Molecular Characterization of Two High Affinity Sulfate Transporters in *Saccharomyces cerevisiae*

Hélène Cherest,* Jean-Claude Davidian,† Dominique Thomas,* Vladimir Benes,‡ Wilhelm Ansorge‡ and Yolande Surdin-Kerjan*

**Centre de Génétique Moléculaire, Centre National de la Recherche Scientifique, 91198 Gif-sur-Yvette Cedex, France, †Laboratoire de Biochimie et Physiologie Moléculaire des Plantes, Ecole Nationale Supérieure Agronomique, INRA, CNRS (URA 2133), 34060 Montpellier Cédex I, France and ‡ EMBL Biochemical Instrumentation Programm, D-69117 Heidelberg, Germany*

Manuscript received July 26, 1996
Accepted for publication November 19, 1996

ABSTRACT

Strains resistant to the toxic analogues of sulfate, selenate and chromate have been isolated. Their genetic analysis allowed us to identify four genes. One, called *MET28,* encodes a transcriptional factor. The three other genes, called *SUL1, SUL2* and *SUL3,* encode proteins involved in sulfate transport. The sequence of Sul1p and Sul2p indicate that they are integral membrane proteins exhibiting, respectively, 11 and 10 transmembrane domains. Moreover, Sul1p and Sul2p share a high degree of similarity. Sulfate transport kinetic studies made with parental and mutant strains show that, as expected from genetic results, *Saccharomyces cerevisiae* has two high affinity sulfate transport systems. Sul3p has been shown to be involved in the transcriptional regulation of the *SUL2* gene.

SULFATE is among the most abundant anion macronutrients found after phosphate in cells, and it is the major sulfur source in many organisms. Like all the inorganic nutrients, sulfate is taken up and accumulated in cells through highly specific membrane transport systems. After it is accumulated as an inorganic anion, sulfate is enzymatically reduced to sulfide and then incorporated into organic compounds by the sulfate assimilation pathway.

Sulfate transport has been studied in whole plants (LEE 1982; CLARKSON *et al.* 1983; LASS and ULLRICH-EBERIUS 1984), in excised organs (CRAM 1983; CLARKSON *et al.* 1992) and in isolated cells (SMITH 1976; RENNENBERG *et al.* 1989). More recently, kinetic studies of sulfate transport into plant right-side-out purified plasma membrane vesicles have shown that it occurs by sulfate/proton cotransport (HAWKESFORD *et al.* 1993), which agrees with the work on the sulfate uptake system of *Penicillium notatum* (CUPPOLETTI and SEGEL 1975) and of *Saccharomyces cerevisiae* (ROOMANS *et al.* 1979). These results differ, however, from what is observed in animal plasma membrane vesicules in which sulfate transport has been shown to be mediated via an anion-exchange process (MEIER *et al.* 1987) or a sodium/sulfate cotransporter (BUSCH *et al.* 1994).

The recent cloning of plant potassium transporters by heterologous complementation of yeast mutants (ANDERSON *et al.* 1992; SENTENAC *et al.* 1992) has shown that this technique may offer possibilities to isolate the plant genes implicated in sulfate transport. Several cDNAs coding for sulfate transport systems have been cloned in lower eukaryotes (KETTER *et al.* 1991), animals (MARKOVICH *et al.* 1993; BISSIG *et al.* 1994) and man (HÄSTBACKA *et al.* 1994). Aiming to clone the plant sulfate transporters, we initiated the screening of yeast mutants impaired in sulfate transport. A preliminary genetic work on sulfate transport in yeast had shown that it could be mediated by two enzymatic species, although the biochemical results obtained at that time were not easily explained by this simple hypothesis (BRETON and SURDIN-KERJAN 1977) and clearly, only a molecular study would shed light on sulfate transport in *S. cerevisiae.* While our work was in progress, cloning and analysis of cDNAs from the tropical forage legume *Stylosanthes hamata* encoding sulfate transporters has been performed (SMITH *et al.* 1995a) by complementation of a high affinity sulfate transporter mutant of *S. cerevisiae* (SMITH *et al.* 1995b). However, although the yeast mutant used by these authors has proven to be useful, no genetic analysis of sulfate transport in yeast was performed in this case.

We report here genetic and molecular analysis of sulfate transport in *S. cerevisiae* showing that sulfate uptake is mediated by two high affinity transporters. We have cloned these two genes and analysis of their sequences shows that they encode proteins sharing a high degree of similarity to a growing family of proteins active in sulfate uptake. In addition, our screening method allowed us to isolate strains impaired in a factor regulating the expression of the gene encoding one of these transporters.

MATERIALS AND METHODS

Strains, plasmids and media: The strains of *S. cerevisiae* and plasmids used in this work are listed in Table 1. YPG, YNB

Corresponding author: Yolande Surdin-Kerjan, Centre de Génétique Moléculaire, CNRS, 91 198 Gif-sur-Yvette Cedex, France.
E-mail: yolande.kerjan@cgm.cnrs-gif.fr

and the sulfur-less B media were as described in CHEREST and SURDIN-KERJAN (1992). Minimal medium was YNB medium supplemented according to the auxotrophic requirements of the strains, with adenine (40 μg/ml), uracil (20 μg/ml), histidine (200 μg/ml), leucine (100 μg/ml) and tryptophan (20 μg/ml). S6 medium was YNB medium in which the glucose concentration has been raised to 3% and containing 0.1% of casamino acids. This medium contains neither uracil nor adenine, and tryptophan must be added when needed. Genetic crosses, sporulation, dissection and scoring of nutritional markers were done as described in SHERMAN *et al.* (1979).

Mutagenesis and isolation of mutants: Strain W303-1A was transformed with the multicopy plasmid pM3-32 that was constructed by inserting the *Bam*HI-*Sal*I fragment of pM3-4 in plasmid pEMBLYe23 (CHEREST *et al.* 1985). Stationary phase cells of transformed W303-1A grown on S6 medium were washed once with water and suspended in 100 mM sodium phosphate buffer pH 8.0 at a density of 10^8 cells/ml. Cells were exposed to 10 μl/ml EMS at 30° for 70 min (FINK 1970). In these conditions 30% of cells survived the treatment. Selection of mutants was made by plating on B medium containing 0.2 mM DL-homocysteine as a sulfur source and 2 mM sodium selenate, 0.1 mM potassium chromate or both 0.1 mM chromate and 2 mM selenate. After incubation for 3 days at 30°, the resistant colonies were picked for further analysis.

