Genetic Analysis of a New Mutation Conferring Cysteine Auxotrophy in *Saccharomyces cerevisiae*: Updating of the Sulfur Metabolism Pathway

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ABSTRACT

We have identified a mutation in a gene of *Saccharmyces cerevisiae*, *STR1*, that leads to a strict nutritional requirement for cysteine. The *str1-1* mutation decreases to an undetectable level the cystathionine γ-lyase activity. This enzyme catalyzes one of the two reactions involved in the transsulfuration pathway that yields cysteine from homocysteine with the intermediary formation of cystathionine. The phenotype induced by this mutation implies that, in *S. cerevisiae*, the sulfur atom of sulfide resulting from the reductive assimilation of sulfate is incorporated into a four carbon backbone yielding homocysteine, which, in turn, is the precursor of the biosynthesis of both cysteine and methionine. This also reveals that the direct synthesis of cysteine by incorporation of the sulfur atom into a three carbon backbone as found in *Escherichia coli* does not occur in *S. cerevisiae*. The study of the meiotic progeny of diploid strains heterozygous at the *STR1* locus has shown that the *str1-1* mutation undergoes a particularly high frequency of meiotic gene conversion.

THE biosynthesis of the sulfur amino acids, cysteine and methionine, requires first the concentration of sulfate from the medium and its reduction into sulfide. This set of reactions is followed by the incorporation of the sulfur atom of sulfide into a three or a four carbon chain amino acid. Whereas the series of reactions allowing the reduction of sulfate into sulfide appears to be identical in all microorganisms, the assimilation of the sulfur atom in the amino acid backbone is achieved differently, depending on the organism. Indeed, sulfur can be incorporated into a three carbon derivative (a serine ester) yielding cysteine, or in a four carbon derivative (a homoserine ester) yielding homocysteine, or these two mechanisms can exist simultaneously in the same organism.

Homocysteine and cysteine can be converted one into the other by the so-called transsulfuration pathways that transfer the sulfur atom with the intermediary formation of cystathionine. In the C3 to C4 transsulfuration pathway (reactions 3 and 4, Figure 1) cystathionine is synthesized from cysteine and a homoserine ester in a reaction catalyzed by cystathionine γ-synthase. Its cleavage is then catalyzed by cystathionine β-lyase, yielding homocysteine. In the C4 to C3 transsulfuration pathway (reactions 1 and 2, Figure 1), cystathionine is synthesized from homocysteine and serine in a reaction catalyzed by cystathionine β-synthase. It is cleaved by the action of cystathionine γ-lyase, yielding cysteine.

In *Escherichia coli*, in which the mechanism of sulfur incorporation was clearly established by the study of mutants, sulfide is condensed with a serine ester (*O*-acetylserine) to yield cysteine (KREDICH 1987) which is then transformed into homocysteine by the C3 to C4 transsulfuration pathway. The homoserine ester used for the synthesis of cystathionine in this organism is *O*-succinylhomoserine (COHEN and SAINT-GIRONS 1987).

By contrast, in lower eukaryotes, the reactions allowing the incorporation of sulfur are to date less clearly established. For example, in *Aspergillus nidulans*, study of mutants has shown that sulfur is incorporated in the C3 backbone yielding cysteine, homocysteine being formed by the C3 to C4 transsulfuration pathway (BALBIN and STEPIEN 1974). Nevertheless, direct sulfhydrylation of *O*-acetylhomoserine by acetylhomoserine sulfhydrylase has also been suggested in *A. nidulans* (PASZEWSKI and GRABSKI 1974; PIENIAZEK *et al.* 1974). In *Neurospora crassa*, another ascomycete, results of regulatory studies favor the incorporation of sulfur at the level of homocysteine (PIOTROWSKA, KRUSZEWSKA and PASZEWSKI 1980).

In *Saccharomyces cerevisiae*, it is generally admitted that sulfur is incorporated both in *O*-acetylserine and in *O*-acetylhomoserine (Figure 1). This arises mainly from the results of YAMAGATA and co-workers, who purified a protein exhibiting both *O*-acetylhomoserine sulfhydrylase and *O*-acetylserine sulfhydrylase activities (YAMAGATA, TAKESHIMA and NAIKI 1974, 1975; YAMAGATA 1989). *MET25*, the structural gene for this *O*-acetylhomoserine-*O*-acetylserine sulfhydrylase (reaction 6, Figure 1), has been isolated and studied in our laboratory (SANGSODA, CHEREST and SURDIN-KERJAN 1986; KERJAN, CHEREST and SURDIN-KERJAN 1987; THOMAS, CHEREST and SURDIN-KERJAN 1989). We have reported that a *met25* mutant strain is able to grow on homocysteine as well as on cysteine, indi-

