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PHYSIOLOGY

The Complete Pathway for Thiosulfate Utilization in *Saccharomyces cerevisiae*

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ABSTRACT *Saccharomyces cerevisiae* is known to grow with thiosulfate as a sulfur source, and it produces more ethanol when using thiosulfate than using sulfate. Here, we report how it assimilates thiosulfate. *S. cerevisiae* absorbed thiosulfate into the cell through two sulfate permeases, Sul1 and Sul2. Two rhodaneses, Rdl1 and Rdl2, converted thiosulfate to a persulfide and sulfite. The persulfide was reduced by cellular thiols to H_2S, and sulfite was reduced by sulfite reductase to H_2S. Cysteine synthase incorporated H_2S into *O*-acetyl-L-homoserine to produce L-homocysteine, which is the precursor for cysteine and methionine in *S. cerevisiae*. Several other rhodaneses replaced Rdl1 and Rdl2 for thiosulfate utilization in the yeast. Thus, any organisms with the sulfate assimilation system potentially could use thiosulfate as a sulfur source, since rhodaneses are common in most organisms.

IMPORTANCE The complete pathway of thiosulfate assimilation in baker's yeast is determined. The finding reveals the extensive overlap between sulfate and thiosulfate assimilation. Rhodanese is the only additional enzyme for thiosulfate utilization. The common presence of rhodanese in most organisms, including *Bacteria*, *Archaea*, and *Eukarya*, suggests that most organisms with the sulfate assimilation system also use thiosulfate. Since it takes less energy to reduce thiosulfate than sulfate for assimilation, thiosulfate has the potential to become a choice of sulfur in optimized media for industrial fermentation.

KEYWORDS sulfur starvation, thiosulfate permease, rhodanese, cysteine synthesis, budding yeast

Sulfur is an essential element in all organisms, being present in organic compounds like L-cysteine. Animals obtain organic sulfur from food (1), while plants and microorganisms can assimilate sulfate (2–4). The pathway for sulfate assimilation has been well characterized. In *Saccharomyces cerevisiae* (yeast), sulfate is transported into the cell by two H^+-dependent symporters, Sul1 and Sul2 (5, 6), and reduced to H_2S, which is incorporated into *O*-acetyl-L-homoserine to produce L-homocysteine, which is the precursor for cysteine and methionine (7). Yeast has recently been reported to produce more biomass and ethanol when growing with thiosulfate than with sulfate, because less energy is needed to reduce it to H_2S (8).

Thiosulfate assimilation has been comprehensively characterized in *Escherichia coli*. It is transported into the cell through thiosulfate permease, a sulfate/thiosulfate ABC-type transporter (9). Inside the cell, it is directly combined with *O*-acetyl-L-serine to generate *S*-sulfocysteine by cysteine synthase B (CysM) (10), and the product is then reduced to cysteine and sulfite by glutaredoxin (11). *E. coli* also contains cysteine synthase A (CysK) (12, 13). CysK and CysM are homologous and both synthesize cysteine from sulfide and *O*-acetyl-L-serine, but only CysM can use thiosulfate as a substrate (10). It is unknown whether the yeast cysteine synthase (Met15) could use thiosulfate as a substrate to synthesize *S*-sulfohomocysteine, which could be converted

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to L-homocysteine by glutaredoxin. Alternatively, yeast may reduce thiosulfate to sulfide and sulfite by thiosulfate reductase (14). The latter was recently shown to be a rhodanese, also known as thiosulfate sulfurtransferase, that converts thiosulfate and glutathione (GSH) to oxidized glutathione (GSSH) and sulfite (15). GSSH spontaneously reacts with GSH to produce H_2S and glutathione disulfide (GSSG) (15). Met15 potentially can use the produced sulfide for the synthesis of L-homocysteine.

Since it has been both theoretically and practically proven that thiosulfate is a better sulfur source than sulfate for yeast during the production of biomaterials (8), it is important to have a detailed understanding of how thiosulfate is used by yeast. In the present work, both genetic and biochemical data support that *S. cerevisiae* BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) uses sulfate permeases, Sul1 and Sul2 (5, 16), to transport thiosulfate into the cell. Inside the cell, the rhodaneses Rdl1 and Rdl2 convert thiosulfate to sulfide and sulfite; the latter is also reduced to sulfide by sulfite reductase. Met15 uses sulfide for L-homocysteine synthesis. The pathway of thiosulfate assimilation extensively overlaps with that of sulfate assimilation. The only additional enzyme is a rhodanese that is widely present in *Bacteria*, *Archaea*, and *Eukarya*. Thus, organisms with the sulfate assimilation ability may also use thiosulfate as a sulfur source for growth.