Sulfate uptake assay: Sulfate uptake was measured on whole cells. They were grown at a cell density of 10^7 cells/ml in sulfur-less B medium containing 0.2 mM DL-homocysteine as sulfur source. Such growth conditions were made necessary by the impairment of sulfate uptake activity after growth in sulfate-containing media. The uptake assay was performed on two cell concentrations. For 1 ml final volume, 2.5×10^6 and 5×10^6 growing cells were added to 500 μl of twofold concentrated B medium containing 0.1 mM $Na_2^{35}SO_4$ (~5000 cpm/nmol). Uptake was started by the addition of the cell suspension and the mixture was incubated at 20° for different times (1, 2, 3 and 4 min). For each time and cell density, the reaction was stopped by the addition of 10 ml of ice cold 0.1 mM Na_2SO_4 and cells were filtered on a GF/C filter, washed with 3 × 10 ml of cold 0.1 mM Na_2SO_4, and each filter was transferred in a counting vial. For kinetic analysis of sulfate transport, the concentration range of sulfate was 0.001–0.05 mM. It appears that the Michaelis-Menten equation can give an adequate description of transport processes. However, the uptake being measured on whole cells, the K_m value cannot have the same meaning as in the case of an enzymatic reaction. We have thus referred to the apparent K_m values calculated from double reciprocal plots as K_T.

Derepression kinetics: Strains CP36-7C (*sul3*) and W303-1A (parental) were grown in 100 ml of sulfur-less B medium in the presence of a repressing amount of methionine (0.1 mM). When the cells reached a density of ~10^7 cells/ml, they were quickly filtered, washed and transferred into B medium without methionine. Cells were harvested at different time intervals after the shift and total RNAs were extracted. For each time point 10 μg of total RNA was electrophoresed on a 1% agarose gel and then transferred to a nylon membrane. The transferred RNAs were hybridized to probes specific to the *SUL1* and the *SUL2* genes.

Recombinant DNA methods: Plasmid pEMBLYe23 (BALDARI and CESARINI 1985) was used as a shuttle vector between *S. cerevisiae* and *Escherichia coli*. The *S. cerevisiae* genomic library used for the cloning of the *SUL1* and *SUL2* genes was constructed by inserting the product of a partial *Hin*dIII digest from the wild-type strain X2180-1A in the *Hin*dIII site of pEMBLYe23. Plasmid purification was performed as described in ISH-HOROWICZ and BURKE (1981).

The sequencing of the *SUL2* gene was as described in SCHOLLER *et al.* (1996). The construction of disrupted alleles followed the strategy of ROTHSTEIN (1983). To disrupt *SUL1*, the *Hin*dIII-*Xho*I fragment of the *SUL1* region was inserted in plasmid pUC19 digested by *Hin*dIII-*Sal*I. The *Eco*RV fragment of the *SUL1* insert was removed and replaced by the *Bam*HI-*Bam*HI fragment of plasmid pYD-LEU bearing the *LEU2* gene. The resulting plasmid was cut by *Xba*I and was used to transform W303-1A to leucine prototrophy yielding strain CD131 (*sul1::LEU2*). The disruption was verified by Southern blotting (not shown). To disrupt *SUL2*, plasmid pSUL2-1 was digested by *Sma*I and religated, thus eliminating the *URA3* gene and the 2μ sequences. In the resulting plasmid, the *Bam*HI-*Eco*RI fragment of the *SUL2* gene was replaced by a *Bgl*II-*Bgl*III fragment bearing the *URA3* gene. The resulting plasmid was cut by *Pst*I and *Bgl*II and used to transform strain W303-1A to uracil prototrophy yielding strain CD141 (*sul2::URA3*). The disruption was verified by Southern blotting (not shown).

RESULTS

Characterization of the mutants: Previous results (BRETON and SURDIN-KERJAN 1977) had shown that selection of strains resistant to toxic analogues of sulfate (selenate or chromate) led mainly to strains mutated in the *MET3* gene encoding ATP sulfurylase, catalyzing the first step of sulfate reduction. To avoid this issue, we mutagenized strain W303-1A harboring additional copies of the *MET3* gene on the multicopy plasmid pM3-32 (see MATERIALS AND METHODS). Mutagenesis was performed as described in MATERIALS AND METHODS, and 39 strains resistant to chromate, to selenate or to selenate and chromate were further studied. As mutants impaired in sulfate uptake are likely to be auxotrophic for methionine, selenate- and chromate-resistant strains were tested on minimal medium with or without methionine. This allowed us to classify the resistant strains into two classes, one comprising strains requiring methionine for growth, the other one containing prototrophic strains. Twenty-nine strains auxotrophic for methionine and 10 prototrophic strains were cured of the pM3-32 plasmid by growth for 25 generations on nonselective medium and selection for colonies requiring uracil for growth.

As previously shown, cells from strains bearing *met4*, *met14* or *met16* mutations are capable of growing in the presence of selenate or chromate, as sulfate uptake is absent in these mutants (BRETON and SURDIN-KERJAN 1977; THOMAS *et al.* 1990; THOMAS *et al.* 1992). To determine if the isolated methionine auxotrophic strains could be impaired in *MET4*, *MET14* or *MET16* genes, the 30 auxotrophic mutants were transformed with plasmids pYe(MET14)-2, pM16-2 and pM4-4 (Table 1), and the transformants were tested for methionine requirement. This analysis showed that 23 mutants were complemented by one of the three plasmids bearing the *MET4*, *MET14* or *MET16* genes, indicating that these mutants were impaired in one of these three genes. The six remaining auxotrophic strains (CrSe922, 91, 752, 503, 412 and Cr602) that were not complemented by one of the plasmids were thus further analyzed.

TABLE 1

Yeast strains and plasmids

	Genotype	Origin
Strains		
W303-1A	*MAT***a** *his3 leu2 ura3 ade2 trp1*	R. ROTHSTEIN
W303-1B	*MATα his3 leu ura ade trp1*	R. ROTHSTEIN
CP60-4A	*MAT***a** *his3 leu2 ade2 trp sul2-1*	This work
CP60-4C	*MAT***a** *his3 leu2 ura ade trp1 sul1-1*	This work
CP60-1C	*MAT***a** *his3 leu2 ura3 trp1sul1-1 sul2-1*	This work
CP36-7C	*MATα leu2 ura3 lys2 sul3-1*	This work
CD131	*MAT***a** *his3 leu2 ura3 ade2 trp1 sul1::LEU2*	This work
CD141	*MAT***a** *his3 leu2 ura3 ade2 trp1 sul2::URA3*	This work
CP156-10B	*MATα his3 leu2 ura3 ade2 trp1 sul2::URA3 sul3-2*	This work
CP157-13B	*MAT***a** *his3 leu2 ura3 ade2 trp1 sul1::LEU2 sul3-2*	This work
CP154-7A	*MATα his3 leu2 ura3 ade2 trp1 sul1::LEU2 sul2::URA3*	This work
Plasmids		
pM3-32	Expresses *URA3* and *MET3*	CHEREST *et al.* (1985)
pYe(MET14)2	Expresses *TRP1* and *MET14*	FITZGERALD-HAYES *et al.* (1982)
pM16-2	Expresses *URA3* and *MET16*	THOMAS *et al.* (1989)
pM4-4	Expresses *URA3* and *MET4*	THOMAS *et al.* (1992)

