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MOLECULAR GENETICS OF TRANSPORT AND METABOLISM OF SULFUR COMPOUNDS IN YEAST

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Abstract

Sulfur compounds have many biochemical functions including protein structure, enzyme catalysis, biological oxidation and detoxification. Glutathione is an essential reductant that protects against oxidative damage. In addition, glutathione may be conjugated with toxic substances such as cadmium ions and arsenite to produce compounds of low toxicity. A mutant of the yeast *Saccharomyces cerevisiae* defective in transport of L-cysteine was isolated utilizing a strain already deficient in the general amino acid permease and the transport of inorganic sulfate. Cells were mutagenized with ethylmethanesulfonate, and mutants capable of utilizing methionine but not cysteine as sulfur source were selected. Quantitative growth studies showed that one mutant exhibited defective growth on low cysteine, but normal growth on high cysteine. The mutant gave normal growth when glutathione was used as sole sulfur source. Results suggest that *Saccharomyces cerevisiae* contains two cysteine permeases, one of high affinity and one of low affinity. The mutant described appears to lack only the high-affinity permease. Cloning of the putative cysteine permease gene is in progress.

Key words: Yeast, Cysteine transport, Glutathione.

Sulfur in protein structure and enzyme catalysis

Sulfur compounds have many very important functions in biology and biochemistry. Two protein amino acids, methionine and cysteine are found in almost all proteins. Cysteine is particularly important in many structural proteins because two cysteine residues residing on different polypeptide chains or at different locations on the same chain can cause cross linking by forming disulfide bonds. For example, the A and B chains of the hormone insulin are covalently linked by two disulfide bonds. In addition, two cysteine residues in the A-chain form a loop (Figure 1).

Fibrous proteins like hair are cross linked by many such disulfide bonds.

The sulfhydryl groups of cysteine residues are key components of the catalytic sites of many enzymes. The -SH group is a nucleophile, and a number of enzymes catalyzing hydrolysis and acyl group transfer utilize this property of sulfhydryl groups. Moreover, sev-

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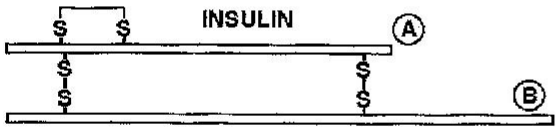

Fig. 1. Sulfur in protein structure.

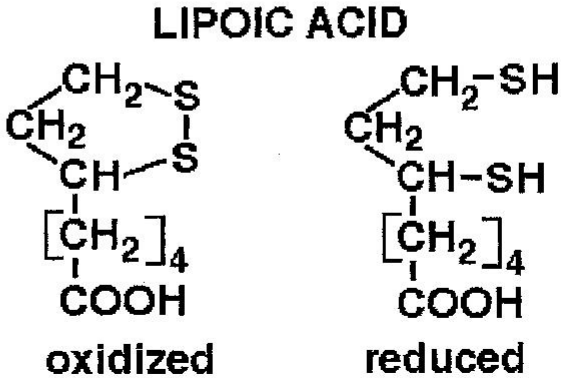

Fig. 2. Sulfur in biological oxidation.

eral coenzymes, such as coenzyme A and thiamine pyrophosphate, contain reactive sulfur groups. Frequently, thiolesters are produced as intermediates in enzyme-catalyzed reactions.

Sulfur in biological oxidation

Sulfur compounds are also central to many key reactions in biological oxidation (Figure 2). For example, the disulfide coenzyme lipoic acid undergoes cycles of oxidation and reduction during oxidation of pyruvate and 2-ketoglutarate by their respective dehydrogenases. These reactions are central to the operation of the citric acid cycle. Proteins containing complexes of iron and sulfur in equimolar concentrations are also essential to biological oxidation. Another citric acid cycle enzyme, succinate dehydrogenase, contains two of these complexes. The electron transport chain found in mitochondria contains seven proteins with such iron-sulfur centers: NADH dehydrogenase has four, cytochrome *b* is associated with two, and cytochrome c_1 with one.

Sulfur in detoxication reactions

Sulfur-containing compounds are also important in detoxication reactions. Proteins called metallothioneins can sequester substantial amounts of toxic metals such as mercury, copper and zinc, rendering them harmless to cells. These proteins are very rich in cysteine, which complexes the metal irons.