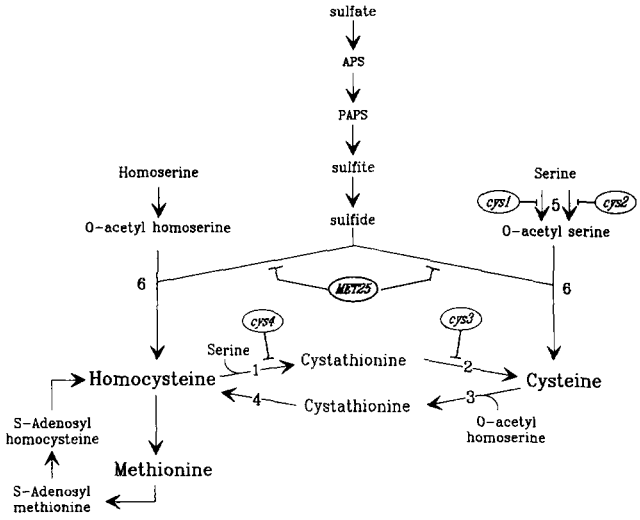

FIGURE 1.—Former pathway for the biosynthesis of sulfur amino acids in *S. cerevisiae*. The genes encoding the enzymes catalysing the different reactions are indicated. *CYS1*, *CYS2*, *CYS3* and *CYS4* are indicated as described in HALOS, cited in JONES and FINK (1982) and in ONO *et al.* (1984, 1988). APS, adenylylsulfate; PAPS, phosphoadenylyl sulfate.

cating that the two transsulfuration pathways (C3 to C4 and C4 to C3) are functional in *S. cerevisiae* (CHEREST, EICHLER and DE ROBICHON-SZULMAJSTER 1969; MASSELOT and DE ROBICHON-SZULMAJSTER 1975). In addition, only two mutant strains the growth requirement of which can be satisfied only by cysteine have been described up to now. They have been shown to belong to two complementation groups, *CYS1* and *CYS2*. First biochemical analysis indicated that *cys1* or *cys2* mutations result in the loss of serine transacetylase activity (HALOS cited in JONES and FINK 1982). This result was in contradiction with the pathway outlined in Figure 1, because a strain devoid of serine transacetylase activity should be able to synthesize cysteine by the C4 to C3 transsulfuration pathway, unless the *cys1* and *cys2* mutations have a pleiotropic effect impairing this pathway. Recently, ONO and co-workers (1984, 1988) have studied these *cys1* and *cys2* mutants and found that the *cys1* mutant lacked cystathionine γ-lyase activity (catalyzing reaction 2, Figure 1), whereas the *cys2* mutant was devoid of cystathionine β-synthase activity (catalyzing reaction 1, Figure 1). They thus concluded that the *cys1* mutant strain carried a second mutation affecting the gene encoding cystathionine γ-lyase that they called *CYS3* and that the *cys2* mutant strain carried a mutation affecting the structural gene of cystathionine β-synthase that they called *CYS4*. Such an hypothesis explained, in accord with the metabolic pathway presented in Figure 1, that the strains isolated by HALOS require cysteine for growth. But, to account for their phenotypic results, ONO and co-workers (1984, 1988) concluded that *CYS1* and *CYS3* on the one hand and *CYS2* and *CYS4* on the other hand were tightly linked.

By using a strain bearing a *met25* mutation we anticipated that we could isolate mutant strains impaired in the transsulfuration pathways, by selecting for strains growing exclusively either on cysteine or on homocysteine. A genetic study of these mutants has shown that, in *S. cerevisiae*, cysteine is exclusively synthesized from homocysteine by the C4 to C3 transsulfuration pathway. In addition, this study led to the identification of a genetic locus undergoing meiotic gene conversion with a high frequency.

TABLE 1

Strains used

Strains	Genotype	Source
EY9	*Matα,met25*	YGSC
X2180-1A	*MAT***a**	YGSC
S288C	*MATα*	YGSC
R280	*MAT***a***,leu2,his3*	R. ROTHSTEIN
CC359-OL2	*MATα,his3,leu2,ura3*	H. CHEREST
MT1	*MATα,met25,str-1-1*	This study
MT2	*MATα,met25,str2-1*	This study
CC554-17	*MATα,str1-1*	This study
CC554-12	*MAT***a***,str1-1*	This study
CC577-2C	*MATα,str1-1,leu2*	This study
CC555-3B	*MATα,met25,str2-1*	This study
CC555-2C	*MAT***a***,met25*	This study
CC591-7	*MAT***a***,his3,leu2,cys2-1*	This study
JW1-1C	*MATα,cys2-1,CUP1, cys4-1*[a]	YGSC
JW4-5C	*MAT***a***,cys1-3,CUP1, cys3-1*[b]	YGSC

[a] Mutation identified by ONO *et al.* (1984).
[b] Mutation identified by ONO *et al.* (1988).

MATERIALS AND METHODS

Strains: *S. cerevisiae* strains used in this work are listed in Table 1.