Genetic analysis of the methionine-requiring mutants: The six methionine-requiring strains were crossed to the wild-type strain (W303-1B); the resulting diploids were sporulated and their progeny analyzed. In most cases, germination was very poor. A spore from each cross exhibiting the same phenotype as the resistant parental strain (methionine auxotrophy and resistance to chromate and/or selenate) was backcrossed to the wild-type strain. For four original mutants (CrSe91, 922, 752, and 503), the auxotrophy for methionine and the resistance to the sulfate analogues cosegregated 2+:2− in 12 tetrads examined. In one mutant (Cr602), the auxotrophy for methionine segregated 2+:2−, but the resistance was lost and for another mutant (CrSe412) the resistance to selenate and the methionine auxotrophy were the result of the mutation of two different genes.

Complementation tests classified the mutants into three groups. In the first one were strains CrSe91, 922, 752 and 503 defining a new gene, *MET28*. The cloning and sequencing of the *MET28* gene has shown that it encodes a transcription factor involved in sulfur metabolism (KURAS *et al.* 1996). The second group comprised strain Cr602, which was eventually shown to bear a mutation in the *MET1* gene (THOMAS *et al.* 1992). The mutation leading to methionine auxotrophy found in strain CrSe412 was shown to be a *met22* mutation (THOMAS *et al.* 1992; GLASER *et al.* 1993). The other mutation harbored by strain CrSe412 and leading to selenate resistance defined a yet unknown gene that we called *SUL3*.

Analysis of the methionine prototrophs: The 10 mutants that did not require methionine for growth were tested again for their resistance to selenate. Four mutants were selected for their easily scored resistance to selenate and further studied. These strains (CrSe702, 661, 431 and 121) were backcrossed to the wild-type strain (W303-1B) and the resulting diploids were subjected to tetrad analysis. Diploids involving mutants CrSe702 and CrSe661 display a 2+:2− segregation for the resistance to selenate in 18 tetrads examined for each diploid. Strain CrSe702 was shown to bear a mutation in the *SUL3* gene already defined and strain CrSe661 carried a mutation in a new gene that we called *SUL2*. For the other two mutants (CrSe121 and CrSe431) the resistance to analogues was the result of two different mutations in two independent genes. Strain CrSe121 was shown to bear a mutation in the *SUL2* gene already defined and in another gene that we called *SUL1*. Strain CrSe431 was shown to bear also a mutation in the *SUL1* gene and in a gene that we called *CHR1* as its mutation leads to an easily scored resistance to chromate. The *CHR1* gene was shown to be identical to the *MET14* gene encoding APS kinase, the enzyme catalyzing the second step of sulfate reduction. As already stated, a mutation in the *met14* gene leads to a loss of sulfate uptake activity. The *chr1* mutation isolated here does not lead to methionine auxotrophy. However, this mutation impairs sulfate uptake as other mutations in the *MET14* gene and thus results in a chromate-resistant strain. The genotypes of the different studied mutants are summarized in Table 2. It is noteworthy that the different mutated alleles in

TABLE 2

Mutated alleles in the mutants resistant to chromate + selenate

Strain	Mutated allele
1-methionine auxotrophs	
CrSe 141, 223, 352, 444, 563, 871, 883, 903	*met4*
Se 2111, 1515, 1615, 1625, 2614, 2131, 1126, 1141, 11110	*met4*
CrSe 71, 211, 794, 993	*met14*
Cr691, Se2017, Se394	*met16*
CrSe91, 922, 752, 503	*met28*
Cr602	*met1*
CrSe412	*met22 sul3*
2-methionine prototrophs	
CrSe121	*sul1 sul2*
CrSe702	*sul3*
CrSe661	*sul2*
CrSe431	*chr1 sul1*

the *SUL3* gene are dominant with respect to selenate resistance.

Sulfate uptake in the different mutants: The genetic analysis of selenate-resistant mutants had defined three new genes *SUL1, SUL2* and *SUL3* that encode proteins that could be involved in sulfate transport. We thus measured the sulfate transport in the different mutant cells. Results (Table 3) showed that cells from single mutants exhibited only a small decrease in sulfate uptake as compared to cells of the parental strain. Strain CP60-1C bearing a mutation in each gene, *SUL1* and *SUL2*, had no sulfate transport activity. We then examined the kinetics of sulfate uptake in strains CP36-7C (*sul3*), CP60-4A (*sul2*), CP60-4C (*sul1*) and W303-1A (parental strain), in response to different sulfate concentrations. The double reciprocal plot of sulfate uptake data for the different strains are shown in Figure 1. For all strains, the plot is linear. The calculated K_T for sulfate was 4.5 μM for the parental strain and strains bearing *sul2* and *sul3* mutations. However, for the strain bearing the *sul1* mutation the K_T was reproducibly found to be 10 μM. These kinetics results and the lack of sulfate uptake activity in the double mutant CP60-1C (*sul1 sul2*) were an indication that sulfate uptake in *S. cerevisiae* is mediated by two transport systems both exhibiting a high affinity for sulfate. The double reciprocal plot of sulfate uptake in the parental strain shows only the higher affinity transport (4.5 μM). The inactivation of one of these transporters by the *sul1* mutation (strains CD131 and CP60-1C) uncovers the system exhibiting the lowest affinity for sulfate (10 μM).