Aerobic cells are constantly exposed to highly toxic by-products of oxidation reactions, including peroxides and free-radicals. Experiments by Grant et al. [1] have shown that in the yeast, *Saccharomyces cerevisiae*, the tripeptide, glutathione (GSH) is required for resistance to oxidative stress. Normal yeast cells contain a high concentration of GSH, accounting for about 1% of the dry weight [2]. These investigators disrupted the *GSH1* gene which encodes the enzyme γ-glutamylcysteine synthetase (Gsh 1 p) to create a mutant that was completely unable to synthesize GSH, and required added GSH in order to grow on minimal medium (Figure 3). Surprisingly, growth could be restored by replacing GSH with cysteine or dithiothreitol, but not by ascorbate. Apparently, GSH is providing an essential reductant which protects against oxidative damage. Other thiol compounds can serve this purpose, but non-thiol reductants cannot. When the mutant was grown on complete medium, which contains a low concentration of GSH, it exhibited snsitivity to hydrogen peroxide. This indicated that GSH normally acts to protect cells from free radicals produced from peroxide, but when the GSH concentration drops, these reactive oxygen species can reach toxic levels.

We have studied a *gsh 1* disruption strain that also contains an *ade 2* mutation. Ordinarily, when an *ade 2* strain is grown on medium containing a low concentration of adenine, one of the intermediates of adenine biosynthesis accumulates in the cells. As the concentration of the intermediate rises, it forms a polymer which accumulates in the

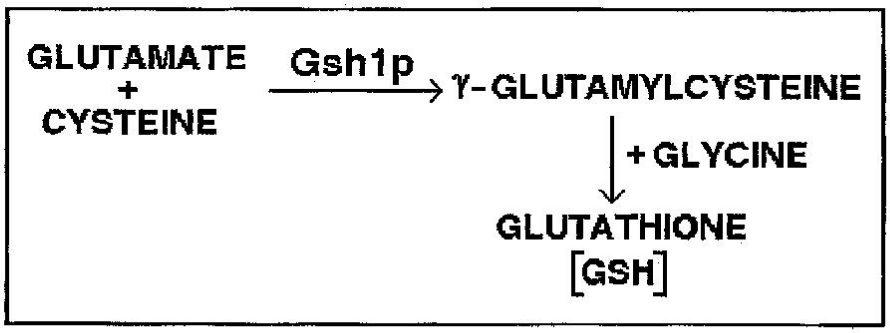

Fig. 3. Glutathione biosynthesis.

vacuole, causing the cells to turn red. However, the added presence of the *gsh 1* disruption results in a major reduction in the pigment accumulation (Figure 4). The probable explanation for this effect is that accumulation of intermediate requires conjugation with GSH in the cytoplasm, followed by ATP-dependent pumping of the GSH conjugate across the vacuolar membrane (Figure 5).

Yeast uses this same system for removing toxic Cd^{++} ions from the cytoplasm and sequestering them in the vacuole [3]. Two molecules of glutathione conjugate a single cadmium ion, in this case. It has also been shown that the same ATP-dependent pump can cause detoxication of arsenite [As(III)] by causing its accumulation in the vacuole, probably as $As[GS]_3$. The pump is know as an ABC transporter, and is encoded by the *YCF1* (yeast cadmium factor) gene [4].

From the point of view of mass, the major sulfur-containing compounds in yeast are the amino acids cysteine, methionine and, glutathione. When grow on minimal medium lacking these substances, yeast grows quite well, indicating that it has the biosynthetic capacity to produce all three compounds using inorganic sulfate as the source of the sulfur atoms required (Figure 6). The pathway of sulfate utilization has been well studied by Yolande Surdin-Kerjan and colleagues in France [5]. The pathway involves an eight-electron reduction of sulfate to inorganic sulfide, which is then incorporated into organic form as homocysteine. Homocysteine is methylated to give methionine. Two reactions are required to produce cysteine. First, a three-

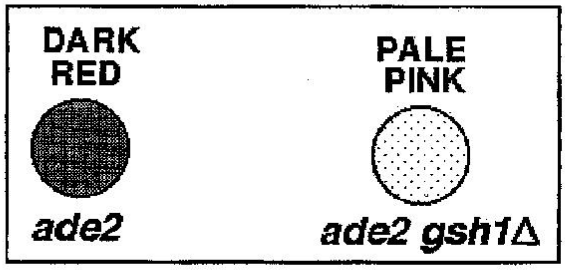

Fig. 4. Glutathione requirement for red pigment production in an ade2 mutant of *Saccharomyces cerevisiae*.

