The nuclear gene encoding the Sit4 protein phosphatase was identified in the budding yeast *Kluyveromyces lactis*. *K. lactis* cells carrying a disrupted *sit4* allele are resistant to oligomycin, antimony, ketoconazole, and econazole but hypersensitive to paromomycin, sorbic acid, and 4-nitroquinoline-N-oxide (4-NQO). Overexpression of *SIT4* leads to an elevation in resistance to paromomycin and to lesser extent tolerance to sorbic acid, but it has no detectable effect on resistance to 4-NQO. These observations suggest that the Sit4 protein phosphatase has a broad role in modulating multidrug resistance in *K. lactis*. Expression or activity of a membrane transporter specific for paromomycin and the ABC pumps responsible for 4-NQO and sorbic acid would be positively regulated by Sit4p. In contrast, the function of a Pdr5-type transporter responsible for ketoconazole and econazole extrusion, and probably also for efflux of oligomycin and antimony, is likely to be negatively regulated by the phosphatase. Drug resistance of *sit4* mutants was shown to be mediated by ABC transporters as efflux of the anionic fluorescent dye rhodamine 6G, a substrate for the Pdr5-type pump, is markedly increased in *sit4* mutants in an energy-dependent and FK506-sensitive manner.

The occurrence of multidrug resistance (MDR) is one of the main obstacles to the successful treatment of cancer. When treated with chemotherapeutic drugs, many cancer cells develop resistance to a variety of structurally and functionally unrelated compounds (for review, see Ref. 1). In most cases, MDR is mediated by an increased expression of the integral membrane multidrug transporters (reviewed in Refs. 2–4). These membrane proteins, known as ATP-binding cassette (ABC) transporters, function in an ATP-dependent way probably by increasing drug efflux and consequently lowering their intracellular accumulation. In a similar manner, many pathogenic microorganisms such as *Candida albicans* and *Plasmodium falciparum* can use the ABC transporter-mediated drug efflux mechanism to evade chemotherapy (5–8). However, despite the rapidly growing number of ABC transporters identified in various organisms, little is known about how activities of the drug transporters are modulated and how aberrant regulation of the expression of ABC transporter genes contributes to the acquisition of MDR in *vivo*.

The ABC transporter-mediated drug efflux mechanism is evolutionarily conserved and occurs in a variety of living organisms ranging from bacteria to humans. An example is a recent work demonstrating that the *Lactococcus lactis* ABC transporter LmrA is able to confer MDR in human cells (9). The recently completed genome sequencing project of *Saccharomyces cerevisiae* has revealed the presence of as many as 29 ABC proteins belonging to the ubiquitous ABC superfamily (10) that transport a wide range of chemical compounds (11, 12). The yeast ABC proteins so far characterized, such as Pdr5, Sqq2, Ycf1 and Yor1, confer MDR with physiological and biochemical properties very similar to the human *MDR1*-encoded P-glycoprotein (P-gp) and to Mrp1, which is known as a multidrug resistance-associated protein (13–22). Functional similarities between yeast and human ABC transporters have also been shown by a number of studies showing that expression of human P-gp and Mrp1 confers drug resistance in yeast (23–27).

Extensive efforts have been directed to understanding the regulation of ABC transporter activity at both the transcriptional and post-translational levels. Three transcriptional activators of the Cys6 zinc finger type have been genetically identified in *S. cerevisiae* (28–32). The Pdr1 and Pdr3 proteins control the transcriptional levels of *PDR5*, *SNQ2*, *YOR1*, *PDR10*, and *PDR15* by direct binding to DNA in the promoter of the target genes (33–37). The Yrr1 protein is involved in the regulation of *SNQ2* (29). However, the mechanism of the regulatory pathway upstream of *PDR1*, *PDR3*, and *YRR1* remains elusive. Two recent studies have revealed a functional link of the Pdr1 and Pdr3 transcriptional factors to the yeast homologues of the stress-dependent transcriptional factor AP1 and the heat shock protein Hsp70 (38, 39). These observations suggest the presence of a signaling pathway upstream of Pdr1 and Pdr3 in the cellular response to drug stress.