Media: YPG medium contained 0.5% yeast extract, 0.5% Bacto-peptone and 3% glucose. YNBG medium contained 7 g/liter of Yeast Nitrogen Base without amino acids and 2% glucose. According to the auxotrophic requirements of strains, uracil (20 μg/ml), histidine (200 μg/ml) and leucine (100 μg/ml) were added to the growth media. B medium was a synthetic medium without any sulfur source. The composition of this medium was the following: (i) mineral salts: 15 mM ammonium chloride, 6.6 mM monopotassium phosphate, 0.5 mM dipotassium phosphate, 1.7 mM sodium chloride, 0.7 mM calcium chloride and 2 mM magnesium chloride; (ii) oligo elements: 0.5 μg/ml boric acid, 0.04 μg/ml copper chloride (1 H_2O), 0.1 μg/ml potassium iodide, 0.19 μg/ml zinc chloride and 0.05 μg/ml ferric chloride (6 H_2O); and (iii) vitamins and growth factors: 2 μg/ml calcium pantothenate, 2 μg/ml thiamine, 2 μg/ml pyridoxine, 0.02 μg/ml biotin and 20 μg/ml inositol. Glucose was added to 2% final concentration. This medium was filter sterilized and a sulfur source was added before use. Inorganic sulfur sources were added at 0.5 mM final concentration. When used as sulfur sources, amino acids were used at the following concentrations: 0.5 mM for L-cysteine, 0.5 mM for DL-homocysteine. To obtain solid B medium, 1% agarose was used instead of agar to minimize the addition of uncontrolled sulfur sources to the medium.

Genetic methods: Genetic crosses, sporulation, dissection and the scoring of nutritional markers were as described by SHERMAN, FINK and HICKS (1987).

Growth and acellular extracts: Cells were grown in 10 ml of YNBG medium supplemented to meet the auxotro-

phies of the strains. When the cell concentration reached 10^7 cells/ml, the cultures were centrifuged and washed in potassium phosphate buffer, 100 mM, pH 7.5. The extracts were performed in 100 mM potassium phosphate buffer, pH 7.5, using glass beads as described previously in THOMAS, CHEREST and SURDIN-KERJAN (1989). The beads and the cell debris were eliminated by centrifugation at 4°. For the serine transacetylase assay, we tried three different extraction buffers: buffer A contained 100 mM potassium phosphate pH 7.5, 1 mM phenylmethylsulfonylfluoride (PMSF), and 10% glycerol (v/v). Buffer B contained 100 mM potassium phosphate pH 7.5, 0.1 mM EDTA and 0.05 mM pyridoxal phosphate (PLP). Buffer C contained 100 mM potassium phosphate, pH 7.5, 1 mM PMSF, 0.1 mM EDTA, 0.05 mM PLP and 10% glycerol. To test serine transacetylase, acellular extracts were made in an Eaton Press from 250-ml cultures.

Enzymatic assays: *O*-Acetylhomoserine sulfhydrylase was assayed as described by WIEBERS and GARNER (1967) and the homocysteine formed was estimated according to the method described by KREDICH and TOMKINS (1966). For each extract, four different protein concentrations (2–10 μg) were used. Cystathionine β-synthase was measured on 250 and 500 μg of protein in a final volume of 250 μl by the method described by KASHIWAMATA and GREENBERG (1970); cystathionine γ-lyase was assayed on 50 and 100 μg of protein in a final volume of 250 μl by the method described in PASZEWSKI and GRABSKI (1974) and the cysteine synthesized in the reaction was measured as described by GAITONDE (1967). For cystathionine β-lyase assay, we took advantage of the fact that propargylglycine inhibits *in vitro* cystathionine γ-lyase activity (PIOTROWSKA and PASZEWSKI 1986). Cystathionine β-lyase activity was measured on 50 and 100 μg of protein in a final volume of 100 μl. The reaction mixture contained: 200 mM potassium phosphate buffer, pH 7.5, 12 mM L-cystathionine, 0.5 mM pyridoxal phosphate and 1 mM propargylglycine. The incubation was for 30 min at 37° and the homocysteine formed was estimated by the method of KREDICH and TOMKINS (1966). Serine transacetylase was assayed by a modification of the methods described by KREDICH and BECKER (1971) and by YAMAGATA (1987). The reaction mixture contained in a total volume of 1 ml, 100 mM potassium phosphate buffer, pH 7.5, 10 mM L-serine, and 0.15 mM acetyl-CoA. For each extract, four different protein concentrations were assayed and a blank without serine was made for each protein concentration. After 30 min of incubation at 30°, 0.65 mM 5,5′-dithiobis-(2-nitrobenzoic acid) was added. Absorbance was measured at 412 nm. The same assay was run to determine homoserine transacetylase activity with homoserine in place of serine. The specific activity was determined, using E (mM) for 1 cm light path = 13.6.

Serine transacetylase has also been assayed by a radioactive assay. The assay mixture was as described above except it was run in 100-μl final volume and serine was radioactive (10 mM final concentration, 2700 cpm/nmol). After incubation, the reaction was stopped by 5 min at 95°. After centrifugation, 20 μl of the supernatant was layered on a cellulose thin layer chromatography plate. Chromatography was performed in butanol/acetic acid/water (12/30/50/in volume) and radioactive spots were revealed by autoradiography.

Protein concentrations were estimated by the method described by LOWRY *et al.* (1951).