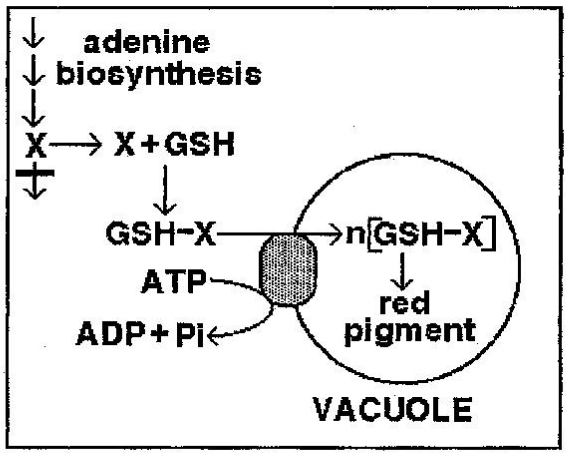

Fig. 5. Red pigment formation requires conjugation of accumulated intermediate X with GSH and ATP-dependent pump which transports the conjugate to the vacuole.

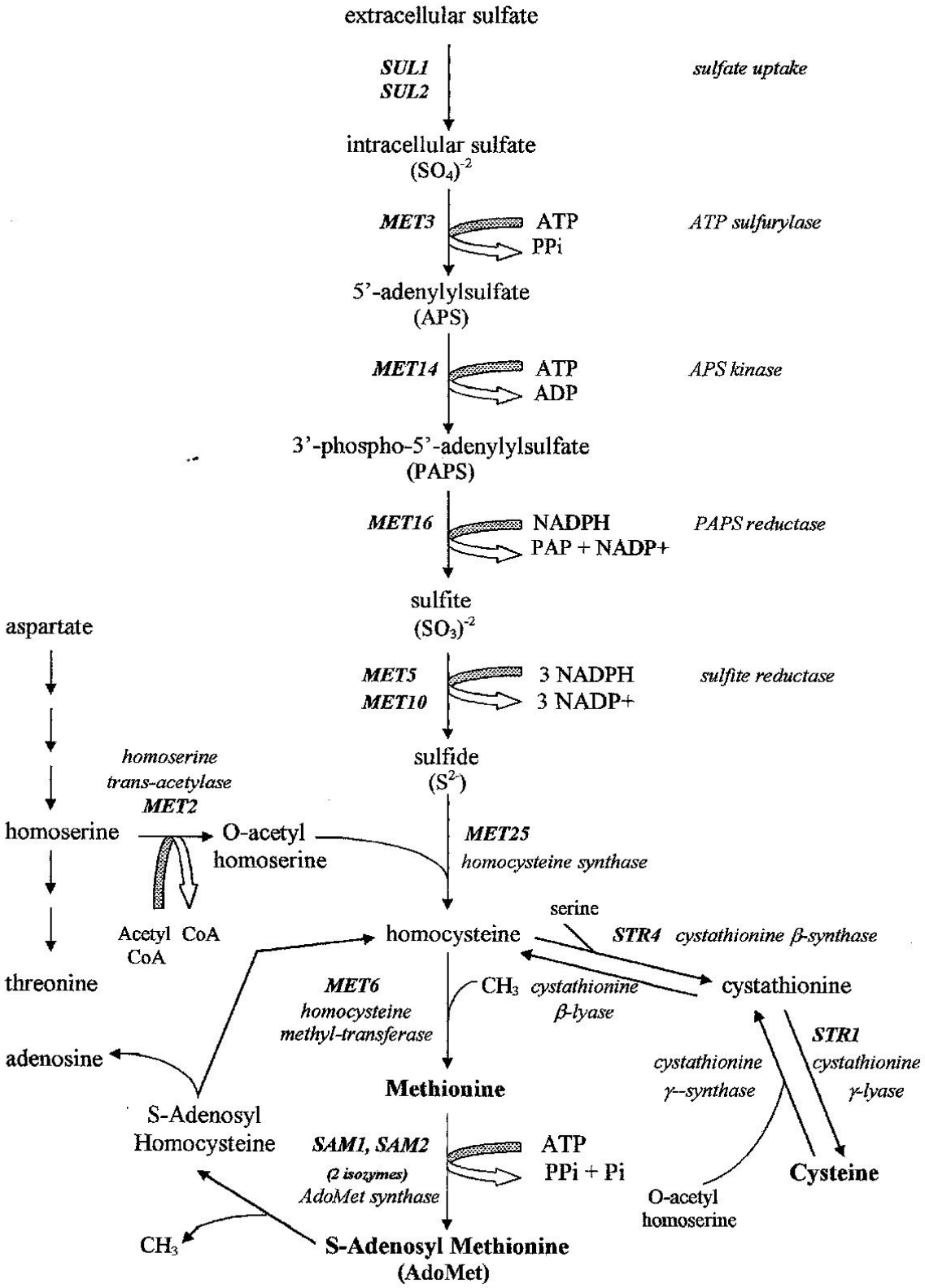

Fig. 6. Metabolism of sulfur compounds in *Saccharomyces cerevisiae*.

carbon fragment, derived from serine, reacts with homocysteine to produce the thioether, cystathionine. Second, cystathionine is cleaved, so that the sulfur atom is transferred to the three-carbon chain, forming cysteine and releasing the four-carbon unit as α-ketobutyrate + NH_3. This process is called the C4 to C3 transsulfuration pathway. Yeast also produces the enzymes that catalyze transfer of sulfur atoms from cysteine to homocysteine in a C3 to C4 transsulfuration pathway.