At a post-translational level, attention has been directed to a possible role of protein phosphorylation/dephosphorylation in the modulation of ABC transporter activity. Because the human P-gp is phosphorylated *in vivo*, an approach is to develop chemosensitizers that inhibit P-gp function at the level of phosphorylation and reverse the MDR phenotype in tumor cells. Early studies have demonstrated that a change in the state of phosphorylation of P-gp has been associated with differences in relative drug resistance of mammalian cells, suggesting that the phosphorylation/dephosphorylation mechanisms may be...
involved in the regulation of the efflux activity of the drug transporter (40, 41). More recently, similar results were obtained with the human Mrp1 transporter. By using protein kinase inhibitors, it has been shown that phosphorylation of the serine residues of Mrp1, probably by protein kinase C, plays an important role in modulating drug accumulation in resistant cells (42, 43). Biochemical and genetic studies identified four serine residues in a basic domain of the linker region of the human P-gp that are accessible and recognized as major targets for phosphorylation by protein kinase C or protein kinase A. However, recent mutational analysis showed that a mutant P-gp, with the putative phosphorylation sites for protein kinase C within the linker region changed to non-phosphorylatable alanine residues, or to aspartic acid residues to mimic permanently dephosphorylated serine residues, is still functionally active to diminish drug accumulation within cells (44, 45). This suggests that phosphorylation by protein kinase C may not play a role in regulating drug transport of P-gp. Whether the phosphorylation of an acidic domain of the linker region is involved in the modulation of drug transport activity remains to be established (46). By contrast, in S. cerevisiae, a phosphorylation site has been proposed in the Ycf1 protein (21), which upon transcription, complements the SIT4-1 mutant CW2-8B, indicating that phosphorylation of the serine residues of Mrp1, probably by protein kinase C, may not play a role in regulating drug transport of P-gp.

**EXPERIMENTAL PROCEDURES**

**Strains and Media**—Table I lists the yeast strains used in this study. Complete medium (YPD) contains 0.5% Bacto-yeast extract, 1% Bacto-peptone, and 2% glucose. YPD medium (YPD) contains 2% glycerol in place of glucose. Glucose minimal medium (GMM) contains 0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, and 2% glucose. Nutrients essential for auxotrophic strains were added at 25 μg/ml for bases and 50 μg/ml for amino acids. ME medium used for sporation of K. lactis contains 5% malt extract and 2% Bacto-agar.

**Resistance to antimycins A, oligomycin, and paromomycin** was tested on GlyYP plates. Resistance to ketoconazole and 4-nitroquinoline-N-oxide (4-NQO) was tested on GMM plates supplemented with appropriate amino acids to meet auxotrophic requirements. Resistance to econazole was tested on GYP. For testing sensitivity to sorbic acid, the GYP medium was adjusted to pH 4.5 according to Piper and co-workers (47). The drugs antimycin A, oligomycin, econazole, and rhodamine 6G used in this study were all purchased from Sigma and dissolved in ethanol. Paromomycin (Parke-Davis) was dissolved in sterile water. 4-NQO (Sigma) was dissolved in acetone, whereas the stock solution for ketoconazole (ICN Pharmaceuticals) was prepared with Me2SO.

**Manipulation of K. lactis—**Transformation of K. lactis was performed by the lithium acetate/dimethyl sulfoxide method (48) as described in detail (49). Genomic DNA was extracted from protoplasts obtained by Zymolyase treatment (50). Sporulation of K. lactis diploid strains was induced on ME agar for 2–3 days. Tetrad analysis was performed using a de Fonbrune micromanipulator following treatment of ascis with Zymolyase 20T (0.7 mg/ml in 1 M sorbitol, Seikagaku Co., Tokyo, Japan) for 10 min at 30 °C. Spores were germinated at 28 °C for 2–3 days on GYP medium.

**Isolation of K. lactis Oligomycin-resistant Strains**—To obtain oligomycin-resistant mutants, strain PM6-7A was grown overnight in liquid GYP medium, and approximately 6 × 10^7 cells were spread on GlyYP plates containing 1.0 μg/ml oligomycin. After 1 week at 28 °C papillae were transferred to oligomycin-GlyYP plates. Oligomycin-resistant clones were then grown in liquid GYP for 48 h before being streaked onto drug-free GYP plates. After 2 days at 28 °C colonies were replica-plated onto oligomycin-GlyYP plates to examine the stability of the oligomycin-resistant phenotype. Stable oligomycin-resistant clones were retained for further analysis.

**Nucleic Acid Manipulations**—Standard techniques were used for generating recombinant DNAs and performing DNA blot hybridizations as described by Sambrook et al. (51). The integrative plasmid pURA-oli was constructed by the insertion of a 1.8-kb HindIII-SalI fragment, containing the K. lactis SIT4 gene, into the HindIII and SalI sites of the URA3-based integrative vector pUC-URA3/4 (52). The same fragment was cloned into the HindIII and SalI sites of the K. lactis vector pUK-S11 (52) to produce pUK-oli. The 3.1-kb KpnI-SalI fragment containing KISIT4 was cloned into the KpnI and SalI sites of the K. lactis overexpression vector pCXJ3 (53), the K. lactis/s. cerevisiae multicopy vector pCXJ15, and the S. cerevisiae centromeric vector pFL39 (54), to produce pCXJ3-KISIT4, pCXJ15-KISIT4, and pFL39-SIT4, respectively, constructs pCXJ22-ScSIT4, a 1.98-kb SacI-I fragment, containing the S. cerevisiae SIT4 (kindly provided by K. Arndt), was blunt-ended with T4 DNA polymerase and ligated to the SmaI-digested S. cerevisiae K. lactis shuttle vector pCXJ22 (53). A 1.98-kb HindIII-EcoRI fragment containing ScSIT4 was cloned into the HindIII and EcoRI sites of pCXJ15 to produce pCXJ15-ScSIT4.