RESULTS

***S. cerevisiae* uses thiosulfate as a sole source of sulfur.** *S. cerevisiae* grew at similar rates to an optical density at 600 nm (OD_{600}) of 2.8 ± 0.1 in the modified SD medium with either 100 μM $Na_2S_2O_3$ or Na_2SO_4 as a sole sulfur source, but it grew to an OD_{600} of 1.4 ± 0.1 in the sulfur-free medium, indicating that *S. cerevisiae* can utilize thiosulfate as a sole sulfur source for growth (see Fig. S1 in the supplemental material). *S. cerevisiae* was further transferred twice in the medium without added sulfur, and the final OD_{600} values were 0.85 ± 0.03 and 0.61 ± 0.02 for the 2nd and 3rd transfers, respectively, suggesting that *S. cerevisiae* carries over some intracellular sulfur, and the medium may contain trace amounts of sulfur. When 100 μM thiosulfate and 100 μM sulfate were both included in the medium, yeast grew to an OD_{600} of 2.8 in 21 h, and it consumed 25 ± 2 μM thiosulfate and did not consume sulfate (Fig. S2), suggesting that yeast prefers to use thiosulfate instead of sulfate.

Phenotypic study of *SUL1*- and *SUL2*-disrupted mutants. Thiosulfate and sulfate are structurally similar (Fig. 1A). Whether sulfate permeases (Sul1 and Sul2) could transport thiosulfate in *S. cerevisiae* was tested. The genes were disrupted. When the WT, Δ*sul1*, Δ*sul2*, and Δ*sul1* Δ*sul2* strains grew in the modified SD medium with 100 μM thiosulfate, the mutants bearing disruptions in *SUL1* or *SUL2* grew as well as the wild type, and only the double mutant (Δ*sul1* Δ*sul2*) did not grow (Fig. 1B), indicating that both Sul1 and Sul2 transported thiosulfate, and one of them was sufficient for thiosulfate uptake. The lack of growth by the Δ*sul1* Δ*sul2* strains also suggests that the mutant contained minimal intracellular sulfur, and the modified SD medium without added sulfur contained some $Na_2S_2O_3$ or Na_2SO_4, which was likely from the impurities of the used chemicals and was able to support minimal growth of the wild-type strain (Fig. S1). However, the Δ*sul1* Δ*sul2* strain was able to grow in the modified SD medium with 500 μM thiosulfate (Fig. 1C). The Δ*sul1* Δ*sul2* Δ*soa1* triple mutant did not grow with 1 mM thiosulfate but grew with 5 and 10 mM thiosulfate (Fig. S3). The data implied that Sul1 and Sul2 were high-affinity transporters, Soa1 was a low-affinity transporter, and at very high concentrations thiosulfate still was able to enter the organism either via diffusion or mediated by another transporter.

The uptake of thiosulfate via Sul1p and Sul2p in yeast. WT, Δ*sul1*, Δ*sul2*, and Δ*sul1* Δ*sul2* strains were subjected to sulfur starvation for 2 days, and the cells were harvested and suspended at an OD_{600} of 2 in 50 mM potassium phosphate buffer (pH 6) with 2% glucose. Thiosulfate was added to the cell suspensions at 200 μM. The wild type was the most efficient in absorbing thiosulfate, removing about 100 μM thiosulfate from the medium in 90 min, while the Δ*sul1* Δ*sul2* strain did not take up thiosulfate (Fig. 2). The cellular concentration of the absorbed thiosulfate in the wild type was