Sulfur compounds in brewing and wine production

Two by-products of the sulfur assimilation pathway which are important in the brewing and wine industries are sulfite and hydrogen sulfide. The amounts of these substances that accumulate during fermentation vary from one yeast strain to another, as well as with the composition of the medium being fermented to beer or wine. For example, when high concentrations of fermentable sugars are used, substantial amounts of acetaldehyde are formed which can result in undesirable flavors in the beverage. However, if the yeast is also producing substantial amounts of sulfite, it reacts with acetaldehyde to form a relatively tastless complex. Sulfite also acts as a natural antioxidant and can preserve beverage flavor which gradually deteriorates as trace compounds undergo oxidation during storage. Therefore, many wine yeasts have been selected which accumulate relatively high concentrations of sulfite. If yeast over-produces hydrogen sulfide, trace amounts of other organic sulfur compounds can be produced from it which affect flavor adversely.

Genetics of amino acid transport in yeast

Nearly fifty years ago it was recognized that permeases are specific proteins, and that the structure of these proteins is encoded by specific genes. This recognition naturally led investigators to devise ways to study the effects of mutations on transport of important nutrients. In the 1960's Marcelle Grenson in Belgium began an extensive series of investigations of the transport of amino acids in yeast. In many of these studies she was able to obtain mutants defective in genes encoding specific transporters by screening for resistance to toxic analogs of individual amino acids. Initially, she identified a gene encoding an arginine permease by selecting for mutants resistant to the arginine analog, canavanine [6]. The gene was named *CAN1*. By employing classical genetic techniques, the genes encoding several different amino acid permeases were identified. Many of these permeases have quite narrow specificities, but one of them, named the General Amino acid Permease, or GAP, was found to carry all the L-amino acids found in proteins as well as many D-amino acids [7]. The gene encoding GAP was designated *GAP1*. Of course, this work was done well before methods for gene cloning were developed, so the mutant genes, *can1* and *gap1*, were described only by their inheritance and their phenotypes. The normal *CAN1* gene was cloned and sequenced in 1985 [8] and the GAP1 gene in 1990 [9].

Energetics of amino acid transport

Cells of both humans and yeast carry out active transport of amino acids (Figure 7). In human cells, the sodium-potassium ATP-ase in the plasma membrane creates a chemical gradient of Na^+ ions, by pumping them out of the cytoplasm. This gradient is used to drive amino acid uptake though carriers that co-transport Na^+ ions and amino acids from outside into the cell interior. In yeast, a very similar system is employed except that it is a gradient of H^+ ions that provides the driving force. In this case, the proton pump that provides the proton gradient is an ATPase in the plasma membrane.

Molecular genetics of yeast permeases

The yeast *Saccharomyces cerevisiae* is extremely well suited for molecular genetics. It has a relatively small genome with about 6200 genes. Only a very small fraction of these genes contain introns, so most yeast genes can be cloned directly from a genomic library. In addition, yeast has very little repetitive DNA.

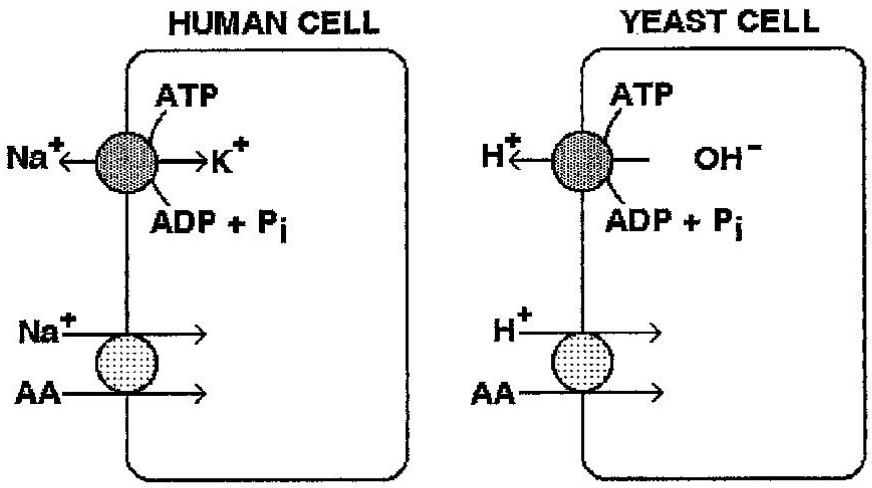

Fig. 7. Energetics of amino acid transport in human and yeast cells. In humans, a Na^+ gradient drives amino acid uptake. In yeast, a proton gradient drives transport.