**Cloning and Sequencing of KlSIT4**—The K. lactis sit4 mutant CW2-8B was transformed with a K. lactis partial Sac3A1 genomic library cloned in the K. lactis Escherichia coli shuttle vector KEp6 (55). The vector contains the S. cerevisiae URA3 gene, which upon transformation, complements the ura1 defect of K. lactis. Approximately 8,300 Ura" transformants were scored and replica-plated onto GlyYP. After incubation at 37 °C for 2 days, 11 Gly" colonies were obtained. Further analysis confirmed that all 11 clones show segregation of the Ura" and the Gly" phenotypes at 37 °C after growth in non-selective medium. Total DNA was extracted from the transformants, and plasmids were rescued into E. coli. Restriction enzyme analysis showed that the 11 plasmids are identical and derived from a single chromosomal locus. A physical map was established from one of the complementing plasmids, pOli2/1, which contains an insert of 7.05 kb (Fig. 2A). Subcloning of KISIT4 was performed as follows. The plasmid pOli2/1 was digested by BanIII, EcoRI, HindIII, KpnI, and SalI plus XhoI, respectively, followed by self-ligation that created a series of internal deletions in the insert DNA of pOli2/1. The resulting plasmids were re-introduced into CW2-8B by transformation and examined for complementation of the sit4 mutation on GlyYP at 37 °C. This enabled us to localize the KISIT4 gene on a 1.8-kb HindIII-SalI fragment on the right end of the insert DNA as shown in Fig. 2A. The nucleotide sequence of KISIT4 was determined by the dideoxy chain termination method (56) after subcloning the 1.8-kb DNA fragments into pTZ18U and 19U (Amersham...
Pharmacia Biotech). Templates for sequencing were obtained by using DNase I to create a series of nested deletions from the ends of yeast DNA insert. The deduced protein sequence was compared with sequences in the Swiss-Prot data bases.

Disruption of KlsIT4—The 1.8-kb HindIII-SalI fragment containing KlsIT4 was cloned into pTZ18U to produce pTZ18-oli. The plasmid pTZ18-oli was digested with BclI that creates an internal deletion within the SIT4 coding region (Fig. 2A). A 1.05-kb BglII fragment containing the URA3 gene of S. cerevisiae was then inserted to produce pKlsIT4::URA3. Disruption of the chromosomal SIT4 gene was achieved by one-step gene replacement (57) with a 2.05-kb EcoRI-HindIII fragment containing the disrupted gene isolated from pKlsIT4::URA3. Following transformation of the haploid strain PM6-7A, Ura+ colonies were screened and tested for stability of uracil independence by growth on non-selective medium. Genomic DNA from stable strains was examined for disruption of the resident gene by Southern blot analysis.

ATPase Activity Determination—Preparation of K. lactis mitochondrial extracts has been described in a previous paper (58). The mitochondrial ATPase activity assay was performed essentially according to Law et al. (59).

Flow Cytometry of Yeast Cells—K. lactis strains were grown in complete medium to an A600 of about 1.0. For steady state rhodamine 6G accumulation assays, rhodamine 6G was added to a final concentration of 5 μM, and dye-loading was allowed for 60 min at 30 °C. Rhodamine 6G efflux was stopped by a 1:10 dilution of the cells in ice-cold water. For the measurement of dye efflux, cells were washed, resuspended in phosphate-buffered saline (PBS) containing 10 mM sodium azide, and loaded with rhodamine 6G (5 μM) for 60 min at 30 °C, with or without a 30-min preincubation with the inhibitor FR506 (10 μM final concentration). After dye-loading, cells were harvested, washed, and resuspended in prewarmed PBS containing 1% glucose to initiate rhodamine 6G efflux. Efflux was allowed for 15 min at 30 °C and then stopped by dilution of the cells in ice-cold water. Intracellular rhodamine fluorescence was analyzed with a FACSCalibur flow cytometer (Becton Dickinson Medical Systems) using Cell Quest software.

RESULTS

Identification of K. lactis Nuclear Mutations Conferring Oligomycin Resistance—In the course of our studies on the K. lactis mitochondrial F1-ATPase that is involved in the maintenance of mitochondrial genome integrity (58, 60, 61), we sought K. lactis mutants resistant to the antibiotic oligomycin. This screening procedure was based on observations that the majority of oligomycin-resistant mutants in S. cerevisiae and K. lactis occur in the mitochondrial DNA-encoded subunits 6 and 9 of the ATP synthase F0 complex (62–65). By this means we hoped to examine whether any structural and functional alterations in the F0 subunits would affect the integrity of the mitochondrial genome. From these studies we have identified two mutants that are resistant to oligomycin due to a mutation in a nuclear gene.