RESULTS

Isolation of mutants impaired in the transsulfuration pathways: To isolate mutants impaired in the transsulfuration pathways, we mutagenized a strain bearing a *met25* mutation. Such a mutation inactivates the synthesis of the sulfur amino acids from the sulfur arising from the reduction of sulfate.

The strain EY9 was submitted to UV mutagenesis to obtain a 10% survival. Then 15,000 colonies were tested for their ability to grow on cysteine or homocysteine. One strain was found to grow exclusively on cysteine (strain MT1) and one was shown to grow exclusively on homocysteine (strain MT2).

TABLE 2

Analysis of the meiotic progeny of diploids CC555 and CC579

		Addition to minimal YNBG medium					
		None		Homocysteine	Cysteine		
Diploid	No tetrads tested	$0^+/4^-$	$2^+/2^-$	$4^+/0^-$	$2^+/2^-$	$3^+/1^-$	$4^+/0^-$
CC555	15		15	15	1	11	3
CC579	16	16		16	16		

The concentration of DL-homocysteine was 0.2 mM and of L-cysteine was 0.5 mM. CC555 = MT2 × X2180-1A (*met25,str2/MET25,STR2*); CC579 = CC555-3B × CC555-2C (*met25,str2/met25,STR2*).

Genetic and phenotypic analysis of the mutant strain MT2: The mutant MT2 which grows on homocysteine but not on cysteine was crossed to the wild type strain X2180-1A. The diploid strain (CC555) was sporulated and 15 tetrads were analyzed. The inability to grow on cysteine segregated as a two gene trait (Table 2). These results show that probably the isolated mutation results in the inability to grow on cysteine only in the presence of a *met25* mutation. A segregant of cross CC555, exhibiting the same phenotype as strain MT2 was thus backcrossed to a *met25* mutant strain. The resulting diploid (CC579) was sporulated and 16 tetrads analyzed. Results (Table 2) show that, in this background, the inability to grow on cysteine segregates perfectly in a $2^+/2^-$ pattern, proving that the inability to grow on cysteine segregates as a monogenic trait. The gene, the mutation of which leads to a strict homocysteine requirement in the presence of a *met25* mutation, has been called *STR2* (for *S*ulfur *TR*ansfer in accord with R. K. MORTIMER).

Phenotypic analysis of a strain bearing the *str2-1* mutation has been made on YNBG and B media (Table 3). It must be recalled that in the B medium, the added amino acid is the only sulfur source. The results show that such a strain is able to use homocysteine as a sulfur source (B medium + homocysteine). By contrast, a *str2-1* mutant cannot use cysteine as a sulfur source (B medium + cysteine) proving that the C3 to C4 transsulfuration pathway (cysteine to homocysteine) is impaired in this mutant. Moreover, the *str2* mutant grows on YNBG medium without any addition, as this medium contains sulfate that can be

TABLE 3

Phenotypic analysis of different strains

Strain	Relevant genotype	Addition to YNBG minimal medium			Addition to B medium		
		None	Hcyst	Cyst	None	Hcyst	Cyst
X2180-1A		+	+	+	−	+	+
CC359-OL2		+	+	+	−	+	+
R280		+	+	+	−	+	+
EY9	*met25*	−	+	+	−	+	+
CC554-17	*str1-1*	−	−	+	−	−	+
CC554-12	*str1-1*	−	−	+	−	−	+
CC555-1D	*str2-1*	+	+	+	−	+	−
MT1	*str1-1,met25*	−	−	+	−	−	+
MT2	*str2-1,met25*	−	+	−	−	+	−

Growth was noted after 48 hr at 30°. + indicates good growth, − indicates no growth. Hcyst, DL-homocysteine 0.2 mM; Cyst, L-cysteine 0.5 mM.

reduced and as the sulfur atom can be incorporated into homocysteine by this strain.

Genetic and phenotypic study of the mutant strain MT1: The mutant MT1 which grows on cysteine but not on homocysteine was crossed to the wild type strain X2180-1A. The diploid (CC554) was sporulated but no complete tetrad germinated. Among 31 random spores, fourteen could grow on minimal medium supplemented with homocysteine or with cysteine, which is the phenotype of *met25* bearing strains, while seventeen spores grew only on cysteine supplemented medium, which is the phenotype of the strain MT1 bearing both a *met25* mutation and a mutation that allows growth on cysteine and not on homocysteine. However, some of these 17 strains could carry only the newly isolated mutation if this mutation resulted by itself in a cysteine requirement. To determine if the strains that grow only on cysteine carried the *met25* mutation, complementation tests were made. Diploids were constructed between the 17 strains growing exclusively on cysteine and a *met25* mutant. Among the 13 strains that gave diploids with the *met25* mutant, ten did not complement with the *met25* mutant whereas three did. Two of the three strains that required cysteine and complemented the *met25* mutation were crossed to wild-type strains, yielding the diploid strains CC575 and CC577, which were sporulated. Phenotypic analyses of 48 tetrads from diploid CC575 and 61 tetrads from diploid CC577 are shown in Table 4. In the case of diploid CC575, the His^-, Ura^- and Leu^- characters showed a perfect $2^+/2^-$ segregation. Likewise, the Leu^- and His^- characters involved in cross CC577 segregated perfectly $2^+/2^-$. As expected, all segregants from CC575 and from CC577 grow on a cysteine supplemented medium. For the cysteine requirement, 43 tetrads out of 48 in cross CC575 and 50 tetrads out of 61 in cross CC577 exhibit a $2^+/2^-$ segregation. Nevertheless, in both crosses an equal number of $3^+/1^-$ and of $1^+/3^-$ tetrads were found. This segregation pattern is typical of