Yeast can be transformed readily, so that direct complementation cloning is easily carried out. Homologous recombination occurs with very high efficiency in yeast. This enables one to insert genes that have been modified in vitro directly into the correct locus on the appropriate chromosome. This technique is widely used to produce mutants bearing large disruptions or deletions in specific genes, so that the consequences of total loss of function can be studied. It also allows site-specific mutagensis to be carried out readily. Another technique that is useful both in biotechnology and in research is to overexpress genes by replacing the normal promoter of a gene with a promoter of a highly expressed gene. This allows the over-production of useful recombinant proteins and permits the investigation of over-expression of normal yeast proteins, including permeases.

In 1988 André Goffeau in Belgium organized a project that involved about fifty labs, mostly in Europe, to carry out the sequencing of the entire yeast genome, each lab taking responsibility for a specific segment of one of the 16 chromosomes in a haploid yeast cell. In early 1996, the project was complete. Stanford University now maintains an excellent database of the entire yeast genome which is free on the internet [10]. Included are tools for working with the database. For example, you can pick any gene you want, and ask the program to make a restriction map of it using one or several rectriction enzymes. One of the surprises that came out of the sequencing results was that only about half of the genes encoded yeast proteins of known function.

Use of the yeast genome to identify transporter genes

Goffeau and investigators from various labs in the U.S., Australia, and Belgium have been using the yeast genome to develop an inventory of the all the known and suspected transporter proteins in *Saccharomyces cerevisiae* [11]. They have asked the computer to group all the proteins according to the number of transmembrane spanners (TMSs) they contain, deduced from the protein sequences. The following table shows the results. 5,301 proteins (85.1%) have zero or only one membrane-spanning region, and can be considered as soluble or essentially so. Of the 850 remaining proteins, 258 have been tentatively identified as transmembrane solute transporters. These have been classified into 42 families based on sequence comparisons to other known transporters in yeast and

Table 1. Inventory of yeast proteins by number of transmembrane spanners *

No. of TMSs	No. of Proteins	% of Total
0	4364	70.8
1	937	15.3
2-3	390	6.5
4-6	185	3.1
7-9	144	2.3
>10	121	2.0
Total	6141	100.0

* *From reference [11].*

in other organisms, especially *E. coli* (Table 1).

We have been interested in one of these 42 families called the APC family. This group includes 24 permeases, including permeases transporting many of the amino acids. The substrates of some of the members of this group have not been identified, and some of the permeases can transport a few, or many, different amino acids. One of the protein amino acids which has not been matched with a specific permease genes is the sulfur amino acid cysteine. José Antonio Chávez, a graduate student from Peru, and I have been studying cysteine transport in yeast, and have made significant progress in isolating a clone which we hope will be the gene encoding a specific cysteine permease.

Although kinetic studies by Ono's group in Japan indicated that yeast produces a single cysteine permease [12], so far, no cysteine transport mutants have been described, and the gene encoding the permease has not been cloned directly.

Construction of *sul1Δ gap1Δ* parental strain

To approach the problem of isolating cysteine permease mutants we first had to construct a special yeast strain with two characteristics: (1) the inability to utilize inorganic sulfate and (2) the inability to transport cysteine by the nonspecific general amino acid permease (Figure 8). Such a strain could use cysteine as sole sulfur source without interference from sulfur derived from sulfate. Without the general amino acid permease, the cells would be forced to utilize the more specific cysteine permease which we designate Str5p, in order to obtain sulfur for biosynthesis of methionine and glutathione. We constructed this strain by a standard genetic cross between two haploid strains containing deletion mutations in the *SUL1* gene, encoding sulfate permease, and the *GAP1* gene, encoding the general amino acid permease. The diploid

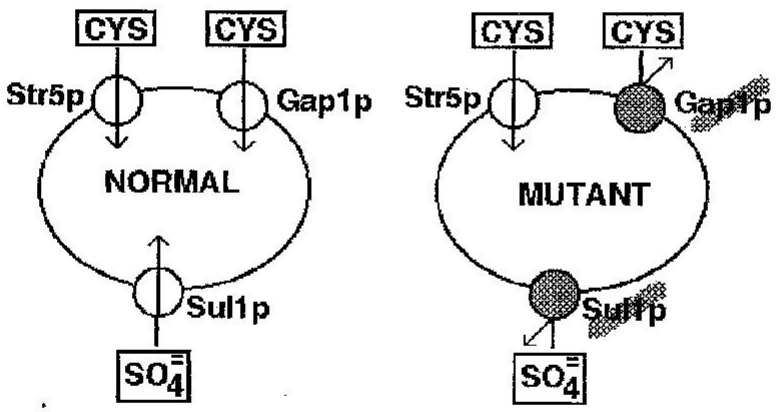

Fig. 8. Strategy for making yeast dependent upon specific cysteine transporter(s). Deletion of functional *SUL1* (sulfate permease) prevents cells from utilizing sulfate as sulfur source. Disruption de *GAP1* eliminates cysteine transport through the general amino acid permease.

