2-112 his3-11 his3-15* Gal$^+$	NCYC1445[b]
LL20-1	*MAT*α *leu2-3 leu2-112 his3-11 his3-15 ura3* Gal$^+$	Butler *et al.* (1994)
LL20-2	*MAT***a** *leu2-3 leu2-112 his3-11 his3-15 ura3* Gal$^+$	Vera Martin
LL20-3	*MAT***a***/MAT*α LL20	LL20-1 × LL20-2
LY	*MAT*α *leu2-3 leu2-112 his3-11 his3-15 ura3::*YIplac211 Gal$^+$	From LL20-1
TS54-1A	*MAT***a** *leu2-3, 112 ura3-52 rme1 HML***a** *tap42::kanMX* Gal$^+$ [YCplac111::*tap42-11*]	M. Hall

[a] W303 genetic background is *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* Gal$^+$ and, normally, *ssd1-d2*.
[b] National Collection of Yeast Cultures, Colney, Norwich, UK.

tions of strains (grown under selection for plasmids as appropriate) were spotted out onto YPD agar with or without culture supernatant from AWJ137 as a source of toxin [65% (v/v) unless otherwise stated]. Growth was compared after 2 days incubation at 26°. To test plasmids for conferring resistance to intracellular expression of the zymocin γ-subunit, they were introduced into strains LA and LY (Table 1) with selection on SD medium with appropriate supplements, and then transformants were tested for growth on S agar containing 2% raffinose and 2% galactose. Other strains were similarly tested by introduction of pLF16 (encoding a *GAL* promoter-zymocin γ-subunit fusion) or YCplac111 (as a control).

RESULTS

Isolation of sequences conferring resistance to *K. lactis* zymocin in high copy: We previously isolated two *S. cerevisiae* genes that conferred resistance to *K. lactis* zymocin when introduced in high copy into sensitive strains of *S. cerevisiae* (Butler *et al.* 1994). One of these encoded a $tRNA_3^{glu}$ while the other encoded *KTI12*, a gene previously defined by a recessive, zymocin-resistant mutation and recently shown to encode a component of the RNAPII Elongator complex (Frohloff *et al.* 2001). Since the previous screen was far from saturated we examined a different high-copy library for additional sequences that could confer zymocin resistance and that might, like *KTI12*, also correspond to genes defined by our collection of zymocin-resistant mutations (Butler *et al.* 1994). To avoid obtaining spontaneous zymocin-resistant mutants among our transformants, we conducted the present screen in a homozygous diploid strain (LL20-3) transformed with a yeast genomic library in pMA3a, selecting Leu$^+$ transformants directly. Since pMA3a carries the *leu2*^{d} marker it is expected to be present at very high copy number when transformants are grown under selection for Leu$^+$ (Erhart and Hollenberg 1983). Cells from ~12,500 independent transformants (~1.5 genome equivalents) were recovered from the selective plates, resuspended, and plated on

TABLE 2

Plasmids

Name	Description	Source
pARB1	1.3-kb *Eco*RI-*Sal*I fragment carrying the *GAL1*-zymocin γ-subunit gene fusion in YIplac211	Butler *et al.* (1994)
pARB7	4.8-kb *Eco*RI-*Sal*I fragment from pARB106 in YEplac181	This study
pARB15	3.5-kb *Sal*I fragment from pARB106 in YEplac181	This study
pARB18	8.2-kb *Eco*RI-*Sal*I fragment carrying *SAP155* in YCplac111	This study
pARB19	8.2-kb *Eco*RI-*Sal*I fragment carrying *SAP155* in YEplac181	This study
pARB22	5.9-kb *Eco*RI-*Sal*I fragment of pARB100 in YCplac111	This study
pARB23	6.5-kb *Eco*RI-*Sal*I fragment of pARB100 in YEplac181	This study
pARB31	3.0-kb *Pvu*II fragment of pAB106 in YEplac181	This study
pARB36	3.8-kb *Pvu*II fragment of pAB106 in YEplac181	This study
pARB100	5.25-kb fragment carrying *GRX3*/tRNA^{gln} in pMA3a	This study
pARB106	8.4-kb fragment carrying *SAP155* in pMA3a	This study
CB337	*SIS2* in YEp24	Kim Arndt
CB1317	*ADH1-CLN2* in *LEU2*-2μ	Kim Arndt
CB1418	*ADH1-PCL1* in *LEU2-CEN*	Kim Arndt
CB2606	*SAP190* in YEp24	Kim Arndt
CB2643	*SAP155* in YEp24	Kim Arndt
CB2645	*sap155^{fs}* in YEp24 (frameshift mutation in *SAP155*)	Kim Arndt
CB2819	*SAP185* in YEp24	Kim Arndt
CB2925	*SAP4* in YEp24	Kim Arndt
pDG6	3.3-kb *Psp*1406I fragment of *SAP155* in the *Cla*I site of pBluescript II SK+	This study
pDG8	3.8-kb *Xba*I-*Psp*1406I fragment of *SAP155* in YEplac181	This study
pDG11, 12	*sap155::HIS3* versions of pDG8	This study
pDG14	0.62-kb *Xho*I-*Bam*HI fragment of pARB100 in YEplac181 (*Sal*I-*Bam*HI) carrying tRNA^{gln}	This study
pDG15	1.4-kb *Spe*I-*Xho*I fragment of pARB100 in YEplac181 (*Xba*I-*Sal*I) carrying *GRX3* and tRNA^{gln}	This study
pDJ14	1.8-kb *Eco*RI-*Pst*I fragment carrying *TAP42* in YEplac195	This study
pJHW27	2.9-kb *Dra*I fragment carrying *KTI12* in YEplac181	Butler *et al.* (1994)
pLF16	1.45-kb *Eco*RI-*Hin*dIII fragment of pARB1 (carrying the *GAL1*-zymocin γ-subunit gene fusion) in YCplac111	This study
YCplac33	*CEN4 ARS1 URA3* yeast shuttle vector	Gietz and Sugino (1988)
YCplac111	*CEN4 ARS1 LEU2* yeast shuttle vector	Gietz and Sugino (1988)
YEp24	2μ *URA3* yeast shuttle vector	Botstein *et al.* (1979)
YEplac181	2μ *LEU2* yeast shuttle vector	Gietz and Sugino (1988)
YEplac195	2μ *URA3* yeast shuttle vector	Gietz and Sugino (1988)
pYF1	1.9-kb *Hin*dIII-*Bam*HI fragment carrying tRNA$^{glu}_{3}$ in YEplac181	Butler *et al.* (1994)