TABLE 4

Analysis of the meiotic progeny of diploids CC575 and CC577

Diploid	No. of tetrads tested	Types of tetrads, tested on minimal YNBG medium		
		$2^+/2^-$	$3^+/1^-$	$1^+/3^-$
CC575	48	43	4	3
CC577	61	50	6	5

CC575 = CC359-OL2 × CC554-12 (*STR1/str1*); CC577 = R280 × CC554-17 (*STR1/str1*).

meiotic gene conversion and is not compatible with the segregation of two linked genes. All spores from diploids CC575 and CC577 were tested on the sulfurless B medium supplemented with homocysteine. Only strains able to synthesize cysteine from homocysteine, thus having an active C4 to C3 transsulfuration pathway, can grow on such a medium. The segregation on this medium was the same as in the YNBG minimal medium showing that, in the tetrads exhibiting $3^+/1^-$ and $1^+/3^-$ segregation of the cysteine marker, growth corresponds in all cases to a functional C4 to C3 transsulfuration pathway and the absence of growth results from an impaired C4 to C3 transsulfuration pathway.

In conclusion, the analysis of the progeny of diploids CC575 and CC577 proves that the cysteine requirement results from a mutation affecting a single gene. This gene has been named *STR1*. The ability of strains bearing the *str1-1* mutation to grow on media differently supplemented is summarized in Table 3.

Enzymatic analysis: *O*-Acetylhomoserine sulfhydrylase, cystathionine-β-synthase, cystathionine γ-lyase, and cystathionine β-lyase have been assayed in different mutant strains. The main result of this enzymatic study (Table 5) is that the *str1-1* mutants are devoid of cystathionine γ-lyase activity. All strains exhibit comparable activities for the other enzymes with the exception of strains bearing a mutation in gene *MET25*, which are devoid of acetylhomoserine sulfhydrylase activity, *MET25* being the structural gene for this enzyme (KERJAN, CHEREST and SURDIN-KERJAN 1987). These results show also that the strain CC555-1D, bearing a mutation in the *STR2* gene, has a wild-type cystathionine β-lyase activity. According to the results reported above, this strain could be impaired in the C3 to C4 transsulfuration pathway, which is comprised of two steps catalyzed respectively by cystathionine γ-synthase and by cystathionine β-lyase. As the *str2-1* mutant exhibits cystathionine β-lyase activity, this mutation affects probably the gene encoding cystathionine γ-synthase. This point could not be enzymatically verified because no assay conditions have been found for this enzyme in yeast.

Genetic and phenotypic analysis of strain JW4-5C: The results reported here show that a strain bearing the *str1-1* mutation has no cystathionine γ-

TABLE 5

Enzymatic analysis of different strains

Strain	Relevant genotype	Addition to MM	CTT β-synth	CTT γ-lyase	OAH Sulfh	CTT β-lyase
CC359-OL2			18.5	5.9	175	5.8
		L-cys	15.6	6.2	168	—
CC554-17	*str1*	L-cys	18.5	0.60	137	—
CC577-5B	*str1*	L-cys	17.7	0.35	138	—
CC555-1D	*str2*		17.6	6.1	138	7.3
CC555-1B	*met25*	DL-Hcys	30	8.7	0	—
MT1	*met25,str1*	L-cys	26.5	0.50	0	—
MT2	*met25,str2*	DL-Hcys	15	6.3	0	7.2
JW1-1C	*cys2*	L-cys	0	11	243	—
JW4-5C	*cys1*	L-cys	12	0.6	241	—

MM, YNBG-based minimal medium L-cys: 0.2 mM L-cysteine; DL-Hcys, 1 mM DL-homocysteine; CTT β-synth, cystathionine β-synthase; CTT γ-lyase, cystathionine γ-lyase; CTT β-lyase, cystathionine β-lyase; OAH Sulfh, *O*-acetyl homoserine sulfhydrylase. The activities are expressed as nmoles of substrate transformed·min^{-1}·mg $protein^{-1}$. — indicates not determined.

lyase activity. ONO *et al.* (1984, 1988) have reported that strain JW4-5C bearing the *cys1-3* mutation described by HALOS (cited in JONES and FINK 1982), was devoid of cystathionine γ-lyase. For these authors, the cysteine auxotrophy of strain JW4-5C results from simultaneous mutations in gene *CYS1* encoding serine *O*-transacetylase and in a tightly linked gene *CYS3*, which would encode cystathionine γ-lyase. This double mutation is expected to prevent the functioning of the C4 to C3 transsulfuration pathway as well as the direct incorporation of sulfur in *O*-acetylserine yielding cysteine. In view of our data which show that a single mutation in the C4 to C3 transsulfuration pathway leads to a nutritional requirement for cysteine, we decided to perform a genetic analysis of strain JW4-5C.