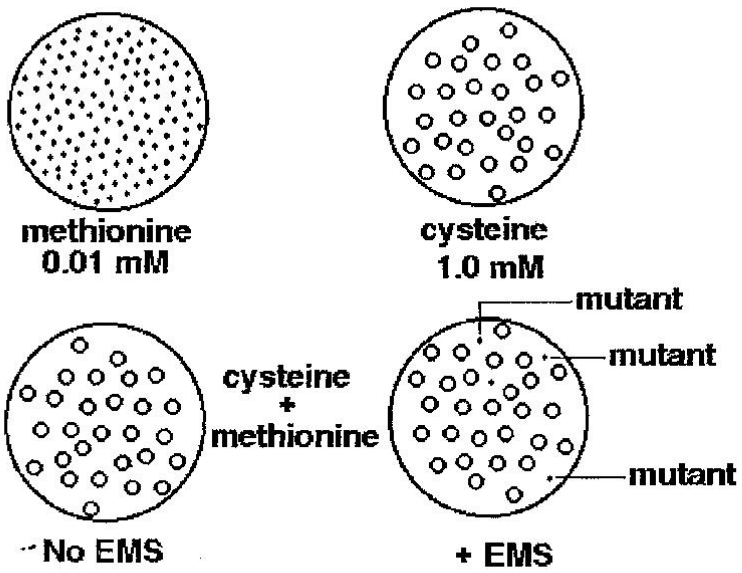

Fig. 9. Screening for potential cysteine transport mutants. EMS-mutagenized yeast cells were plated on minimal medium containing both limiting methionine (0.01 mM) and non-limiting cysteine concentrations. Mutants lacking cysteine transport grow only until methionine is used up, giving very small colonies.

resulting from this cross was then induced to undergo meiosis to produce haploid spores. The spore progeny were then tested, and a strain inheriting both defective genes (*sul1Δ gap1Δ*) was selected. The strain selected also contained the marker *ura3*, which can be used for selection of transformants.

Setting up the genetic screen for potential cysteine permease mutants

Because the *sul1Δ gap1Δ* strain, JAC1-R8, can use either methionine or cysteine as sulfur source, this fact was used to devise a screen for mutants defective in cysteine transport. The strain was grown as single colonies on Petri dishes of minimal medium containing different concentrations of methionine to determine the minimal concentration required to produce only very small colonies. This turned out to be 0.01 mM methionine (Figure 9). Next, a different experiment was made using different concentrations of cysteine as sole sulfur source. In this case, the minimum concentration of cysteine required to produce colonies of normal size was determined to be 1.0 mM. We then prepared a minimal medium containing both sulfur sources at these concentrations. The parental strain was then treated with the mutagen ethylmethanesulfonate, and plated for single colonies on the medium. Mutants unable to use cysteine, but retaining the ability to use methionine, would grow only until methionine became limiting. Several of these mutants were isolated, then tested for growth on various concentrations of cysteine and on 1.0 mM methionine (Figure 10).

Two types of interesting patterns were found. Mutant M5 showed limited growth on all concentrations of cysteine tested, but grew normally on methionine medium. The parental strain, JAC1-R8 and a methionine auxotroph (CKX-4A-8B) were included as controls. Mutant M3, in contrast, showed very limited growth on low concentrations of cysteine, but nearly normal growth when cysteine concentration was increased to 2.0 mM. The behaviour of mutant M3 suggests that the mutation has inactivated a cysteine transporter with high affinity, but that a second low-affinity permease may be acting at higher