a range of concentrations of crude *K. lactis* zymocin in YPD agar. From 5.5×10^6 cells thus screened, ~200 clones that could grow at concentrations of 0.25% crude zymocin or above were selected. From these, 50 plasmids were recovered that conferred resistance to 1.8% crude zymocin when reintroduced into LL20-3. Restriction mapping allowed these plasmids to be classified into several different categories.

Since we had already shown that tRNA$^{glu}_{3}$ genes and *KTI12* could each confer zymocin resistance in high copy, clones from the current screen were examined by Southern blot analysis using tRNA$^{glu}_{3}$ and *KTI12* probes (not shown). This allowed identification of four *KTI12* clones and three clones encoding two tRNA$^{glu}_{3}$ loci [tE(UUC)GL2 and tE(UUC)ER3: see Hani and Feldmann (1998)], which were distinct from the two other tRNA$^{glu}_{3}$ loci we had identified previously [tE(UUC)B and tE(UUC)E2: Butler *et al.* (1994)]. Representative clones from each of the two remaining categories of clone (pARB100 and pARB106) were therefore characterized further.

High-copy *GRX3* confers *K. lactis* zymocin resistance: The entire insert of pARB100 was transferred from pMA3a into both YCplac111 and YEplac181 as an *Eco*RI-*Sal*I fragment (see Figure 1A). Since the YEplac181 derivative (pARB23) conferred zymocin resistance when transformed into LL20, we concluded that the extra high copy number of the original isolate (pARB100) imposed by *leu2^{d}* selection was not essential for the insert to function as a multicopy resistance determinant. The low-copy construct (pARB22) was introduced into a representative strain from each of the 13 complementation groups of *K. lactis* zymocin-resistant mutants described by Butler *et al.* (1994) and several transformants were

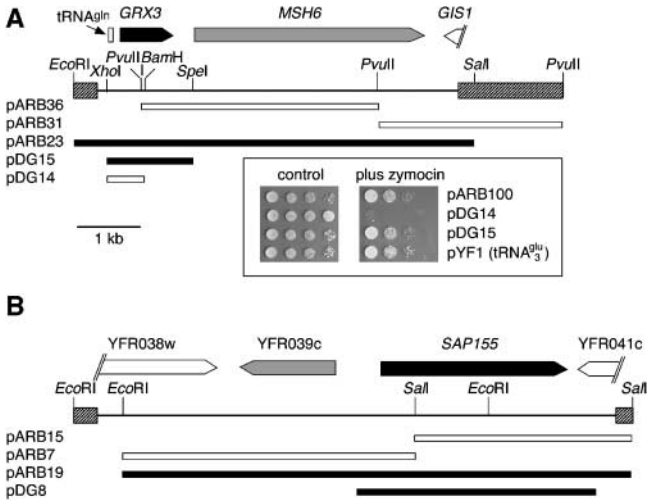

FIGURE 1.—Characterization of clones that promote zymocin resistance in high copy. Maps indicate key restriction sites in both the insert (—) and the flanking pMA3a vector sequences (▨), together with the genes encoded by the insert (▭). Below the maps, open boxes (▭) indicate subclones that failed to confer zymocin resistance in high copy, while solid boxes (▬) indicate subclones that continued to confer zymocin resistance when inserted into YEplac181. (A) pARB100 subclones. The inset compares the zymocin resistance of CY4029 (W303 *SSD1-v1*) carrying pARB100, pDG14, and pDG15 by plating 10-fold serial dilutions of cells onto YPD agar with or without *K. lactis* zymocin with a high-copy $tRNA_3^{glu}$ clone (pYF1) as a control. (B) pARB106 subclones.

tested for zymocin resistance in each case. Since complementation was not observed in any instance (not shown), we concluded that pARB100 does not correspond to any of the previously described *KTI* loci. pARB22 also failed to confer resistance on wild-type strains, demonstrating that the resistance determinant was required specifically in high copy.