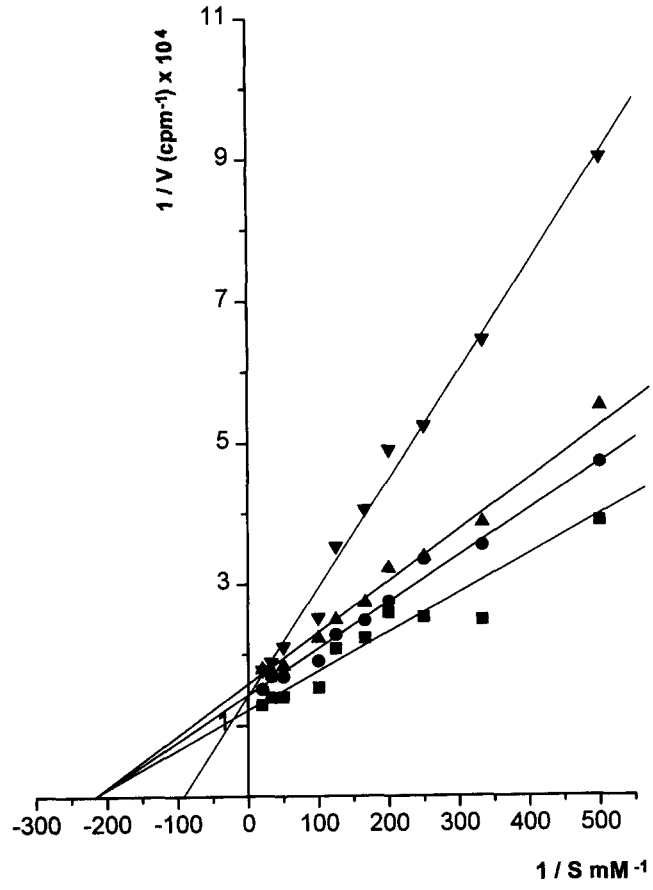

FIGURE 1.—Kinetic analysis of sulfate uptake in different mutants. Sulfate uptake was measured as described in MATERIALS AND METHODS. The incubations were for 3 min. Uptake rates were determined at sulfate concentrations ranging from 0.05 to 0.001 mM. ■, strain W303-1B (*SUL1 SUL2*); ●, strain CP36-7C (*sul3*); ▲, strain CP60-4A (*sul2*); ▼, strain CP60-4C (*sul1*).

TABLE 3

Sulfate uptake activity and K_T for sulfate in different mutants

Strain	Relevant genotype	Sulfate transport[a]	K_T[b]
W303-1A		5.9	4.6
CP36-7C	*sul3*	2.9	4.5
C60-4A	*sul2*	3.4	4.6
CP60-4C	*sul1*	4.4	10
CP60-1C	*sul1 sul2*	<0.1	ND
CD131	*sul1::LEU2*	4.4	10
CD141	*sul2::URA3*	4.1	4.5
CP156-10B	*sul2::URA3 sul3*	2.3	4.1
CP157-13B	*sul1::LEU2 sul3*	<0.1	ND
CP154-7A	*sul1::LEU2 sul2::URA3*	<0.1	ND

The figures are mean values of at least three experiments with a deviation <10%.

[a] Sulfate transport is expressed in nmol of sulfate transported per min per mg of dry weight.

[b] K_T is expressed in μM. ND, not determined.

Cloning of the *SUL1* and *SUL2* genes: Although cells from strain CP60-1C (*sul1 sul2*) are resistant to selenate and express no sulfate transport activity, they are able to grow on YNB-based medium that contains a high concentration of sulfate (~30 mM). However, on B medium complemented with 1 mM sulfate, this strain grows very slowly. To clone the *SUL1* and *SUL2* genes we ex-

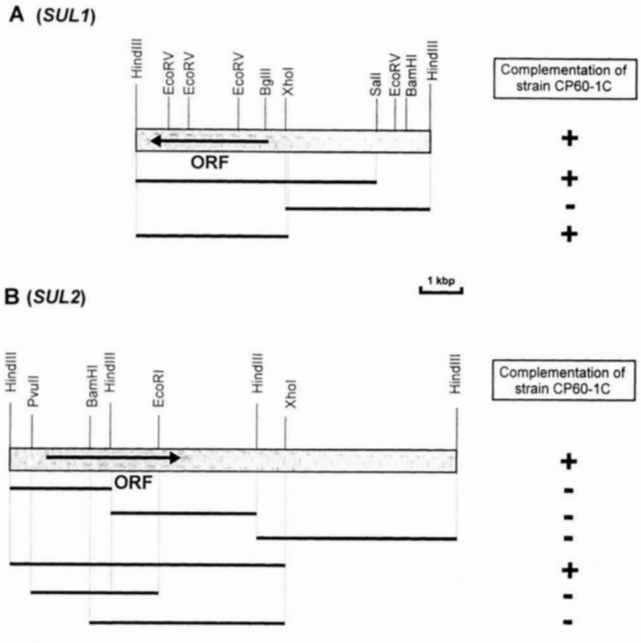

FIGURE 2.—Physical map of the *SUL1* (A) and *SUL2* (B) regions. The fragments subcloned in plasmid pEMBLYe23 and their ability to confer to strain CP60-1C (*sul1 sul2*) the ability to grow on sulfate 1 mM as a sulfur source are shown.

ploited this property and complemented the strain CP60-1C with the pEMBLYe23-based genomic library described in MATERIALS AND METHODS. About 36000 Ura^+ transformants were selected on 24 plates. These transformants could not be replica plated due to the number of transformants on each plate (~1500). They were thus recovered by washing the plates and the resulting cell suspensions were plated after appropriate dilution onto B medium containing 1 mM ammonium sulfate. Cells that grew quickly in these conditions were derived from six original plates. Plasmid DNA was recovered from 12 colonies and used to retransform CP60-1C. Eleven DNA preparations led to transformants able to grow on 1 mM sulfate. Ten clones were found to harbor identical plasmids with a 6.5-kbp insert. The other transformant was found to harbor another plasmid with a 10.2-kbp insert (Figure 2). The determination of the apparent K_T for sulfate of strain CP60-1C (*sul1 sul2*) transformed with one or the other plasmid allowed us to identify the plasmid complementing respectively the *sul1* or *sul2* mutations. Indeed, when transformed by the plasmid bearing the 10.2-kbp insert (putative *SUL2*), the measured K_T is 10 μM as in a *sul1* mutant, and when transformed by the plasmid bearing the 6.5-kbp insert (putative *SUL1*), the apparent K_T is 4.5 μM, as expected in a *sul2* mutant.

We verified that the inserts directed the integration to the *SUL1* or the *SUL2* region of the yeast genome. Plasmids missing an autonomous replicating sequence but bearing the *URA3* gene and the complementing inserts were constructed. For *SUL1,* integration was directed by cleaving the plasmid within the insert with *Xba*I (Figure 2). Strain W303-1B was transformed with the cleaved plasmid, and one Ura^+ transformant was crossed to strain CP60-4C (*sul1*), the diploid was sporulated and 42 tetrads analyzed. The 84 spores showing a Ura^+ phenotype were all sensitive to selenate, indicating that the insert directed integration at the chromosomal *SUL1* locus. For *SUL2,* integration was directed by cleaving the plasmid within the insert by *Bam*HI (Figure 2). Cross of one Ura^+ transformant with CP60-4A (*sul2*) showed that the insert directed integration at the *SUL2* locus.

The position of the *SUL* genes within the cloned DNA of pSUL1 and pSUL2 was determined by subcloning (Figure 2). The different subclones were tested for their ability to restore growth on 1 mM sulfate and the sensitivity to selenate of strain CP60-1C (*sul1sul2*).