K. lactis strains PM6-7A/oli4 and PM6-7A/oli18 are resistant to 1.0 μg/ml oligomycin on GlyYP medium compared with the wild-type parent PM6-7A whose growth is completely inhibited by the drug (Fig. 1). PM6-7A/oli4 was crossed to the wild-type strain CK56-16C to produce the diploid CW2. Sporulation and analysis of 19 tetrads from CW2 showed that the oligomycin-resistant trait segregated (2R):2S) in all tetrads, indicating that the drug resistance was conferred by a single nuclear allele rather than by mutations in mtDNA-encoded genes. Because the mutation can be complemented by the cloned SIT4 gene (see below), we designated the allele from the isolate PM6-7A/oli4 as sit4-1. As a sit4-1/ SIT4 diploid strain is oligomycin-sensitive, the sit4-1 allele is thus recessive. The mutation in the second isolate, PM6-7A/oli18, is likely to be allelic to sit4 as the mutant phenotype can be complemented by SIT4 (data not shown). This allele has been designated sit4-2.

To see whether resistance to oligomycin is related to any structural and functional alterations in the mitochondrial F,F0-ATP synthase as a result of sit4 mutations, ATPase activity was measured in isolated mitochondria. We found that the sit4-1 strain CW2-8B and the sit4 null mutant CK254/1 (see below) retain a high mitochondrial ATPase activity that is as sensitive as in wild-type cells to oligomycin inhibition (data not shown). The sensitivity of the ATP synthase to oligomycin in the mutants suggests that the drug resistance observed is not due to alterations in the biochemical properties of F,F0-ATP synthase. Rather, it raises the possibility that SIT4 controls the function of a membrane transporter that affects accumulation of oligomycin within cells.

sit4 Mutants Are Defective in Respiratory-dependent Growth—Although sit4 mutants can grow on glycerol medium with or without supplementation of oligomycin, a defect in the respiratory-dependent growth is noticeable on GlyYP. Compared with the wild-type strain PM6-7A, the mutants have a significantly slower growth after incubation for 2 days at 30 °C (not illustrated). Moreover, growth on GlyYP can be completely inhibited when cells are incubated at 37 or 19 °C (Fig. 1). The temperature- and cold-sensitive respiratory-deficient phenotype was also observed when cells were grown on plates containing 2% ethanol in place of glycerol.

Cloning of KlsIT4—The temperature-sensitive respiratory growth of sit4 mutants has allowed us to isolate KlsIT4 by complementation on glycerol medium. After transformation of CW2-8B with a K. lactis genomic DNA library, transformants were screened for the ability to grow on GlyYP at 37 °C. Plasmids were rescued from the Gly+ clones, and physical maps were established. All the plasmids from 11 independent transformants contained the same chromosomal locus with an insert size of 7.05 kb. The physical map of the plasmid pOli2/1 is shown in Fig. 2A. When a 1.8-kb HindIII-SalI fragment is subcloned into the K. lactis vector pUK-S11, the resulting plasmid, pUK-oli, was found to complement the Gly- phenotype of

**Fig. 1. Growth and multidrug-resistant phenotypes of K. lactis sit4 mutants.** Strains PM6-7A (wild-type (WT), CW2-8B (sit4-1), PM6-7A/oli (sit4-2), and CK254/1 (sit4-1::URA3)) were diluted to 5 x 10^6 cells/ml, and 10-μl aliquots were applied to GlyYP, GlyYP supplemented with oligomycin (Oli) and antimycin (Ant) at a concentration of 1 μg/ml and 0.2 μM, GMM supplemented with ketoconazole (Keto) at 4 μg/ml, and GYP supplemented with econazole (Eco) at 0.5 μg/ml. The GlyYP plates were incubated at 30 °C for 4 days, 37 °C for 5 days, and 19 °C for 7 days. The plates containing the drugs were incubated at 28 °C for 4–5 days before being photographed.

[Image of growth and multidrug-resistant phenotypes of K. lactis sit4 mutants]
the sit4-1 mutant CW2-8B and the sit4-2 strain PM6-7A/oli18. Confirmation that the cloned DNA fragment contains the same locus as the one defined by the sit4 mutations was performed first by cloning the 1.8-kb HindIII-SalI fragment into the integrative vector pUC-URA3/4 carrying the S. cerevisiae URA3 gene. The resulting plasmid, pURA-oli, was targeted to the chromosomal sit4-1 locus of CW2-8B after digestion with Clal which cuts within the SIT4 coding sequence. The transformant, CK231, grew on Glycerol medium at 37 °C. This implies that this segment of DNA has single copy-complementing activity and is thereby unlikely to be a multicopy suppressor of sit4-1. Second, pURA-oli was targeted onto the chromosomal SIT4 locus of the wild-type strain PM6-7A (uraA1). Two independent transformants, CK230/3 and CK230/4, were crossed to the CW2-8B (sit4-1 and uraA1), and the resulting diploids, CK233 and CK234, were sporulated and dissected for tetrad analysis. The Ura+ and OliK phenotypes segregated 2+/-2- in a total of 28 tetrads analyzed. Moreover, OliK co-segregated with Ura+. These results suggest that the cloned DNA fragment is allelic to sit4-1. Additional evidence supporting this view is the observation that a disrupted allele of the SIT4 gene has the same phenotypes as sit4-1 and sit4-2 (see below).