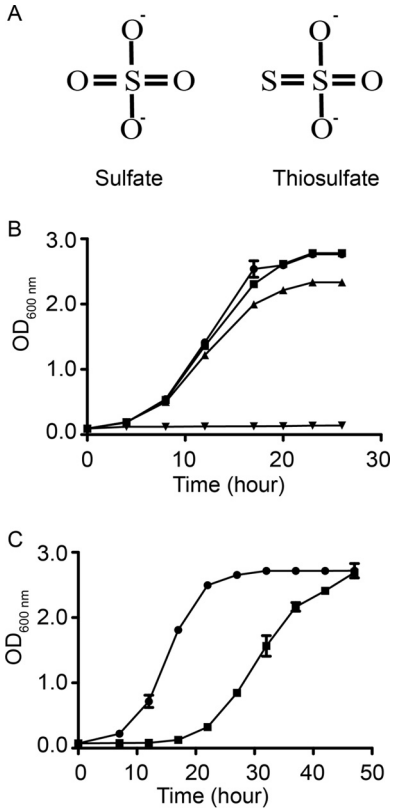

FIG 1 Growth of *S. cerevisiae* and its mutants on thiosulfate. (A) Chemical structure of sulfate and thiosulfate. (B) Growth with 100 μM thiosulfate. Wild-type (●), Δ*sul1* (■), Δ*sul2* (▲), and Δ*sul1* Δ*sul2* (▼) strains are shown. (C) Growth on 500 μM thiosulfate. Wild-type (●) and Δ*sul1* Δ*sul2* (■) strains are shown. The optical density was measured via a spectrophotometer. All data are averages with standard deviations (error bars) from at least three cultures.

calculated to be ~890 μM by using a reported haploid cell volume of 50 fl (17, 18). The Δ*sul1* single-gene deletion mutant absorbed more thiosulfate than the Δ*sul2* mutant did, indicating Sul2 was the major thiosulfate permease in yeast (Fig. 2).

Thiosulfate-mediated downregulation of *SUL1* and *SUL2* transcription and thiosulfate uptake. Sulfur-starved yeast cells strongly expressed *SUL1* and *SUL2*. A sharp drop in both *SUL1* and *SUL2* mRNA levels occurred shortly after the addition of 1 mM thiosulfate to the cells, indicating a tight negative regulation of the two genes at the transcriptional level coupled with mRNA breakdown (Fig. 3A). Moreover, upon the addition of thiosulfate to sulfur-starved cells, the thiosulfate uptake rate also rapidly decreased, with a 50% reduction in 10 min and 90% reduction in 30 min, and then remained constant (Fig. 3B).

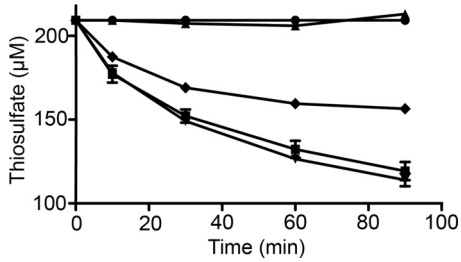

FIG 2 Uptake of thiosulfate by *S. cerevisiae* and its mutants. The sulfur-starved cells were suspended in 50 mM potassium phosphate buffer, pH 6. The amounts of thiosulfate remaining in the medium were determined. Wild-type (▼), Δ*sul1* (■), Δ*sul2* (◆), and Δ*sul1* Δ*sul2* (▲) strains, as well as a control (●), are shown.

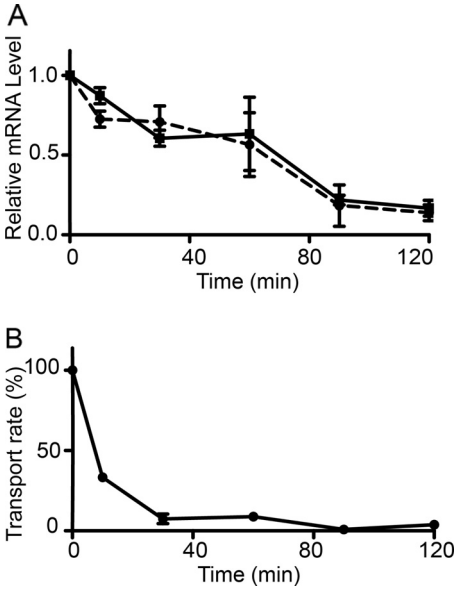

FIG 3 Effects of thiosulfate on Sul1 and Sul2 transcription and thiosulfate uptake rates. (A) Relative RNA levels of *SUL1* (●) and *SUL2* (■) after 1 mM thiosulfate addition to sulfur-starved cells. (B) The uptake rate of thiosulfate by the wild type decreased after the addition of 1 mM thiosulfate to sulfur-starved cells. The cells were harvested at the indicated time point, and the uptake rate was then measured with a 1-h uptake assay.