Sequence of the *SUL1* and *SUL2* genes: Partial sequencing of the cloned *SUL1* region showed that gene *SUL1* was identical to an open reading frame (ORF) of chromosome *II, YBR294,* sequenced as part of the yeast genome sequencing project (FELDMAN *et al.* 1994) and to the *SUL1* gene described by SMITH *et al.* (1995b). It contains an ORF with the potential to encode a protein comprising 859 amino acid residues. Analysis of the protein sequence has shown that it comprises 11 potential helical transmembrane domains. Moreover, the sequence shows significant homology to the polypeptide derived from the *cys-14* gene of *Neurospora crassa* encoding the mycelial sulfate transporter of this organism (KETTER *et al.* 1991). The *SUL2* gene, mapping to chromosome *XII* (*YLR092*) was sequenced on both strands. It has the potential to encode a protein comprising 893 amino acids with a molecular mass of 94,944 and a calculated isoelectric point of 6.16. The hydropathy profile of the Sul2 protein suggests that it contains 10 transmembrane spanning regions. Searches in databases by using the BLAST program (ALTSCHUL *et al.* 1990) reveals that Sul2p is strongly similar to Sul1p (*YBR294*). Alignments obtained with the Clustal V program (HIGGINS 1994) show that the two proteins exhibit 563 identical residues (62%) and 95 conservative replacements (11%) (Figure 3). Figure 3 shows also that 10 putative transmembrane domains of Sul1p and Sul2p are superimposable, Sul1p having one additional transmembrane domain in the N-terminal part of the protein.

Phenotypic study of *sul1* and *sul2* disrupted strains and of different double mutants: Gene disruption alleles were constructed as described in MATERIALS AND METHODS. The specific activity of sulfate transport was measured in the *sul1* and *sul2* disrupted strains as well as in different double mutants (Table 3). The strains bearing disruptions in the *SUL1* (CD131) or in the *SUL2* (CD141) genes exhibit a sulfate transport activity comparable to the parental strain W303-1A. The specific activity measured in strain CP156-10B (*sul3 sul2::URA3*) is comparable to that found in strain CP36-

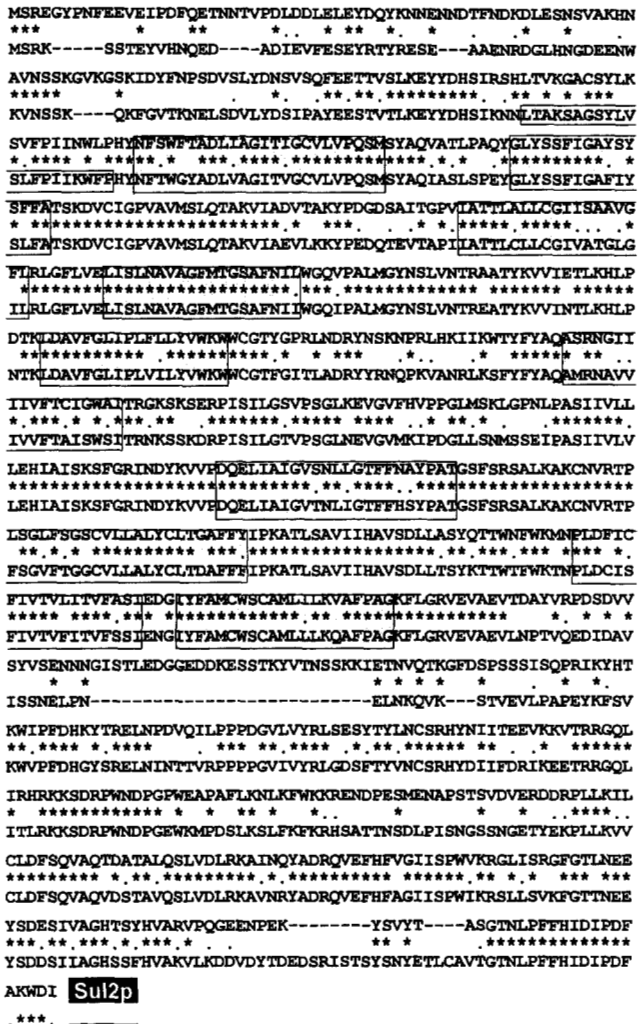

FIGURE 3.—Alignment of the Sul1 and Sul2 proteins. The two proteins were aligned using the CLUSTAL V program (HIGGINS 1994). * indicate identities and · indicate conservative replacements. The putative membrane spanning domains are boxed.

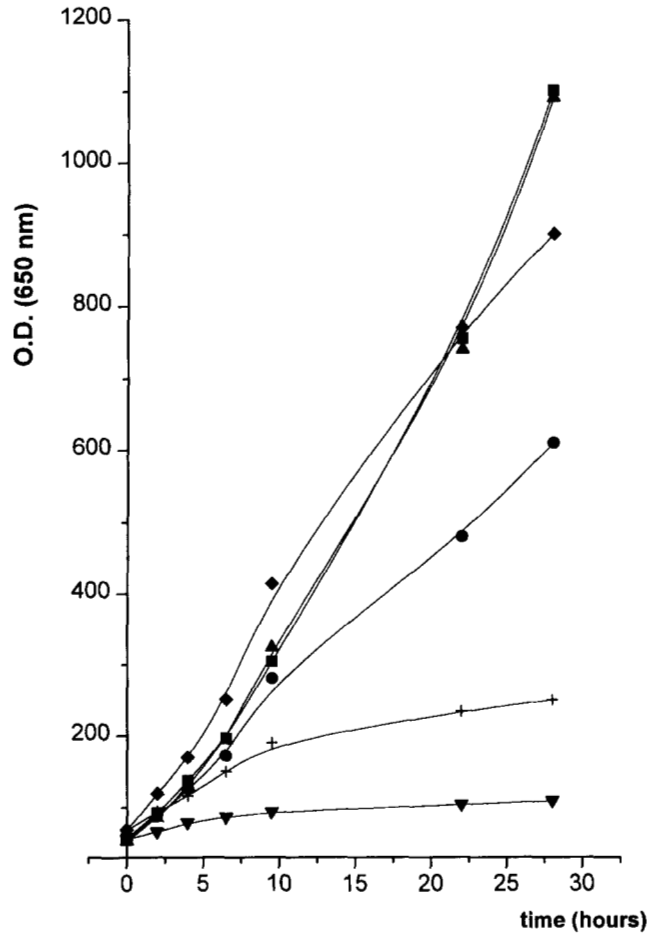

FIGURE 4.—Growth on 1 mM sulfate of the parental and different mutant strains. The optical density was measured at 650 nm (10 mm light path) in a Hitachi 2000 spectrophotometer. ■, strain W303-1B (*SUL1 SUL2*); ●, strain CD131 (*sul1::LEU2*); ▲, strain CD141 (*sul2::URA3*); ▼, strain CP154-7A (*sul1::LEU2 sul2::URA3*); ♦, strain CP156-10B (*sul2::URA3 sul3*); +, strain CP157-13B (*sul1::LEU2 sul3*).