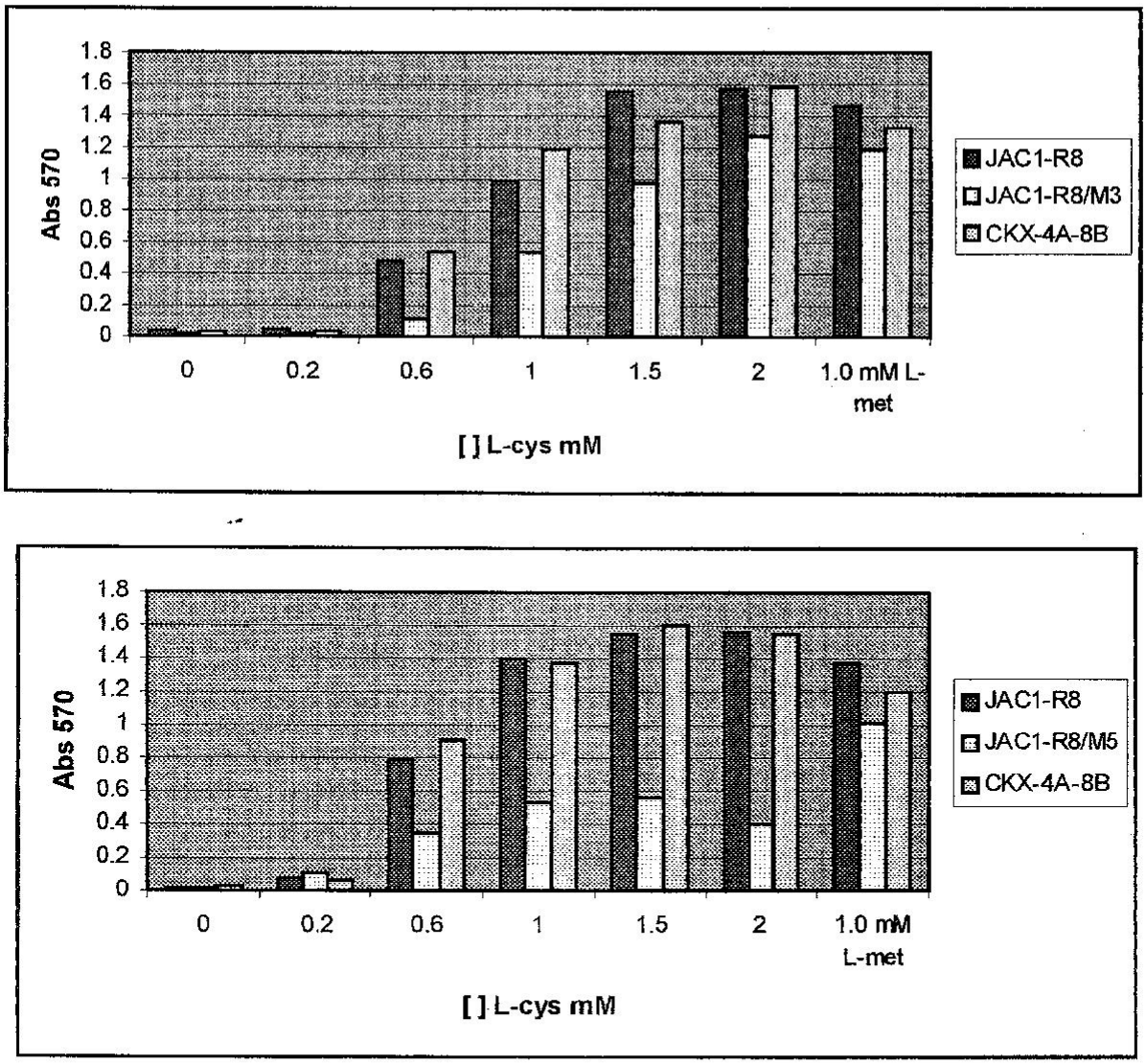

Fig. 10. Relative growth of two mutants, M3 and M5, on increasing concentrations of cysteine. Parental strain, JAC1-R8 was disrupted in both SUL1 and GAP1. Strain CKX-4A-6B is a methionine auxotroph defective in the met5 gene. Cells were cultured in minimal medium lacking added sulfate ion at 30°C for three days in shake flasks on a rotary shaker operated at 250 rpm.

cysteine concentrations. Alternatively, there is only one cysteine permease, but the mutation has decreased its affinity for cysteine. Because mutant M5 is defective in cysteine utilization at all concentrations tested, it may be suggested that instead of an alteration in cysteine permease, one of the metabolic enzymes involved in transfer of sulfur from cysteine to methionine could be partially defective (Figure 6).