Nucleotide sequence analysis of the 1.8-kb HindIII-SalI DNA fragment revealed the presence of a 927-bp open reading frame capable of encoding a protein of 309 amino acids with a predicted molecular mass of 35,285 daltons. Comparison of the deduced protein with sequences in the Swiss-Prot data bases revealed that it has high homology to a large group of serine/threonine protein phosphatases from various sources. The protein showing the highest level of homology is the gene product of the S. cerevisiae SIT4 gene (66) which shares 92.9% identical residues (not illustrated). The Sit4 protein together with its homologues, Ppe1 protein of the fission yeast Schizosaccharomyces pombe (67–69), PPV of Drosophila melanogaster (70), and the human PP6 enzyme (71), have been thought to be the catalytic subunit of a type 2A-related serine/threonine protein phosphatase. The proteins Ppe1, PPV, and PP6 have 70.2, 62.7, and 62.3% residues identical to the K. lactis protein. Phylogenetic tree analysis also indicates that the cloned gene most likely belongs to the Sit4/PPE1/PPV group of phosphatases.3 By analogy to S. cerevisiae, the gene is termed KISIT4.

KISIT4 Is Not Essential for Cell Viability—The chromosomal SIT4 gene was disrupted by replacing a 306-bp BclI fragment within the coding region by the URA3 gene in the K. lactis haploid PM6-7A (Fig. 2A). Correct disruption of the gene was confirmed by comparing genomic Southern blots of the disrupted CK254/1 and PM6-7A (Fig. 2B). Digestion with the restriction enzymes EcoRI and HindIII, followed by hybridization with a 32P-labeled 1.25-kb EcoRI-HindIII fragment containing the SIT4 coding sequence, yielded bands of 2.05 kb for CK254/1 and 1.25 kb for PM6-7A. This is the expected result for a SIT4-disrupted allele with an internal deletion within the SIT4 gene and replacement by the insertion of URA3. The successful disruption of SIT4 indicates that the gene is not essential in K. lactis strains with the PM6-7A background.

The sit4 null mutant, CK254/1, displayed the same phenotypes as sit4 alleles. These include 1) a slightly slower growth on GYP compared with the parental strain PM6-7A (not illustrated), 2) resistance to oligomycin on Glycerol medium (Fig. 1), and 3) a respiratory-deficient phenotype on glycerol at 37 and 19 °C (Fig. 1). Because sit4-1 and -2 alleles confer similar phenotypes as the null mutant, they probably cause loss of Sit4 function.

Complementation between KISIT4 and ScSIT4—The S. cerevisiae strain CY144 carries the sit4-102 allele which is temperature-sensitive for growth on GYP (72). CY144 was transformed with the plasmid pFL39-KISIT4, and the transformants were examined for growth at 37 °C. sit4-102 cells carrying the K. lactis sit4 gene can grow at the restrictive temperature (Fig. 3A), indicating that KISIT4 can functionally complement the sit4 mutation of S. cerevisiae. Reciprocally, the temperature-sensitive phenotype of K. lactis sit4-1 on glycerol medium can be complemented by ScSIT4 when the plasmid pCXL22-ScSIT4 was introduced into the K. lactis sit4-1 mutant CW2-8B (Fig. 3B). Functional inter-complementation between KISIT4 and ScSIT4 was further supported by the experiments showing that ScSIT4 can complement the paromomycin- and sorbic acid-hypersensitive phenotype of K. lactis sit4 mutants (see below).

K. lactis sit4 Mutants Have a Typical MDR Phenotype—The observation that resistance of K. lactis sit4 mutants to oligomycin is not due to functional modification of the F0,FATP synthase (see above) suggested a role for KISIT4 in the control of drug transport activities. Resistance to oligomycin could be a consequence of decreased drug accumulation within cells mediated by increased activity of membrane drug pumps. Consequently it was decided to ascertain whether K. lactis sit4 mutants have an altered sensitivity to drugs that are structurally unrelated to oligomycin.