7C (*sul3*). In strains CP157-13B (*sul1::LEU2 sul3*) and CP154-7A (*sul1::LEU2 sul2::URA3*) no sulfate transport activity was found. These results are in accord with the growth studies (Figure 4) that show that the strains bearing single disruptions or the double mutation *sul2::URA3 sul3* are able to use sulfate 1 mM as a sole sulfur source, while strains bearing the double mutations *sul1::LEU2 sul2::URA3* or *sul1::LEU2 sul3* are unable to do so. The kinetic parameters of the disrupted strains CD131 (*sul1::URA3*) and CD141 (*sul2::URA3*) were determined. The K_T for sulfate of strains bearing disrupted alleles was the same as that of strains bearing mutated alleles (Table 3).

Sul3p is involved in the regulation of expression of the *SUL2* gene: The results presented above show that yeast cells contain two high affinity sulfate transporters encoded by the *SUL1* and the *SUL2* genes. However, the role of the *SUL3*-encoded product was still unclear. Results reported in Table 3 and in Figure 4 show that strains CP157-13B (*sul1::LEU2 sul3*) and CP154-7A (*sul1::LEU2 sul2::URA3*) exhibit the same phenotype (no sulfate uptake activity and no growth on B medium complemented with 1 mM sulfate). This was an indication that Sul3p could be involved in the regulation of the expression of the *SUL2* gene or of the activity of Sul2p. Previous results had shown that sulfate uptake is negatively coregulated with the other enzymes of the sulfate assimilation pathway in response to an increase in the intracellular concentration of S-adenosylmethionine obtained by growth in the presence of methionine (BRETON and SURDIN-KERJAN 1977). However, in contrast to the genes of the sulfate assimilation pathway, the expression of which is completely repressed by growth in the presence of 1 mM methionine (KURAS and THOMAS 1995), sulfate uptake was shown to be completely repressed in the parental strain by growth of the cells in the presence of 0.1 mM methionine. This was an indication that the regulation of the expression

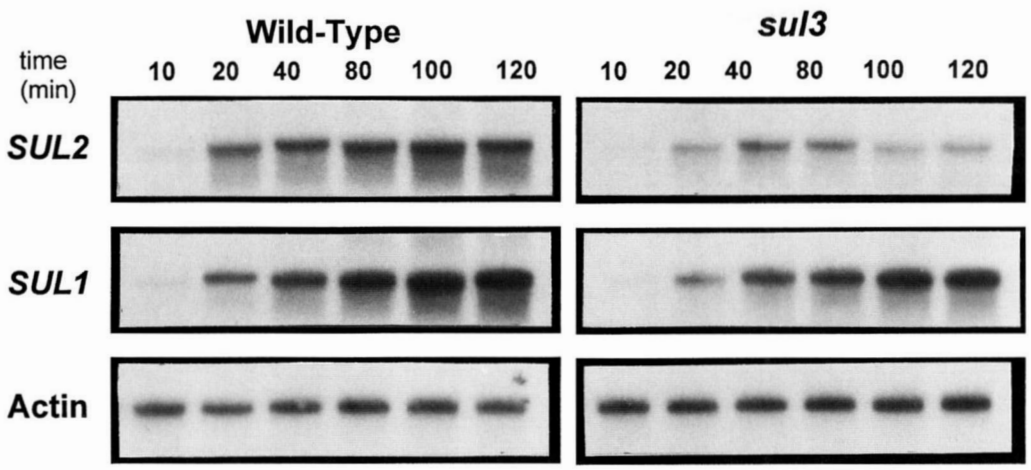

FIGURE 5.—Effect of the mutation in the *SUL3* gene on the transcription of the *SUL1* and *SUL2* genes. Derepression kinetics of the transcription of the *SUL1* and *SUL2* genes were monitored in wild-type cells (strain W303-1A) and in cells mutated in the *SUL3* gene (strain CP36-7C) as described in MATERIALS AND METHODS.

of the *SUL1* and *SUL2* genes could show some differences with that of the other genes of the sulfate assimilation pathway.

We thus analyzed the derepression kinetics of the transcription of the *SUL1* and *SUL2* genes in the parental strain and in a *sul3* mutant. This was done by shifting the two strains W303-1A (wild type) and CP36-7C (*sul3*) from a medium containing 0.1 mM methionine to a medium without methionine and extracting the RNAs at different times after the shift. The RNAs were then analyzed by Northern blotting and hybridizing to probes specific of the *SUL1* and *SUL2* genes. Results (Figure 5) show that in strain W303-1A, *SUL1*- and *SUL2*-specific mRNAs are first detected 20 min after the shift and attain their maximal abundance at 100 min, which could be an indication that the expression of the two genes is coordinately regulated. In cells of the *sul3* mutant, the same derepression pattern is found for the expression of the *SUL1* gene. In contrast, although the *SUL2* mRNA is also first detected 20 min after the shift, it reaches its maximal abundance 40 min after the shift. This maximal level represents ~30% of that observed in the wild-type strain. In addition, after 80 min, the level of *SUL2*-specific mRNA decreases. These results show that when Sul3p is absent, only a transient low level of *SUL2*-specific mRNA can be seen on derepression while the *SUL1*-specific mRNA expression pattern is not modified. This indicates that Sul3p is involved in the regulation of the transcription of the *SUL2* gene. The low level of transcription of *SUL2* in a *sul3* mutant explains why the double mutants *sul1 sul3* and *sul1 sul2* exhibit the same phenotype.

DISCUSSION

Using toxic analogues of sulfate (selenate and chromate) a specific genetic screen allowed us to identify three genes involved in sulfate uptake, *SUL1, SUL2* and *SUL3*. Our results show that the *SUL1* and *SUL2* genes encode highly similar transmembrane proteins bearing, respectively, 11 and 10 putative transmembrane domains. The findings reported here, and more particularly the fact that the absence of sulfate uptake activity and high selenate resistance occurs only in double mutants, are in agreement with a model where two transport systems for sulfate exist in *S. cerevisiae*. Our kinetic results show moreover that both sulfate transport systems are high affinity systems (see Table 3 and Figure 4). However, it is still questionable if a low affinity sulfate transport system exists in yeast as a strain disrupted in both *SUL1* and *SUL2* genes is still capable to grow on YNB-based medium that contains a very high sulfate concentration (30 mM). Sulfate uptake was measured in strain CP154-7A (*sul1::LEU2 sul2::URA3*). Although the determination of the K_T for sulfate was imprecise, it was calculated to be 6 mM. This indicates that sulfate is transported in the double *sul1 sul2* mutant with a very low affinity and the specificity of this transport can be questioned.