Strain JW4-5C was crossed to strain CC359-OL2, a diploid (CC626) was sporulated and its meiotic progeny was analyzed in 76 tetrads. The His^- Leu^- and Ura^- characters showed a perfect $2^+/2^-$ segregation. For the cysteine requirement, 72 tetrads showed a $2^+/2^-$ segregation while 2 tetrads were of the $3^+/1^-$ type and 2 tetrads were of the $1^+/3^-$ type. This type of segregation is typical of meiotic gene conversion but is not compatible with the segregation of two linked genes as reported by ONO *et al.*(1984). The cysteine requirement of strain JW4-5C appears thus to be the result of a single mutation (called here *cys3* according to ONO *et al* 1984) affecting a gene undergoing a high level of meiotic gene conversion, as it has been shown for gene *STR1*. To investigate whether this mutation is in the *STR1* gene, strain JW4-5C was crossed to strain CC577-2C bearing the *str1-1* mutation. The resulting diploid (CC620) was found to have no nutritional requirement. Study of the progeny from CC620 showed that among 40 tetrads tested, 34 exhibited $0^+/4^-$ segregation for the cysteine requirement and 6 tetrads showed a $1^+/3^-$ pattern. The

TABLE 6

Cystathionine γ-lyase activity in different diploid strains

Strain	Relevant genotype	Cystathionine γ-lyase activity
CC630	*STR1,CYS3/STR1,CYS3*	6.8
CC619	*str1,CYS4/STR1,cys4*	3.6
CC620	*str1,CYS3/STR1,cys3*	0.9

segregation of the cysteine requirement in the progeny of diploid CC620 is not compatible with the segregation of two independent genes. Such a segregation could nevertheless result either from the segregation of two linked genes or from meiotic gene conversion events between two alleles of the same gene as we have shown that both the *str1-1* allele and the *cys3* mutation present in strain JW4-5C exhibit a high frequency of gene conversion. Moreover, all spores derived from diploid CC620 were tested on B medium supplemented with homocysteine. The segregation was the same as on the sulfate containing medium (YNBG medium, see MATERIALS AND METHODS), showing that the cysteine requirement results in all cases from an impaired C4 to C3 transsulfuration pathway.

By genetic analysis we could not formally show the identity of the *STR1* and *CYS3* genes. We thus assayed cystathionine γ-lyase activity in an extract of diploid CC620. Indeed intragenic functional complementation between two different mutated alleles of the same gene results frequently in an inactive enzyme *in vitro*. Results reported in Table 6 show that diploid CC620 has a very low cystathionine γ-lyase activity, which is in favor of *STR1* and *CYS3* being the same gene. This table shows also a perfect gene dosage of this enzymatic activity in the *STR1/STR1* and in the *STR1/str1* diploids, which is a good indication that *STR1* is the structural gene for cystathionine γ-lyase. Another implication of these experiments is that cystathionine γ-lyase encoded by *STR1* that displays intragenic complementation, is likely to be at least a dimer

Strain JW4-5C bears a single mutation that results in (1) a strict cysteine auxotrophy, (2) a lack of cystathionine γ-lyase activity and (3) a high frequency of meiotic gene conversion. Moreover, diploid CC620 (*str1/cys3*), although prototrophic, exhibits a very low cystathionine γ-lyase activity *in vitro*. It thus appears that strain JW4-5C bears a mutation affecting the *STR1* gene, which we will indicate as *str1-2*.

Genetic analysis of strain JW1-1C: Strain JW1-1C has been shown by ONO *et al.* (1988) to be devoid of serine transacetylase activity and of cystathionine β-synthase, which catalyses the first reaction of the C4 to C3 transsulfuration pathway. One prediction from the results we report in the preceding sections is that a single mutation in the gene encoding cystathionine β-synthase should result in a strict requirement for cysteine. We performed a genetic analysis of strain

TABLE 7

Enzymatic analysis of a tetrad from diploid CC604

Strain	Growth on B medium + homocysteine	Cystathionine β-synthase
CC604-18A	+	16
CC604-18B	+	30
CC604-18C	−	0
CC604-18D	+	15

The relevant genotype of diploid CC604 (CC591-7 × S288C) is: *cys4/CYS4*.