DNA sequencing revealed that pARB100 carried an insert from chromosome IV that carried three genes (Figure 1A). Subclones from pARB100 were made in YEplac181 and tested for their ability to confer *K. lactis* zymocin resistance (Figure 1A). A YEplac181 subclone carrying *GRX3* (pDG15) was an effective zymocin resistance determinant (Figure 1A, inset). Since this region encodes a $tRNA^{gln}$ in addition to *GRX3* and because $tRNA_3^{glu}$ genes confer zymocin resistance in high copy, we tested a YEplac181 subclone carrying the $tRNA^{gln}$ alone (pDG14). pDG14 was unable to confer zymocin resistance (Figure 1A, inset), thereby ruling out the $tRNA^{gln}$ as the critical factor.

GRX3 is one of three related yeast glutaredoxin genes that differ from classical glutaredoxins by having a single cysteine residue at the putative active site (GRANT 2001). To determine the level at which *GRX3* functions, pARB100 was tested for its ability to protect yeast cells from growth arrest by conditional intracellular expression of the zymocin γ-subunit from the *GAL* promoter. This test has previously been used to distinguish two classes of zymocin-resistant mutant, namely those in which the zymocin's intracellular target is altered to render it insensitive and others that are resistant to exogenous zymocin but still contain a sensitive intracellular target (BUTLER *et al.* 1994). Since presence of pARB100 did not protect cells from intracellular expression of the zymocin γ-subunit (not shown), we conclude that high-copy *GRX3* is unlikely to function at the level of the zymocin's intracellular target but is more likely to operate by impairing entry of native zymocin. By comparison, high-copy *SAP155* (see below), $tRNA_3^{glu}$, or *KTI12* (BUTLER *et al.* 1994) can each confer protection from zymocin γ-subunit expression.

The zymocin resistance determinant on pARB106 is *SAP155*: DNA sequencing demonstrated that pARB106 encoded two complete genes on chromosome VI, YFR039c and *SAP155*. *SAP155* is one of four related *SAP* genes (*SAP4*, *SAP155*, *SAP185*, and *SAP190*) that show a functional relationship with the Sit4p protein phosphatase (LUKE *et al.* 1996). pARB106 subclones were made in YEplac181 (Figure 1B) and tested for their ability to confer zymocin resistance. Neither the *Eco*RI-*Sal*I nor the *Sal*I-*Sal*I fragments derived from the insert could confer zymocin resistance, although a reconstruction of these two fragments (pARB19) was completely functional in the assay. This demonstrated that the extra high copy number of pMA3a was not essential for conferring resistance and also ruled out YFR039c as the resistance determinant. In contrast, *SAP155* alone was capable of conferring resistance when subcloned into YEplac181 (Figure 1B, pDG8), confirming it as the resistance determinant on pARB106. Consistent with this conclusion, pDG8 derivatives in which the *SAP155* coding region was replaced by *HIS3* or a construct that carried a frameshift mutation in *SAP155* were unable to confer zymocin resistance (not shown). Unlike pARB100, pARB106 could protect cells from growth arrest by intracellular expression of the zymocin γ-subunit, allowing growth of strain LA on galactose-containing medium (not shown). Thus, unlike *GRX3*, high-copy *SAP155* confers zymocin resistance at the level of its intracellular target. The pARB19 insert was also cloned into YCplac111 and introduced into the representative *K. lactis* zymocin-resistant mutant strains described above. This construct (pARB18) failed either to confer zymocin resistance on wild-type strains or to complement zymocin resistance in any of the mutants (not shown), indicating that *SAP155* is needed in high copy and that it does not correspond to any of the previously described *KTI* genes.

In the process of identifying *SAP155* as the zymocin resistance determinant, a large section of the pARB106 insert surrounding the *Sal*I site was sequenced on both strands (EMBL accession no. AJ318331). The sequenced region (corresponding to chromosome VI bases 233710–237771) differs in six positions from the current sequence of chromosome VI in the *Saccharomyces* Genome

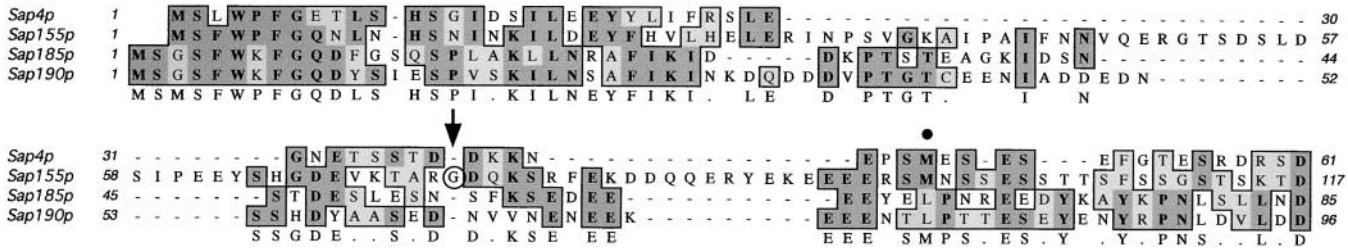

FIGURE 2.—Alignment of the predicted amino-terminal sequences of the four SAP proteins. The similarity of the amino-terminal extension to Sap155p predicted by this and other work (LUKE *et al.* 1996) to Sap4p, Sap185p, and Sap190p is indicated. • indicates the start of the Sap155p ORF predicted from current database entries. The arrow and circled amino acid denote the position of the frameshift (in the glycine codon) that extends the ORF as shown.

Database. One of these differences (deletion of G_{234450} in our sequence) extends the *SAP155* open reading frame by 97 codons, in agreement with LUKE *et al.* (1996). The putative amino-terminal extension generated by this change shows strong similarity to the amino-terminal sequences of the other three SAPs (Sap4p, Sap185p, and Sap190p; Figure 2) and is therefore likely to be correct.