Molecular characterization of sulfate transport has been reported for different microorganisms. The sulfate transport in *E. coli* and in a cyanobacterium (synechococcus sp) has been shown to be composed of five polypeptides: the thiosulfate and the sulfate binding proteins, two inner membrane transport proteins and a hydrophilic membrane associated ATP binding protein, probably an energy-coupling component (GREEN *et al.* 1989; SIRKO *et al.* 1990; LAUDENBACH and GROSSMAN 1991). In *N. crassa*, two sulfate transporters are encoded by two unlinked genes. The *Cys14* gene encoding permease II has been cloned and sequenced. The analysis of the sequence of the *Cys-14* gene has shown that sulfate permease II is probably a carrier protein with 12 putative helical membrane spanning domains (KETTER *et al.* 1991). The two sulfate permeases from *N. crassa* are expressed at different developmental stages of the fungus and are under the control of a complex regulatory system (MARZLUF 1994). In *S. cerevisiae*, we have also found two different sulfate transporters.

Sul1p and Sul2p belong to a family of related proteins known to be sulfate transporters (SANDAL and MARCKER 1994). These comprise the Cys14 gene from *N. crassa*

(KETTER *et al.* 1991), the sat-1 gene from rat hepatocytes encoding a bicarbonate/sulfate anion exchanger (BISSIG *et al.* 1994) and the human DTDST gene, the mutation of which results in diastrophic dysplasia and which has been shown to encode a sulfate transporter (HASTBACKA *et al.* 1994). Amino acid sequence similarities were also found to sequences encoded by the human DRA gene (SCHWEINFEST *et al.* 1993) and a nodulin gene from soybean (SANDAL and MARCKER 1994). The alignments of these different sulfate transporters have been recently updated (SANDAL and MARCKER 1994; SMITH *et al.* 1995). They show moderate levels of homology between the complete sequences of these proteins. However, invariant residues and especially the YGLY motif pointed out by SANDAL and MARCKER (1994) could be the signature of sulfate transporters. In addition, it must be noted that a search against the sequence data bases showed the presence on the yeast genome of one sequence whose product exhibits moderate similarities to Sul1p and Sul2p (~20% of conserved residues) and that corresponds to the *YPR003c* ORF from the chromosome *XVI*. However, nothing is known about the function of this gene.

While this work was in progress SMITH *et al.* (1995) isolated a cDNA from *S. cerevisiae* encoding a high affinity sulfate transporter. As already stated, this *SUL1* gene and the *SUL1* gene described here are identical. These authors disrupted the *SUL1* gene and their resulting mutant was unable to use 1 mM sulfate as a sulfur source, as if their parental strain was devoid of Sul2p activity. In contrast with the results of these authors, we found that the strain bearing the disrupted allele of the *SUL1* gene could still grow on sulfate even at 1 mM, as expected from our finding that *S. cerevisiae* has two high affinity sulfate transporters. We have no explanation for these discrepancies.

The *SUL3* gene encodes a factor involved in the transcriptional regulation of the *SUL2* gene as shown by derepression kinetics of the expression of *SUL2* in a *sul3* mutant. However, the fact that a *sul3* mutant is resistant to selenate while a *sul2* mutant exhibits lower resistance to this analogue seems to indicate that Sul3p could be involved in the regulation of the expression of other genes of the sulfate assimilation pathway. More work is needed to determine if *SUL2* is the only target of Sul3p.

We are indebted to DENISE HENRY for skilfull technical assistance. This work was supported by the Centre National de la Recherche Scientifique.

LITERATURE CITED

ALTSCHUL, S. F., W. GISH, W. MILLER, E. W. MYERS and D. J. LIPMAN, 1990 Basic local alignment search tool. J. Mol. Biol. **215:** 403–410.

ANDERSON, J. A., S. S. HUPRIKAR, L. V. KOCHIAN, W. J. LUCAS and R. F. GABER, 1992 Functional expression of a probable *Arabidopsis thaliana* potassium channel in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **89:** 3736–3740.

BALDARI, C., and G. CESARINI, 1985 Plasmids pEMBL: new single stranded shuttle vectors for the recovery and analysis of yeast DNA sequences. Gene **35:** 27–32.

BISSIG, M., B. HAGENBUCH, B. STIEGER, T. KOLLER and P. J. MEIER, 1994 Functional expression cloning of the canalicular sulfate transport system of rat hepatocytes. J. Biol. Chem. **269:** 3017–3021.

BRETON, A., and Y. SURDIN-KERJAN, 1977 Sulfate uptake in *Saccharomyces cerevisiae:* biochemical and genetic study. J. Bacteriol. **132:** 224–232.

BUSCH, A. E., S. WALDEGGER, T. HERZER, J. BIBER, D. MARKOVICH *et al.*, 1994 Electrogenic cotransport of Na+ and sulfate in Xenopus oocytes expressing the Na^+/SO_4^{2-} transport protein NaSi-1. J. Biol. Chem. **269:** 12407–12409.

CHEREST, H., and Y. SURDIN-KERJAN, 1992 Genetic analysis of a new mutation conferring cysteine auxotrophy in *Saccharomyces cerevisiae:* updating of the sulfur metabolism pathway. Genetics **130:** 51–58.

CHEREST, H., N. T. NGUYEN and Y. SURDIN-KERJAN, 1985 Transcriptional regulation of the MET3 gene of *Saccharomyces cerevisiae*. Gene **34:** 269–281.

CLARKSON, D. T., F. W. SMITH and P. J. VANDENBERG, 1983 Regulation of sulphate transport in a tropical legume. *Macroptilium atropurpureum*, cv Siratro. J. Exp. Bot. **34:** 1463–1483.

CLARKSON, D. T., M. J. HAWKESFORD, J.-C. DAVIDIAN and C. GRIGNON, 1992 Contrasting responses of sulfate and phosphate transport in barley (Hordeum vulgare L.) roots to protein-modifying reagents and inhibition of protein synthesis. Planta **187:** 306–314.

CRAM, L. J., 1983 Characteristics of sulphate transport across plasmalemma and tonoplast of carrot root cells. Plant Physiol. **72:** 204–211.

CUPPOLETTI, J., and I. H. SEGEL, 1975 Kinetics of sulfate transport by *Penicillium notatum*. Interactions of sulfate, protons, and calcium. Biochemistry **14:** 4712–4718.

FELDMAN, H., M. AIGLE, G. ALJINOVIC, B. ANDRE, M. C. BACLET *et al.*, 1994 Complete DNA sequence of yeast chromosome II. EMBO J. **13:** 5795–5809.

FINK, G. R., 1970 The biochemical genetics of yeasts. Methods Enzymol. **17:** 59–78.