3 M. J. R. Stark, personal communication.
SIT4 Controls Multidrug Resistance

On GlyYP medium supplemented with erythromycin, tetracycline, chloramphenicol, or carbonyl cyanide m-chlorophenylhydrazone, and GYP medium plus cryptopleurine, the strains CK254/1 (sit4Δ::URA3) and CW2-8B (sit4-1) did not show an increased or decreased level of resistance compared with the wild-type strain PM6-7A (not illustrated). However, the sit4 mutants showed a significantly elevated level of resistance to antimycin A and the antifungal drugs ketoconazole and econazole (Fig. 1). At the concentrations of 0.2 μg/ml for antimycin A on GlyYP medium, the wild-type strain PM6-7A failed to grow, whereas the drug does not inhibit the growth of the sit4 mutants. The sit4-1 mutant CW2-8B seems to have a higher resistance level to antimycin compared with the sit4-2 strain PM6-7A/oli18. Likewise, the sit4 mutants are resistant to ketoconazole and econazole at a concentration as high as 4.0 and 0.5 μg/ml, respectively, whereas growth of the wild-type PM6-7A is clearly inhibited by these drugs at the same concentrations (Fig. 1). As antimycin and the antifungal drugs have cellular targets different from oligomycin, these experiments demonstrate that mutation in sit4 leads to a typical MDR in K. lactis.

Expression of SIT4 Is Required for Resistance to Paromomycin, Sorbic Acid, and 4-NQO—In contrast to the negative control of resistance to oligomycin, antimycin, ketoconazole, and econazole, it has been found that Sit4p has a positive control over tolerance to other drugs. As can be seen in Fig. 4, K. lactis strains containing the sit4-1 or the sit4Δ::URA3 alleles are hypersensitive to paromomycin on GlyYP. The wild-type strain, PM6-7A, is able to tolerate paromomycin at a concentration of 0.1 mg/ml, whereas growth of the sit4-1 mutant CW2-8B and the sit4 null mutant CK254/1 is completely inhibited by the drug at 0.05 mg/ml. These results imply that SIT4 is required for paromomycin resistance under normal physiological conditions. Resistance to paromomycin can be restored to the wild-type level when the plasmid pCXJ22-ScSIT4, carrying ScSIT4, is introduced into the K. lactis sit4 mutant (not illustrated).

The above results suggest that the Sit4 protein phosphatase positively regulates drug transporter(s) specific for paromomycin. The dephosphorylating activity of the enzyme may directly or indirectly activate the function of membrane drug transporters. Consistent with this view is the observation that a further increase of Sit4 activity by overexpression of KISIT4 leads to a drastic elevation in resistance to paromomycin (Fig. 4). Wild-type cells are unable to grow on GlyYP supplemented with paromomycin at a concentration higher than 0.2 mg/ml, whereas the wild-type strain, PM6-7A, overexpressing KISIT4 on the multicopy plasmid pCXJ3-KISIT4 can resist paromomycin at a concentration as high as 2.0 mg/ml (not illustrated).

To determine whether overexpression of ScSIT4 also confers high level resistance to paromomycin in K. lactis, PM6-7A was transformed with the K. lactis multicopy plasmid pCXJ22-ScSIT4 bearing ScSIT4. On GlyYP medium supplemented with 0.1 mg/ml of paromomycin, the transformants show an improved growth compared with the control strain PM6-7A (Fig. 4). However, the transformants do not tolerate the drug at a concentration beyond 0.2 mg/ml. The data indicate that ScSIT4 can only partially replace KISIT4 for resistance to paromycin at high concentration.

In addition to paromomycin, it has also been found that K. lactis sit4 mutants are hypersensitive to sorbic acid and the mutagen 4-NQO. Both CW2-8B (sit4-1) and the sit4 null mutant CK254/1 are sensitive to inhibition by sorbic acid at 0.5 mM on GYP medium adjusted to pH 4.5 and by 4-NQO at 0.5 μg/ml on GMM, whereas the wild-type strain PM6-7A can grow under the same conditions (Fig. 4). Unlike the case for paromomycin, overexpression of both KISIT4 and ScSIT4 can only slightly increase tolerance to sorbic acid as estimated by colony size of the strains on plates containing the drugs. PM6-7A overexpressing the SIT4 strain alleles are unable to grow when the sorbic acid concentration is increased to 1 mM (not illustrated). By the same criterion, K. lactis cells overexpressing either KISIT4 or ScSIT4 do not have a detectable resistance to 4-NQO higher than the parental strain PM6-7A. On GMM plates containing 4-NQO at 1.0 μg/ml, both PM6-7A and its transformants carrying the SIT4 overexpression plasmids fail to form colonies (not illustrated).

Mutations in SIT4 Increase Efflux of Rhodamine 6G—Resistance to the antifungal drugs ketoconazole and econazole has been shown to be mediated by the Pdr5 transporter in the yeasts S. cerevisiae and C. albicans (8, 73). The resistance of K. lactis sit4 mutants to these two drugs suggests that the activity of a Pdr5-type ABC transporter might be increased in the mutant cells. To test this hypothesis, accumulation of the anionic fluorescent dye rhodamine 6G in the cells was measured by flow cytometry. Previous studies have established that efflux of rhodamine 6G is mediated by Pdr5 in S. cerevisiae (12). If mutations in SIT4 affect the function of a putative Pdr5 pump in K. lactis, it would be reflected by increased drug efflux activity in the mutants.