Only *SAP155* and none of the other *SAP* genes function as a high-copy zymocin resistance determinant: *SAP4*, *SAP155*, *SAP185*, and *SAP190* encode a family of related proteins that function interdependently with the protein phosphatase Sit4p, each of which can partially suppress the Ts^- growth defect of *sit4-102 ssd1-d* strains (LUKE *et al.* 1996). The four SAP proteins fall into two groups based on sequence similarity, with Sap4p and Sap155p in one group and Sap185p and Sap190p in the other. These two groups are functionally distinct since, for example, Sap185p and Sap190p (but not Sap4p and Sap155p) are together required for a Sit4p function that is essential in the absence of *BEM2* (LUKE *et al.* 1996). Although Sap4p could not be found in Sit4p immune precipitates, Sap155p, Sap185, and Sap190p each form complexes separately with Sit4p and, by several criteria, cells lacking all four *SAP* genes behave very like *SIT4*-deficient cells (LUKE *et al.* 1996). We therefore next tested YEp24 clones of all four *SAP* genes for ability to confer zymocin resistance. Unlike *SAP155*, each of the other three *SAP* genes failed to protect cells from inhibition by zymocin (Figure 3A).

In addition to forming complexes with the SAPs, Sit4p also interacts with Tap42p in a TOR-dependent and rapamycin-sensitive manner (DI COMO and ARNDT 1996). However, high-copy *TAP42* also failed to promote zymocin resistance (Figure 3B). We also tested whether other genes that improve the growth defect of *sit4* mutants in high copy or when overexpressed could promote zymocin resistance. However, neither high-level expression of *CLN2* or *PCL1* (from the *S. cerevisiae ADH* promoter) nor high-copy *SIS2* conferred zymocin resistance (not shown). Thus the effect of *SAP155* in this assay is highly specific.

Since the phenotype of *sit4* mutants is highly dependent on the *SSD1* locus, we also compared isogenic *ssd1-d* and *SSD1-v* strains for zymocin sensitivity and for the effect of high-copy *SAP155*. Both *ssd1-d* and *SSD1-v* strains are inhibited by zymocin (Figure 4), although *ssd1-d* cells are slightly more zymocin sensitive (Figure 4). Furthermore, high-copy *SAP155* could promote zymocin resistance in both genetic backgrounds (Figure 4), while *TAP42* had no effect in either (Figure 3B).

Sit4 phosphatase and the SAP185/SAP190 family are required for zymocin sensitivity: We next tested the zymocin sensitivity of isogenic *SSD1-v* strains that were either wild type or deleted for *SIT4* or various combinations of *SAP* genes. As shown in Figure 5, deletion of no single *SAP* gene conferred significant zymocin resistance. In comparison, loss of *SIT4* rendered cells resistant to zymocin. High-copy *SAP155* was unable to promote additional zymocin resistance in *sit4*Δ strains (Figure 3C), consistent with high-copy *SAP155* acting through Sit4p rather than by some alternative route. Although loss of single *SAP* genes was without effect on zymocin sensitivity, the double *sap185*Δ *sap190*Δ strain was as resistant to zymocin as the *sit4*Δ strain, and all other multiple *SAP* deletion strains that also lacked both *SAP185* and *SAP190* were zymocin resistant (Figure 5). In comparison, cells lacking both *SAP4* and *SAP155* were fully zymocin sensitive. Like high-copy *SAP155*, knockout strains lacking *SIT4* or both *SAP185* and *SAP190* promoted zymocin resistance at the level of the zymocin's intracellular target, since these mutant strains were also resistant to intracellular expression of the zymocin γ-subunit from the *GAL* promoter (Figure 6). Thus zymocin sensitivity requires a Sap185p/Sap190p-mediated Sit4p function.

Since the SAPs have been shown to compete with each other for binding to Sit4p (LUKE *et al.* 1996), our data suggested a simple model for how high-copy *SAP155* might promote zymocin resistance: higher levels of Sap155p in strains with extra copies of *SAP155* might out-compete Sap185p and Sap190p for binding to Sit4p, thereby opposing the Sap185/190p-dependent Sit4p function required for zymocin sensitivity. Such a model predicts that extra copies of *SAP185* or *SAP190* should therefore counteract the effect of *SAP155*. Figure 3D