To further test the idea that mutant M3 is defective in transport and not in the metabolism of cysteine, the growth of the mutants on cysteine was compared with their growth on the cysteine-containing tripeptide glutathione (GSH). Yeast has two specific GSH permeases [13], so external GSH can enter the cell even if specific cysteine transport is blocked. When GSH is used a sole sulfur source, it is hydrolyzed inside the cell, liber-

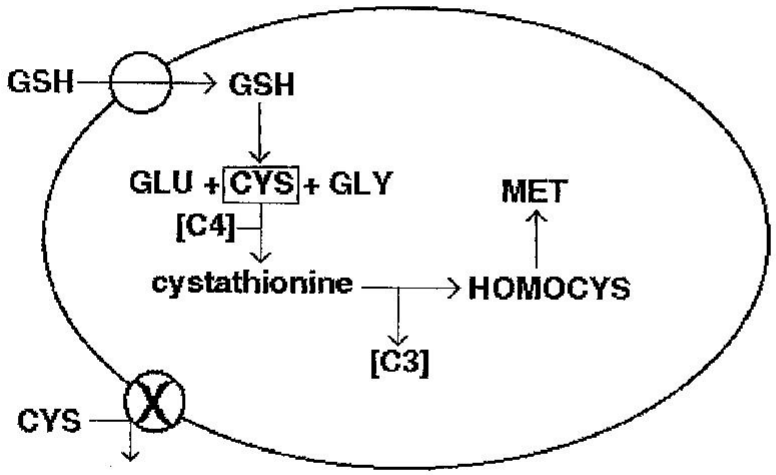

Fig. 11. Glutathione transport and hydrolysis provides cysteine for cells unable to transport cysteine or sulfate.

ating cysteine, which in turn can give rise to homocysteine and methionine by the C3 to C4 transsulfuration pathway (Figure 11).

Although growth of mutant M3 on cysteine is only about 20% of that of the parental strain JAC1-R8, growth of the two strains on glutathione is almost the same (Table 2). This indicates that metabolism of cysteine to homocysteine is essentially normal. This result therefore supports the hypothesis that the mutation in mutant M3 affects cysteine permease rather than an enzyme of cysteine metabolism. Furthermore, the table shows that homocysteine utilization by mutant M3 is also very limited. This result suggests that both cysteine and its structural homolog homocysteine could be transported by the same permease. Since utilization of both cysteine and GSH by mutant M5 is much lower than by the parental strain, this further supports the suggestion that the mutation partially inhibits cysteine metabolism.

Table 2. Sulfur source utilization by putative cysteine permease mutants.

STRAIN	GROWTH [A_{570}] on SULFUR SOURCE			
	MET	CYS	GSH	HCYS
JAC1-R8	1.338	0.602	1.321	1.038
M3	0.570	0.118	1.039	0.189
M5	1.332	0.266	0.465	0.465

Parental strain for mutants M3 and M5 was JAC1-R8. Minimal medium lacking added sulfate ions was supplemented with one of the organic sulfur sources at 1.0 mM. Cultures were grown as indicated for Fig. 10.

However, the lower-than-normal homocysteine utilization remains to be explained.

Complementation cloning experiments

Since the phenotype of mutant M3 indicated that it could well be defective in a cysteine permease gene, we decided to initiate cloning experiments. Since yeast contains very few genes having introns, a yeast genomic library can be used to clone most yeast genes directly. We used a yeast genomic library in the vector YCp50, which produces only one or two copies per cell. This vector can be amplified in *E. coli* and then used to transform yeast cells containing the *ura3* marker, which renders the cells auxotrophic for uracil. Cells were mixed with library DNA, subjected to electroporation, and plated on a minimal medium lacking uracil and containing 1.0 mM cysteine as sole sulfur source. Because the vector contains a normal *URA3* gene, the uracil auxotrophy will be complemented. If the recombinant vector also contains a gene that complements the cysteine utilization defect of mutant M3, the cells will be able to use the cysteine and form transformant colonies.

Thirteen colonies were selected which represent putative clones of the normal allele of the mutant gene in strain M3. These colonies were used to prepare cultures for further study.

To show that these culture represent transformants rather than revertants, a sample of each one was "cured" of plasmid by growing it on medium containing both uracil and 5-fluoroorotic acid (5-FOA). 5-FOA is a chemical analog of orotic acid, which as a nucleotide, is an intermediate in uracil biosynthesis. Any cell that has lost the plasmid spontaneously can give rise to a colony because it cannot convert the fluoro-OMP to the toxic fluoro-UMP. The apparent *curing* is in fact *selection*. In yeast, plasmid loss occurs with a frequency of about 1% per generation. When this procedure was applied to the thirteen transformants, all of them reverted to the M3 phenotype; they required uracil and they grew very poorly when 1.0 mM cysteine was used as sole sulfur source.

The next step will be to prepare DNA from each yeast transformant and to use this DNA to transform *E. coli*, selecting for ampicillin resistance. This should allow us to amplify each plasmid, which may then be purified from the *E. coli* and used for restriction mapping and sequencing. It is highly likely that the gene will be among the twenty-four APC genes identified by Paulsen et al. [11].

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