To address the above question, it is necessary to confirm that K. lactis, like S. cerevisiae, indeed has an active rhodamine 6G efflux system mediated by a Pdr5-type ABC transporter. When fluorescence intensity in energy-deprived and dye-preloaded
cells was measured (Fig. 5), the energy-deprived wild-type PM6-7A strain has a strong accumulation of rhodamine 6G within cells. Following re-energization by glucose, the relative fluorescence intensity is decreased by 4.6-fold. The strong dye efflux activity in the re-energized cells can be largely reversed by addition of the Pdr5-specific inhibitor FK506 (73, 74). The energy dependence and FK506 sensitivity strongly indicate the involvement of a Pdr5-like ABC transporter responsible for efflux of rhodamine 6G in \( K. \) lactis.

A possible difference in rhodamine 6G efflux activity between the wild-type PM6-7A and its isogenic \( sit4 \) mutant CK254/1 was examined. When steady state levels of cellular fluorescence were measured after dye loading for 1 h at 30 °C, it was observed that accumulation of rhodamine 6G is significantly higher in PM6-7A compared with CK254/1 (Fig. 6). The mean fluorescence intensity in the \( sit4 \) mutant is only 31% of the wild-type strain. These results indicate that mutation in \( SIT4 \) increases the efflux of rhodamine 6G. The difference in rhodamine 6G efflux activity between PM6-7A and CK254/1 is even more pronounced when cells are energized by glucose (Fig. 5). Under energized conditions, the \( sit4 \) mutant (CK254/1 + glu) has very low cellular accumulation of fluorescence which is only 7.6% of the wild-type (PM6-7A + glu). Furthermore, the strong dye efflux activity of the energized CK254/1 cells can be inhibited to some extent by addition of FK506. This observation strongly supports the idea that the accelerated rhodamine 6G efflux in \( sit4 \) strains is mediated by a Pdr5-type ABC transporter.

DISCUSSION

The \( SIT4 \) protein is a type 2A-related protein phosphatase (75) that has been originally identified in \( S. \) cerevisiae. Specific mutations in the \( ScSIT4 \) gene restore transcription of the \( HIS4 \) gene in the absence of the \( trans \)-acting DNA binding factors GCN4, BAS1, and BAS2 that are normally required for \( HIS4 \) expression (66). Loss of the \( SIT4 \) gene product was proposed to cause aberrant transcriptional activity of the RNA polymerase II because of accumulation of the phosphorylated form of an unknown transcription factor. Subsequent studies have shown that the \( Sit4 \) protein phosphatase is involved in cell cycle progression and bud formation (72, 76), in control of glycogen synthase and phosphorylase activities (77), and in the ceramide-induced and Tor-signaling pathways (78–80). The \( Sit4 \) function is evolutionarily conserved as \( S. \) cerevisiae \( sit4 \) mutants can be complemented by the \( SIT4 \) homologues from \( D. \) melanogaster and human cells (70, 71).

Reported in this study is the isolation of a \( SIT4 \) homologue from the budding yeast \( K. \) lactis. Several novel functional aspects of the \( Sit4 \) protein phosphatase in \( K. \) lactis are described. First, \( K. \) lactis \( SIT4 \) is not essential as disruption of the gene in several strains did not cause non-viability. In \( S. \) cerevisiae, null mutants are viable only in a genetic background with the \( SSD-v1 \) version of the polymorphic \( SSD1 \) gene (72). Second, \( K. \) lactis \( sit4 \) mutants are clearly impaired in respiratory function. Respiratory growth of \( sit4 \) mutants can be totally abolished when cells are grown at 37 or 19 °C. Although a growth defect on non-fermentable carbon sources was observed in the \( S. \) cerevisiae transcriptional suppressor \( sit4 \) mutants (66), a \( SIT4 \)-disrupted strain with a \( SSD1-v1 \) background is respiratory-competent (72). Third, we find that \( sit4 \) mutants of \( K. \) lactis have an increased formation of specific nuclear mutations on exposure to ethidium bromide that permits the recovery of cells lacking mitochondrial DNA, suggesting that \( Sit4p \) may affect...
susceptibility to a mutagenic agent such as ethidium bromide. Finally, Sit4p has an important role in regulating MDR.

In *S. cerevisiae*, two classes of multidrug resistance genes have been genetically identified. The first class of genes are ones encoding membrane ATP-binding cassette (ABC) transporters such as *PDR5*, *STS1*, *YDR1*, *SNQ2*, *YCF1*, *YOR1*, and *PDR12* that mediate drug efflux out of cells (13–22, 47). The second class of genes are those acting as transcriptional activators for the ABC transporter genes. Among the well characterized transcriptional regulators are *PDR1* and *PDR3*, which control the transcriptional levels of *PDR5*, *YOR1*, *PDR10*, and *PDR15* (28, 30, 35, 37, 81). Thus, the simplest interpretation for the MDR phenotype associated with *K. lactis* sit4 mutants is that the Sit4 protein phosphatase has a regulatory role on either of the two types of genes mentioned above, which ultimately modify the drug efflux capacity of the membrane ABC transporters.