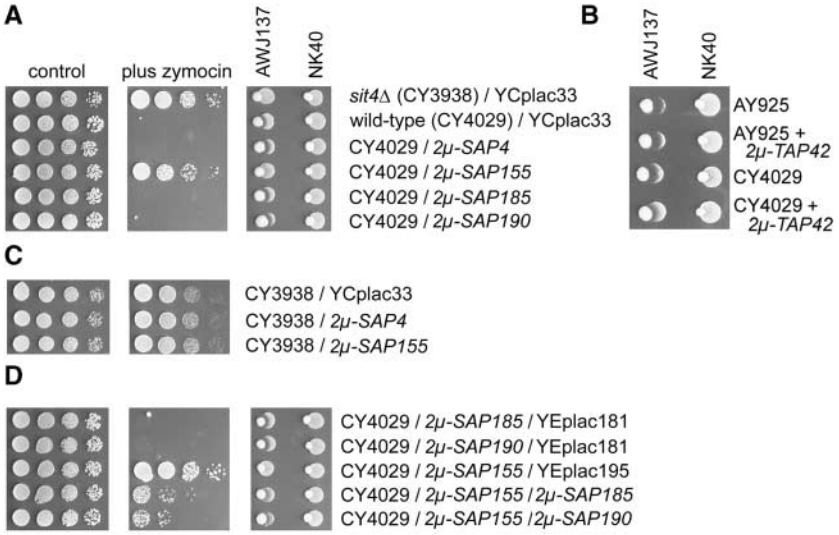

FIGURE 3.—Effect of high-copy genes on zymocin sensitivity. In A, C, and D, 10-fold serial dilutions (right to left) of either CY4029 (W303 *SSD1-v1*) or CY3938 (*sit4::HIS3*) transformed with control *URA3* vectors or 2μ-*URA3* plasmids carrying *SAP* genes were replica plated onto YPD (left) or YPD containing zymocin (middle) or assessed using the eclipse assay (A and D, right). In the latter assay, the size of the growth inhibition halo around the *K. lactis* zymocin-producing colony, inoculated on the edge of a spot of *S. cerevisiae* cells, indicates the level of sensitivity/resistance shown by the latter. In B, the eclipse assay was used to compare sensitivity of *ssd1-d2*(AY925) and *SSD1-v1*(CY4029) strains carrying high-copy *TAP42* with untransformed strains.

confirms this prediction, showing that extra copies of either *SAP185* or *SAP190* greatly reduce the ability of high-copy *SAP155* to promote zymocin resistance.

Loss of Sit4p and SAP function share common phenotypes with Elongator mutations: Since mutations in components of the RNAPII Elongator complex cause zymocin resistance, we next examined whether the *sit4*Δ or zymocin-resistant *sap*Δ strains share any of the other phenotypes shown by Elongator mutations. In addition to zymocin resistance, these phenotypes include slow growth, temperature sensitivity, and hypersensitivity to 6-azauracil. As a control for these experiments we used a strain deleted for *TOT3/ELP3*, which encodes a known component of Elongator (WITTSCHIEBEN *et al.* 1999; FROHLOFF *et al.* 2001). Either loss of *SIT4* or deletion of multiple *SAP* genes has already been shown to confer a slow growth phenotype (LUKE *et al.* 1996) that we have confirmed in our work (not shown). Both *sit4*Δ and the quadruple *sap* deletion strain also conferred temperature sensitivity and 6-azauracil hypersensitivity (Figure 7). Loss of Sap185p and Sap190p also conferred both phenotypes, although the level of 6-azauracil hypersensitivity was lower and comparable to that shown by the *tot3*Δ control strain. Surprisingly, the *sap4*Δ *sap155*Δ double mutant was more hypersensitive to 6-azauracil despite failing to confer temperature sensitivity or zymocin resistance. Because *SIT4* was first identified through mutations that affect RNAPII transcription of a variety of genes (ARNDT *et al.* 1989), it is possible that Sit4p phosphatase is required to activate Elongator function. Such activation might involve effects on either the expression or the activity of the Elongator complex. However, when the mRNA levels of *TOT1/ELP1*, *TOT2/ELP2*, *TOT3/ELP3*, *TOT4/KTI12*, and *TOT5* were examined by RT-PCR, essentially identical levels of mRNA for each of these Elongator components were found in wild-type and *sit4*Δ strains (not shown). Furthermore, comparison of the protein levels of Tot1p to Tot5p in strains with or without high-copy *SAP155* showed essentially identical protein levels (not shown). Thus, if Sit4p does affect Elongator function, it is more likely to result from post-translational effects. If Sit4p and Elongator do function in a common process, then a double mutant would not be expected to show any additional growth defect. Conversely, if they act in distinct processes, then the slow growth of *sit4*Δ strains would be predicted to be additive with that of Elongator deletion mutations. We therefore crossed a *sit4*Δ strain with either *TOT3/ELP3* or *TOT4/KTI12* deletion strains in a common genetic background and compared the growth defect of the single and double knockout progeny. The absence of any additional growth defect when *sit4*Δ was combined with either *tot3*Δ or *tot4*Δ (Figure 8) is consistent with the notion that the phosphatase and the Elongator complex may function in a common pathway.

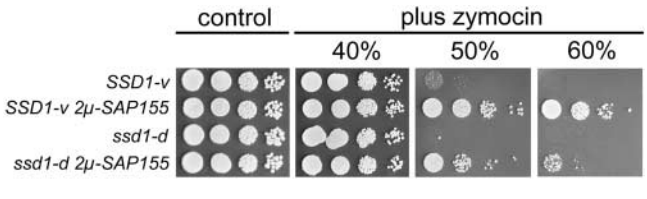

FIGURE 4.—High-copy *SAP155* promotes zymocin resistance in *ssd1-d2* and *SSD1-v1* strains. Tenfold serial dilutions (left to right) of *ssd1-d2* (AY925) or *SSD1-v1*(CY4029) strains with or without high-copy *SAP155* (pARB19) were spotted onto YPD agar containing 65% (v/v) culture supernatant from the zymocin nonproducing *K. lactis* strain NK40 (control) and onto YPD agar containing the indicated concentration of AWJ137 supernatant.