JW1-1C. It was crossed to strain R280, a diploid (CC591) was sporulated and its meiotic progeny was analysed. No complete tetrads were obtained. Nevertheless one spore (CC591-7) exhibiting the same phenotype as JW1-1C was crossed to the wild type strain S288C. One diploid (CC604) was sporulated and 72 tetrads were analyzed. They all segregated perfectly $2^+/2^-$ for the histidine and the leucine requirements. For the cysteine requirement 69 tetrads segregated $2^+/2^-$, 2 tetrads were of the $3^+/1^-$ type and one was of the $1^+/3^-$ type. This segregation pattern is typical of meiotic gene conversion events occurring at a low frequency but not of the segregation of two genes. Moreover, the growth pattern of all spores was the same on B medium containing homocysteine as on the YNBG medium, proving that prototrophy is due in all cases to an active C4 to C3 transsulfuration pathway. Enzymatic analysis of one $3^+/1^-$ tetrad corroborated this finding. Indeed, absence of growth on cysteine corresponds to an absence of cystathionine β-synthase activity (Table 7) and, more important, the presence of cystathionine β-synthase activity, goes always with cysteine prototrophy. In the case of strain JW1-1C, a mutation in one gene appears thus to be responsible for the cysteine auxotrophy of the strain. To avoid confusion, we propose to name this gene *STR4*.

As reported in Table 5, our determination of the different enzymatic activities in strains JW4-5C and JW1-1C are in accord with those of ONO *et al.* (1984, 1988). However, the discrepancies between our genetic results and those of ONO *et al.* (1984, 1988) are difficult to account for. According to these authors, strains JW4-5C and JW1-1C each bear two tightly linked mutations, and cystathionine γ-lyase or cystathionine β-synthase could be inactive without resulting in an auxotrophic requirement. We could not confirm their results. We analyzed 40 tetrads from a heterozygous diploid involving JW4-5C and 72 tetrads in a cross involving JW1-1C and found no indication that these strains carried mutations in two linked genes resulting in a growth requirement for cysteine, nor of prototrophic strains impaired in the C4 to C3 transsulfuration pathway. On the contrary, our results show that a unique impairment in the C4 to C3 transsulfuration pathway always results in a cysteine requirement.

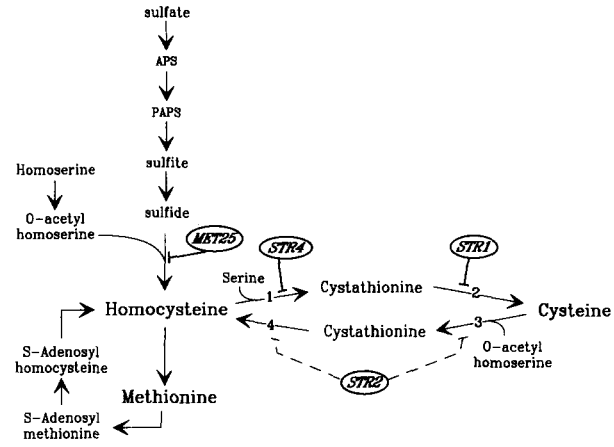

FIGURE 2.—Metabolism of sulfur amino acids in *S. cerevisiae*. For abbreviations see legend of Figure 1.

The metabolism of sulfur amino acids in *S. cerevisiae*: The results reported above show that in a strain of *S. cerevisiae*, a single mutation impairing the C4 to C3 transsulfuration pathway results in a strict growth requirement for cysteine. The consequence of this finding is that in *S. cerevisiae*, the C4 to C3 transsulfuration pathway is the only passage to cysteine biosynthesis. It follows that the pathway for sulfur amino acids biosynthesis in *S. cerevisiae* should be represented as in Figure 2, with the incorporation of the sulfur atom only in the four carbon backbone. One prediction of this model is that serine transacetylase plays no role in the biosynthesis of cysteine. We decided to investigate this hypothesis.

Serine transacetylase: We have assayed serine transacetylase in different strains. The striking result of these enzymatic determinations is that we were unable to find any serine transacetylase activity whatever the strain may be. Even in the wild type strains serine transacetylase was undetectable. To verify these results, we performed different experiments. In the first one, cells from strain CC359-OL2 (wild type for the sulfur amino acids metabolism) were extracted in the three different buffers described under MATERIALS AND METHODS. In the second one, we grew the wild-type strains used in the present work (X2180-1A, CC359-OL2 and R280) and made extracts in the stabilizing buffer of ONO *et al.* (1988) (buffer B described in MATERIALS AND METHODS). In another attempt to find serine transacetylase activity, we devised a new assay using radioactive serine as described under MATERIALS AND METHODS. But in all cases no serine transacetylase activity could be detected. For each acellular extract, homoserine transacetylase activity was assayed as a control by adding homoserine in place of serine in the assay and in each case an homoserine transacetylase activity was measured. The absence of detectable serine transacetylase activity in wild-type strains is in accord with the pathway shown

in Figure 2 based on our finding that cysteine auxotrophy is the result of the impairment of the C4 to C3 transsulfuration pathway.

The absence of serine transacetylase activity *in vitro* cannot be taken as a formal proof of its absence *in vivo*. However, our physiological argument against its existence is that the *str1-1* mutant fails to grow on sulfate. But one possible flaw of this experiment is that the *str1-1* mutation could block sulfate uptake or reduction. To show that sulfate uptake and reduction was active in the *str1-1* mutant, we crossed it to the *str2-1* mutant. The resulting diploid (CC587) was sporulated and 12 tetrads analyzed. The spores that needed both homocysteine and cysteine for growth on B medium, bore simultaneously the *str1-1* and *str2-1* mutations. In all cases, these double mutants could grow on YNBG medium supplemented with cysteine, showing that they could use the sulfate contained in the YNBG medium to synthesize homocysteine.