GLASER, H. U., D. THOMAS, R. GAXIOLA, F. MONTRICHARD, Y. SURDIN-KERJAN *et al.*, 1993 Salt tolerance and methionine biosynthesis in *Saccharomyces cerevisiae* involve a putative phosphatase gene. EMBO J. **12:** 3105–3110.

GREEN, L. S., D. E. LAUDENBACH and A. R. GROSSMAN, 1989 A region of a cyanobacterial genome required for sulfate transport. Proc. Natl. Acad. Sci. USA **86:** 1949–1953.

HASTBACKA, J., A. DE LA CHAPELLE, M. M. MAHTANI, G. CLINES, M. P. REEVE-DALY *et al.*, 1994 The diastrophic dysplasia gene encodes a novel sulfate transporter: positional cloning by fine-structure linkage disequilibrium mapping. Cell **78:** 1073–1087.

HAWKESFORD, M. J., J.-C. DAVIDIAN and C. GRIGNON, 1993 Sulphate/proton cotransport in plasma-membrane vesicles isolated from roots of *Brassica napus* L.: increased transport in membranes isolated from sulphur-starved plants. Planta **190:** 297–304.

HIGGINS, D. G., 1994 Multiple alignment of DNA and protein sequences. Methods Mol. Biol. **25:** 307–318.

ISH-HOROWICZ, D., and J. F. BURKE, 1981 Rapid and efficient cosmid cloning. Nucleic Acids Res. **9:** 2989–2998.

KETTER, J. S., G. JARAI, Y. H. FU and G. A. MARZLUF, 1991 Nucleotide sequence, messenger RNA stability, and DNA recognition elements of cys-14, the structural gene for sulfate permease II in *Neurospora crassa*. Biochemistry **30:** 1780–1787.

KURAS, L., and D. THOMAS, 1995 Identification of the yeast methionine biosynthetic genes that require the centromere factor 1 for their transcriptional activation. FEBS Lett. **367:** 15–18.

KURAS, L., H. CHEREST, Y. SURDIN-KERJAN and D. THOMAS, 1996 A heteromeric complex containing the centromere binding factor 1 and two basic leucine zipper factors, Met4 and Met28, mediates the transcription activation of yeast sulfur metabolism. EMBO J. **15:** 2519–2529.

LASS, B., and C. I. ULLRICH-EBERIUS, 1984 Evidence for proton-sulfate cotransport and its kinetics in *Lemna gibba* Gl. Planta **161:** 53–60.

LAUDENBACH, D. E., and A. R. GROSSMAN, 1991 Characterization and mutagenesis of sulfur-regulated genes in a cyanobacterium:

evidence for function in sulfate transport. J. Bacteriol. **173:** 2739–2750.

LEE, R. B., 1982 Selectivity and kinetics of ion uptake by barley plants following nutrient deficiency. Ann. Bot. **50:** 429–449.

MARKOVICH, D., J. FORGO, G. STANGE, J. BIBER and H. MURER, 1993 Expression cloning of rat renal Na^+/SO_4^{2-} cotransport. Proc. Natl. Acad. Sci. USA **90:** 8073–8077.

MARZLUF, G. A., 1994 Genetics and molecular genetics of sulfur assimilation in fungi. Adv. Genet. **31:** 187–206.

MEIER, P. J., J. VALENTINAS, G. HUGENTOBLER and I. RAHM, 1987 Bicarbonate sulfate exchange in canalicular rat liver plasma membrane visicles. Am. J. Physiol. **253:** G461-G468.

RENNENBERG, H., O. KEMPER and B. THOENE, 1989 Recovery of sulfate transport into heterotrophic tobacco cells from inhibition by reduced glutathione. Physiol. Plant. **76:** 271–276.

ROOMANS, G. M., G. A. KUYPERS, A. P. THEUVENET and G. W. BORST-PAUWELS, 1979 Kinetics of sulfate uptake by yeast. Biochim. Biophys. Acta **551:** 197–206.

ROTHSTEIN, R. J., 1983 One step gene disruption in yeast. Methods Enzymol. **101:** 202–211.

SANDAL, N. N., and K. A. MARCKER, 1994 Similarities between a soybean nodulin, *Neurospora crassa* sulphate permease II and a putative human tumour suppressor. Trends Biochem. Sci. **19:** 19.

SCHOLLER, P., V. BENES, H. VOSS, W. ANSORGE and J. D. HOHEISEL 1996 Sequencing 39,350 bp of *Saccharomyces cerevisiae* chromosome XII utilising ordered shotgun libraries. DNA Sequence (*in press*).

SCHWEINFEST, C. W., K. W. HENDERSON, S. SUSTER, N. KONDOH and T. S. PAPAS, 1993 Identification of a colon mucosa gene that is down-regulated in colon adenomas and adenocarcinomas. Proc. Natl. Acad. Sci. USA **90:** 4166–4170.

SENTENAC, H., N. BONNEAUD, M. MINET, F. LACROUTE, J. M. SALMON *et al.*, 1992 Cloning and expression in yeast of a plant potassium ion transport system. Science **256:** 663–665.

SHERMAN, F., G. R. FINK and J. B. HICKS, 1979 *Methods in Yeast Genetics: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

SIRKO, A., M. HRYNIEWICZ, D. HULANICKA and A. BOCK, 1990 Sulfate and thiosulfate transport in Escherichia coli K-12: nucleotide sequence and expression of the cysTWAM gene cluster. J Bacteriol. **172:** 3351–3357.

SMITH, F. W., P. M. EALING, M. J. HAWKESFORD and D. T. CLARKSON, 1995a Plant members of a family of sulfate transporters reveal functional subtypes. Proc. Natl. Acad. Sci. USA **92:** 9373–9377.

SMITH, F. W., M. J. HAWKESFORD, I. M. PROSSER and D. T. CLARKSON, 1995b Isolation of a cDNA from Saccharomyces cerevisiae that encodes a high affinity sulphate transporter at the plasma membrane. Mol. Gen. Genet. **247:** 709–715.

SMITH, I. K., 1976 Characterization of sulfate transport in cultured tobacco roots. Plant Physiol. **58:** 358–362.

THOMAS, D., R. BARBEY and Y. SURDIN-KERJAN, 1990 Gene-enzyme relationship in the sulfate assimilation pathway of *Saccharomyces cerevisiae.* Study of the 3′-phosphoadenylylsulfate reductase structural gene. J. Biol. Chem. **265:** 15518–15524.

THOMAS, D., R. BARBEY, D. HENRY and Y. SURDIN-KERJAN, 1992 Physiological analysis of mutants of *Saccharomyces cerevisiae* impaired in sulphate assimilation. J. Gen. Microbiol. **138:** 2021–2028.

Communicating editor: M. D. ROSE