DISCUSSION

***GRX3* and zymocin resistance:** Glutaredoxins and thioredoxins are small, heat-stable oxido-reductases containing two active-site cysteine residues that have a vari-

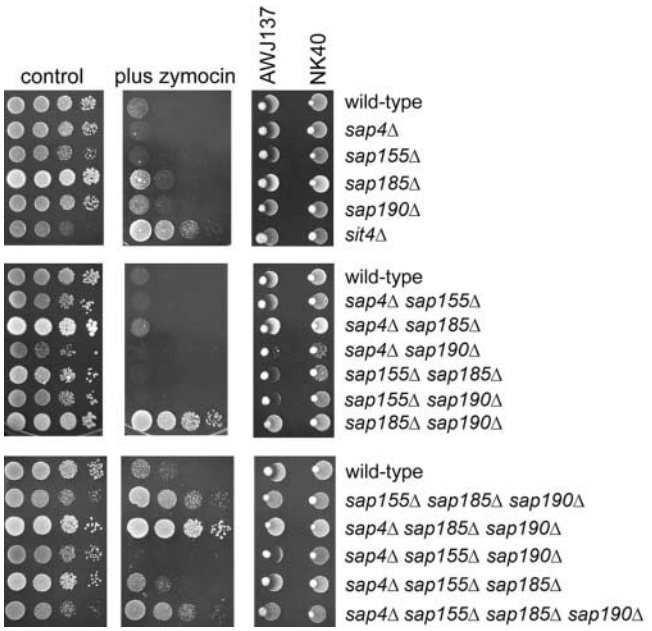

FIGURE 5.—Sit4 phosphatase and either *SAP185* or *SAP190* are required for zymocin sensitivity. Tenfold serial dilutions (left to right) of strains were spotted onto YPD agar (left) or YPD agar containing zymocin (middle) or assessed using the eclipse assay (see Figure 3 legend). All strains were W303 *SSD1-v1* and either wild type (CY4029) or lacking *SIT4* (CY3938) or one or more *SAP* genes as indicated (CY5220, CY5224, CY917, CY4380, and DJY8-DJY17; see Table 1). Note that the resistance of *sit4*Δ and some multiple *sap*Δ strains is evident despite their weaker growth.

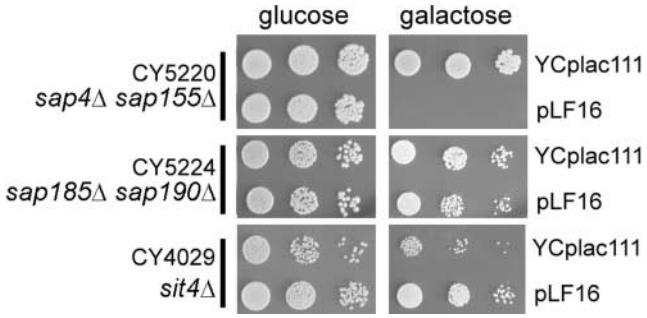

FIGURE 6.—Loss of *SIT4* or combined loss of *SAP185* and *SAP190* is resistant to intracellular zymocin expression. The indicated strains were transformed with either YCplac111 (as a control) or pLF16, which expresses the zymocin γ-subunit from the *GAL* promoter. Tenfold serial dilutions of cells were plated out on YP medium containing either 2% glucose or 2% galactose. While growth of the *sap4*Δ *sap155*Δ strain was inhibited by expression of the zymocin γ-subunit on galactose, strains lacking either *SIT4* or both *SAP185* and *SAP190* were able to grow despite induction of the *GAL*-zymocin γ-subunit construct. The wild-type control strain (CY4029) behaved identically to CY5220 (not shown).

ety of proposed roles, including protein folding and repair of oxidatively damaged proteins (HOLMGREN 1989). Grx3p and Grx4p are two highly related yeast glutaredoxins that belong to a subfamily that also includes Grx5p. Unlike other glutaredoxins and thioredoxins (including yeast Grx1p and Grx2p), Grx3p, Grx4p, and Grx5p have only a single cysteine residue at the active site in place of the usual -C-X-X-C- motif (RODRIGUEZ-MANZANEQUE *et al.* 1999; GRANT 2001). Similar glutaredoxin-like proteins, apparently with a single, active-site cysteine, are also found in other eukaryotic organisms. Like protein disulfide isomerases (FREEDMAN *et al.* 1994), which also contain thioredoxin-like domains, glutaredoxins can catalyze the reduction of disulfide bonds using glutathione, although protein disulfide reduction is generally thought to involve a dithiol mechanism (BUSHWELLER *et al.* 1992). It is therefore unclear whether Grx3p and its paralogues can function in this way, although Grx3p and Grx4p do have an additional cysteine elsewhere in the polypeptide that might conceivably be involved in redox function. We have identified *GRX3* as a sequence that protects cells from *K. lactic* zymocin in high copy, but even on the original pMA3a vector (which uses *leu2*^{d} selection), *GRX3* could not protect cells from induction of the zymocin γ-subunit from the *GAL* promoter. Our data therefore suggest that elevated *GRX3* dosage does not act by protecting the intracellular target from zymocin and that the most likely mode of action therefore concerns entry of zymocin into sensitive cells. Zymocin uptake is a process that is poorly understood at present, although initial binding to sensitive cells requires the chitin binding domain and chitinase activity of the α-subunit (BUTLER *et al.* 1991a; JABLONOWSKI *et al.* 2001). Zymocin entry into the cell may involve the intensely hydrophobic β-subunit, to which the zymocin γ-subunit is known to be disulfide bonded (STARK *et al.* 1990). If this disulfide bond is required for γ-subunit uptake, it is possible that higher-than-normal extracellular levels of Grx3p might promote reduction of this bond and thereby block toxin entry. Since it is most likely that Grx3p is not normally an extracellular protein, it could be released by lysis of some proportion of cells in a colony, thereby protecting the remaining viable cells from zymocin. Alternatively, excess Grx3p within the cell might alter the normal redox balance and interfere with release of the γ-subunit in that way. Finally, high-copy *GRX3* might also function less directly in blocking zymocin entry, for example, by leading to reduced cell-wall chitin levels. A better understanding of the zymocin entry process will be required to determine how *GRX3* might antagonize zymocin function.