*K. lactis* sit4 mutants display altered sensitivity to a wide range of mechanistically unrelated drugs. These drugs include the mitochondrial inhibitors oligomycin, antimycin A, and paromomycin that target to the ATP synthase, the bc1 complex of the respiratory chain, and mitochondrial ribosomes, respectively, the antifungal drugs ketoconazole and econazole, the antimicrobial preservative sorbic acid, and the mutagen 4-NQO. Efflux of most of these compounds has been shown to require specific ABC transporters in yeast. In *S. cerevisiae*, efflux of the antifungal drugs ketoconazole and econazole is mediated by the Pdr5 transporter (8, 73); oligomycin is transported by Yor1 (17); the detoxification of sorbic acid requires the Pdr12 pump (47), whereas 4-NQO is a specific substrate for the ABC protein Snq2 (20). Transporters for antimycin A and paromomycin have not yet been assigned.

In *K. lactis* sit4 mutants, at least four distinct types of transporters appear to be affected. Recent studies have revealed that a single pump is responsible for transport of ketoconazole, econazole, oligomycin, and antimycin. This transporter, most likely corresponding to the Pdr5 homologue in *K. lactis*, is negatively regulated by Sit4p. Rhodamine 6G could also be transported by the same protein. In contrast, a membrane transporter, responsible for detoxification of paromomycin, is positively regulated by Sit4p. *sit4* mutants are hypersensitive to paromomycin, and overexpression of *SIT4* causes a drastic elevation of resistance to the antibiotic. In addition, two other transporters, required for resistance to sorbic acid and 4-NQO, are also positively regulated by Sit4p. Based on the differential responses to overexpression of *SIT4*, these two transporters, together with that for paromomycin, are functionally distinct. In contrast to the paromomycin transporter, the function of the pump for sorbic acid is only slightly augmented in cells overexpressing *SIT4*, whereas the detoxifying capacity of the pump for 4-NQO is not affected by increasing *SIT4* dosage. By analogy to *S. cerevisiae*, the pumps responsible for sorbic acid and 4-NQO might be related to the Pdr12 and Snq2 transporters of *S. cerevisiae* (20, 47).

Strong support for a role of Sit4p in modulating MDR by altering the function of membrane transporters and ultimately the intracellular drug accumulation comes from the drug efflux assay using the fluorescent dye rhodamine 6G. In *K. lactis*, we have shown that an ABC transporter, likely to be of the Pdr5-type, promotes the efflux of rhodamine 6G in an energy-dependent and FK506-sensitive manner. When *sit4* mutants were examined, accumulation of rhodamine 6G was indeed significantly decreased compared with wild-type cells. As the strong rhodamine 6G efflux in *sit4* cells is sensitive to inhibition by the Pdr5-specific drug FK506, we can conclude that the function of a Pdr5-type pump is up-regulated. These results are in accord with the elevated resistance of *sit4* mutants to the antifungal drugs ketoconazole and econazole, which share the same transporter with rhodamine 6G in *S. cerevisiae*. Another line of evidence supporting the involvement of Sit4p in regulating expression/activity of membrane ABC transporters is our preliminary study showing altered protein levels of ABC transporters in *sit4* mutants. Immunoblot analysis using antibodies against *S. cerevisiae* proteins has revealed that the accumulation of Pdr12- and Snq2-like transporters in *K. lactis* is in fact decreased by severalfold in *sit4* cells, which is in agreement with the down-regulation pattern of resistance for sorbic acid and 4-NQO in *sit4* mutants.

As discussed above, the Sit4 phosphatase is involved in the regulation of different types of membrane transporters in both a positive and a negative manner. Mechanistically, control by the Sit4 phosphatase can intervene at different levels as follows: 1) Sit4p could modulate the function of the transcriptional factors that activate expression of particular ABC transporter genes; 2) Sit4p may control membrane targeting and turnover of the drug transporters; and 3) Sit4p could also directly regulate the drug pumping activity or substrate specificity of the membrane transporters by dephosphorylating a phospho-Ser/Thr residue(s). In *S. cerevisiae*, although most multidrug-resistant mutants are confined to mutations in transcriptional factors, some ABC transporters such as Pdr5 are ubiquitinated (82) and subjected to vacuolar degradation (83). It has also been documented that phosphorylation in a hypothetical modular domain of the yeast Ycf1 transporter is important for drug resistance (21). Each type of ABC transporter may thus adopt a different regulatory pattern. In *K. lactis*, because both positive and negative regulation are observed in *sit4* mutants, it can be imagined that more than one pathway operates to achieve the regulation of individual transporters by
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Sit4p. Elucidation of the regulatory mechanisms would first require the isolation and characterization of K. lactis transporter genes, which is currently underway in our laboratories.

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