DISCUSSION

Genetic, phenotypic and enzymatic analyses of a new mutant of *S. cerevisiae* that exhibits a strict nutritional requirement for cysteine and that is impaired in a gene encoding cystathionine γ-lyase that we called *STR1* are reported. The use of a sulfur-controlled medium has confirmed that this cysteine requirement is due to the inactivation of the C4 to C3 transsulfuration pathway. Such a result shows that sulfur is incorporated in a C4 backbone and that cysteine is exclusively synthesized from homocysteine in *S. cerevisiae*. The pathway to sulfur amino acids biosynthesis should thus be represented as in Figure 2.

This report also reveals that the *str2-1* mutation impairing the C3 to C4 transsulfuration pathway does not result in any growth requirement in a minimal medium containing sulfate. This result is also in accord with the pathway outlined in Figure 2. Indeed, as cysteine is not an obligatory intermediary metabolite in the biosynthesis of methionine in *S. cerevisiae*, impairment of the C3 to C4 transsulfuration pathway does not induce a visible phenotype in a wild type background.

Supporting our model of sulfur amino acids metabolism is the action of propargylglycine, an antibiotic that has a lethal effect on *S. cerevisiae* (PIOTROWSKA and PASZEWSKI 1986). Indeed, it has been shown to inactivate specifically cystathionine γ-lyase in rats (BEATTY and REED 1980), although other enzymes can be affected to a lesser extent (BURNETT, MARCOTTE and WALSH 1980; TANASE and MORINO 1976). In *S. cerevisiae*, propargylglycine inhibits the activity of yeast cystathionine γ-lyase *in vitro* (PIOTROWSKA and PASZEWSKI 1986). We have performed experiments showing that it inhibits growth of the wild-type strain X2180-1A and that this effect can be reversed by the addition of cysteine to the medium but not by the addition of methionine (results not shown). These data can be explained by the fact that cysteine is only synthesized by the C4 to C3 transsulfuration pathway.

We also report here that serine transacetylase activity could not be detected in wild type strains. Although not a formal proof of the absence of this enzyme in *S. cerevisiae*, this result is in accord with the cysteine requirement of strains impaired only in the C4 to C3 transsulfuration pathway. We thus propose that serine transacetylase is normally not synthesized in *S. cerevisiae*. Moreover, a strain mutated only in the *STR1* gene does not grow on a medium supplemented with *O*-acetylserine and sulfate, indicating that *O*-acetylhomoserine sulfhydrylase does not function *in vivo* as an *O*-acetylserine sulfhydrylase (results not shown).

The model we propose for sulfur amino acid metabolism in *S. cerevisiae* involves incorporation of sulfur only in homocysteine by a reaction catalysed by the *MET25* encoded enzyme. It follows that the *MET25* encoded enzyme does not catalyse the incorporation of sulfur in a three carbon backbone, as postulated up to now. On the contrary, in *E. coli* the incorporation of sulfur is in O-acetyl serine yielding cysteine. In *E. coli*, two enzymes encoded respectively by *cysK* and *cysM* can catalyse the sulfhydrylation of *O*-acetylserine to cysteine. Comparison of the polypeptide sequences deduced from the gene *MET25* of *S. cerevisiae* (KERJAN, CHEREST and SURDIN-KERJAN 1987) and from genes *cysK* and *cysM* of *E. coli* (BYRNE *et al.* 1988; SIRKO *et al.* 1990) has revealed no similarities between the yeast enzyme and the *E. coli* enzymes, whereas similarities have been found between the *MET25* protein and two enzymes catalysing the C3 to C4 transsulfuration pathway in *E. coli* (BELFAIZA *et al.* 1986). This is in accord with our finding that the MET25 protein (acetylhomoserine sulfhydrylase) does not catalyse *in vivo* the sulfhydrylation of *O*-acetylserine.

The two transsulfuration pathways are functional in *S. cerevisiae*. Nevertheless, we can ask if they can be concomitantly active. This system in which the forward and reverse reactions are catalyzed by different enzymes is not unique but it seems that its study will bring elements to the knowledge in the evolution of pathways. The regulation of the transsulfuration pathways are presently under study in our laboratory.

In the study of the meiotic progeny of heterozygous diploid strains at the *STR1* locus, we have obtained a few tetrads showing irregular segregation for the cysteine requirement. We observed only $3^+/1^-$ and $1^+/3^-$ aberrant tetrad classes, which is typical of gene conversion. (FOGEL, MORTIMER and LUSNAK 1981). The *str1-1* allele that we have described here displays a high conversion frequency (about 15%). Such a frequency of gene conversion is particularly high. The gene conversion frequency for the *str1-2* allele (in

strain JW4-5C) is about 5%, an expression of the well known polarity of conversion (FOGEL, MORTIMER and LUSNAK 1981). Gene *STR1* could thus be a good substrate for a study of gene conversion at the molecular level.

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