The role of Sit4p phosphatase in zymocin sensitivity: Starting with the finding that high-copy *SAP155* confers resistance to *K. lactis* zymocin, we have demonstrated in this work that cells need a functional Sit4p protein phosphatase for zymocin sensitivity and that either Sap185p or Sap190p is also required. Although (like zymocin-treated cells) *ssd1-d2* strains deficient in Sit4p function arrest in the G_1 phase of the cell cycle, Sit4p

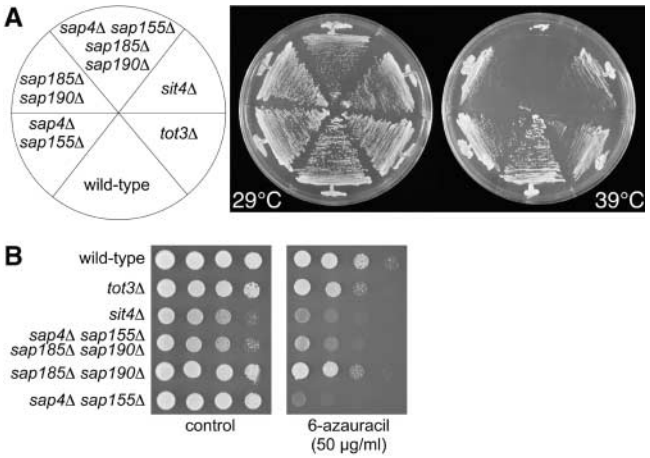

FIGURE 7.—*sit4*Δ zymocin-resistant mutants share other phenotypes with RNAPII Elongator mutants. In A, W303 *SSD1-v1* strains of the indicated genotype were tested for growth at 39°. Only the wild type and *sap4*Δ *sap155*Δ mutant show significant growth at this temperature, as indicated by the presence of individual colonies growing to the center of the plate. (B) Sensitivity of strains toward 6-azauracil. Tenfold serial dilutions of the indicated strains were plated on SD agar without uracil and either containing or lacking 50 μg/ml 6-azauracil. All strains contained YCplac33 (carrying *URA3*).

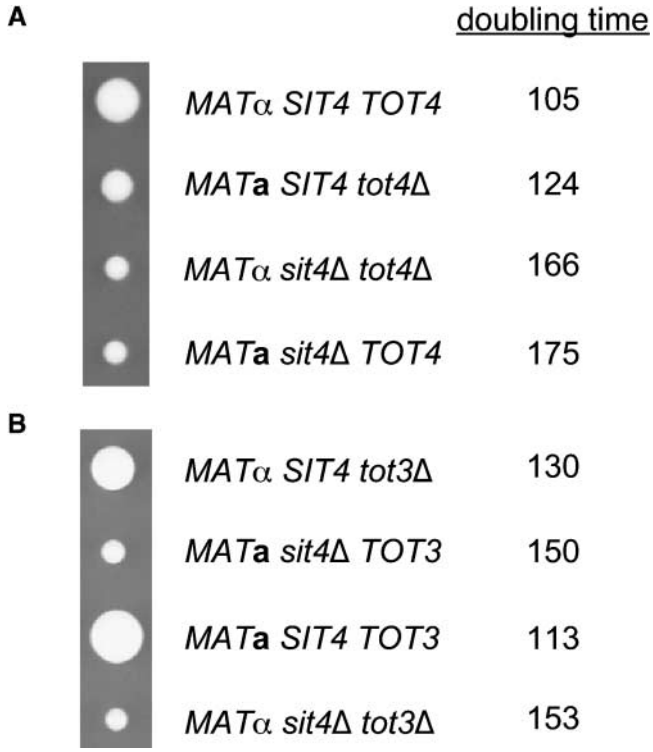

FIGURE 8.—Loss of Elongator function fails to show an additive defect with loss of *SIT4*. W303 *SSD1-v1* strains containing *tot4/kti12*Δ (LFY6; A) or *tot3/elp3*Δ (LFY5; B) mutations were crossed with CY4029 (*sit4*Δ) and sporulated, and tetrads were dissected. The growth of spores from a typical tetrad from each cross is shown, together with the doubling time (in minutes) subsequently determined in liquid YPD medium.

itself cannot be the intracellular target of the zymocin; *SSD1-v1* strains in which Sit4p function is dispensable are sensitive to zymocin inhibition. Sap4p, Sap155p, Sap185p, and Sap190p are Sit4p-associated proteins that could be either regulators or effectors of Sit4p function and our data underscore the division of these four proteins into two families (Sap4p/155p and Sap185p/190p), each of which has at least some unique function. Thus, only combined loss of Sap185p and Sap190p leads to zymocin resistance, while combined loss of Sap4p and Sap155p or triple *sap* deletion strains still containing either Sap185p or Sap190p are zymocin sensitive. This is consistent with previous work showing that *SAP* genes from one family are ineffective in high copy at rescuing the growth defect shown by loss of the other family and that *sap185*Δ *sap190*Δ (but not *sap4*Δ *sap155*Δ) is synthetically lethal with loss of *BEM2*, as is loss of *SIT4*.

High-copy *SAP155* confers zymocin resistance by a mechanism involving competition between the different *SAP* proteins, since its effect is antagonized by elevated dosage of either *SAP185* or *SAP190*. Although we have not looked directly at the effect of *SAP155* on the association of the different SAPs with Sit4p, it has previously been clearly demonstrated that high-copy *SAP155* effectively dissociates Sap185p and Sap190p from Sit4p immunoprecipitates, while, conversely, high-copy *SAP190* greatly reduced the level of Sap155p in Sit4p immune complexes (LUKE *et al.* 1996). Thus, given that the *sap185*Δ *sap190*Δ double mutant is zymocin resistant, all our data are fully consistent with high-copy *SAP155* interfering with the formation of the Sap185p·Sit4p and Sap190p·Sit4p complexes. The alternative model, whereby either elevated dosage of *SAP155* or loss of *SAP185* and *SAP190* promote zymocin resistance by increasing the amount of Sap155p·Sit4p in the cell, is inconsistent with the finding that loss of Sit4p itself also causes resistance.

Another role of Sit4p phosphatase is in the TOR signaling pathway, where its interaction with Tap42p is important (DI COMO and ARNDT 1996). Tap42p is bound to Sit4p and to the catalytic subunit of yeast PP2A ($PP2A_C$) under conditions of nutrient sufficiency, but on starvation or when the TOR kinases are inhibited by rapamycin, this interaction is lost. On release from Tap42p, Sit4p has been proposed to dephosphorylate at least some of the downstream effectors of the TOR pathway (BECK and HALL 1999). Since Tap42p binds to Sit4p in a complex distinct from the Sap·Sit4p complexes we also tested whether high-copy *TAP42* could confer zymocin resistance. However, its failure to do so suggests that it is unable to compete effectively with Sap185p and Sap190p for binding to Sit4p, despite evidence that more Sit4p can associate with Tap42p in *sap*-deficient strains (DI COMO and ARNDT 1996). Since rapamycin also causes cells to arrest in G_1 it is attractive to suppose that zymocin might mimic rapamycin

in blocking growth-promoting signaling through the TOR pathway. Like rapamycin-treated cells, *tap42-11* mutants arrest at their restrictive conditions because they induce a range of responses that is normally inhibited by TOR signaling; at their permissive temperature, however, they show dominant rapamycin resistance because the mutant protein cannot dissociate from Sit4p or $PP2A_C$ when TOR activity is inhibited (Di Como and Arndt 1996). However, since we have found that the *tap42-11* mutant is fully sensitive to zymocin (not shown), this appears to rule out any effect of zymocin either on TOR itself or any upstream activators of TOR. While zymocin might conceivably block the TOR signaling at the level of Tap42p, the failure of high-copy *TAP42* to confer even a low level of zymocin resistance does not support this notion.

Seven genes in addition to *SIT4* and *SAP185/SAP190* that confer zymocin resistance when deleted have now been identified and, of these, at least five encode components of Elongator, a multiprotein complex associated with the elongating form of RNAPII (Butler *et al.* 1994; Frohloff *et al.* 2001; our unpublished data). Elongator mutants share a number of phenotypes in common and we have found that mutants lacking *SIT4* also exhibit these phenotypes. Thus an intriguing possibility is that Sit4p and Elongator are functionally linked and that, for example, Elongator function requires dephosphorylation by Sit4p. This is consistent with previous work showing that *sit4* mutations can affect the transcription of many different genes (Arndt *et al.* 1989) and would provide a mechanism to link Sit4p to the transcriptional machinery. Further investigation will reveal if any of the components of the Elongator complex are phosphorylated and, if so, whether their phosphorylation state changes in strains lacking *SIT4*. Our failure to detect changes in either the mRNA or protein levels of several key components of Elongator would be consistent with such post-translational regulation. Like Sit4p, Elongator function is also dispensable and so it is at first sight difficult to understand how Elongator could be the target of zymocin. One possibility is that zymocin might block the recycling of RNAPII in an Elongator-dependent manner, *i.e.*, by converting Elongator into a cellular poison. In support of this idea, it has been shown that several RNAPII-dependent genes are downregulated by zymocin treatment whereas RNAPI transcription is not affected (Frohloff *et al.* 2001). In addition, cells with reduced levels of Rpb1p are hypersensitive to zymocin (R. Schaffrath, unpublished data), consistent with the notion that RNAPII function becomes limiting in zymocin-treated cells.

We are extremely grateful to Kim Arndt for supplying many of the plasmids and strains used in this study. Thanks are also due to Mick Tuite for providing the pMA3a library, to Vera Martin for generating strain LL20-2, to Evelyn Tait for the *TAP42* clone, to Doug Stirling for critical comments, and to the CRC Nucleic Acid Structure Research Group at Dundee for the synthesis of oligonucleotides. D.J. was supported by a Federation of European Biochemical Societies Fellowship and L.F. by a Federation of European Microbiological Societies Fellowship. This work was supported by project grant FG94/518 from the Agricultural and Food Research council to M.J.R.S. and by grants from Deutsche Forschungsgemeinschaft (Scha 750/2-1 and 2-2) to R.S.

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Communicating editor: P. Russell