***CYS3* and *MET15* were essential genes for thiosulfate utilization.** The *MET15* deletion strain did not grow in the modified SD medium with 100 μM thiosulfate, indicating that *MET15* was an essential gene for thiosulfate utilization (Fig. S4A). *CYS3* encodes cystathionine-γ-lyase, which is involved in converting homocysteine to cysteine (19), and its deletion strain did not grow in the modified SD medium with 100 μM thiosulfate either (Fig. S4B). The complementation restored the growth of the mutants (Fig. S4). The results show that cysteine synthesis from thiosulfate involves homocysteine and cystathionine as intermediates.

Met15p could not directly use thiosulfate as a substrate. We tested whether Met15 could use thiosulfate directly. The recombinant protein with a His tag was produced in *E. coli* and purified (Fig. S5A). Met15 used sulfide and *O*-acetyl-homoserine to produce homocysteine (Fig. S5B); however, the enzyme did not catalyze the reaction between thiosulfate and *O*-acetyl-homoserine. Due to the lack of *O*-acetyl-serine in yeast (20), we did not try *O*-acetyl-serine as a substrate. Met15 shared 29% and 57% sequence identity with *E. coli* CysM and CysK, respectively (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Further bioinformatics analysis revealed that the sequenced fungal genomes from GenBank contained homologous cysteine synthases that grouped together; they were closer to CysK than CysM in terms of sequence similarity (Fig. S6).

The role of rhodaneses in thiosulfate utilization. *S. cerevisiae* has five rhodaneses, *RDL1*, *RDL2*, *TUM1*, *YCH1*, and *UBA4*. Single-deletion mutants grew well in the SD medium containing sulfate except the Δ*uba4* strain (data not shown), perhaps due to its involvement in the thiolation of the wobble base of tRNAs (21, 22). The reduced growth rate prevented testing the mutation for its function in thiosulfate utilization. Only the single deletion of *RDL1* (Δ*rdl1* mutant) showed growth retardation in the modified SD medium containing 100 μM thiosulfate (Fig. 4). The other three single-deletion strains showed the same growth as the wild type in the modified SD medium with thiosulfate (Fig. S7), while the double deletion mutant of *RDL1* and *RDL2* grew more poorly than the Δ*rdl1* mutant and the Δ*rdl1* Δ*rdl2* Δ*tum1* Δ*ych1* (4k) strain grew more poorly than the Δ*rdl1* Δ*rdl2* strain (Fig. 4). On the other hand, the Δ*rdl1* Δ*rdl2* Δ*tum1* strain grew the same as the Δ*rdl1* Δ*rdl2* strain (Fig. S7). Thus, *RDL1* is mainly responsible for thiosulfate utilization in yeast; *RDL2* is dispensable but can partially replace the role of *RDL1*.

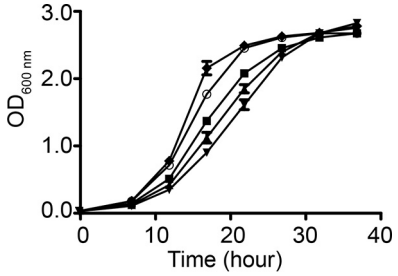

FIG 4 Growth curves of *S. cerevisiae* and its rhodanese mutants. Growth of the wild-type and its rhodanese mutant strains in the modified SD medium with 100 μM thiosulfate. Wild-type (○), Δ*rdl1* (■), Δ*rdl1*::*RDL1* (♦), Δ*rdl1* Δ*rdl2* (▲), and Δ*rdl1* Δ*rdl2* Δ*tum1* Δ*ych1* (4k) (▼) strains were used. All data are averages with standard deviations (error bars) from at least three cultures.

The participation of sulfite reductase (Met5) in thiosulfate utilization. A single-deletion mutant of *MET5* showed growth retardation in the modified SD medium containing 100 μM thiosulfate compared with the wild-type strain (Fig. 5A). The double deletion mutant of *RDL1* and *MET5* (Δ*rdl1* Δ*met5*) resulted in further growth retardation (Fig. 5A). The grown cells were then harvested and resuspended in modified SD

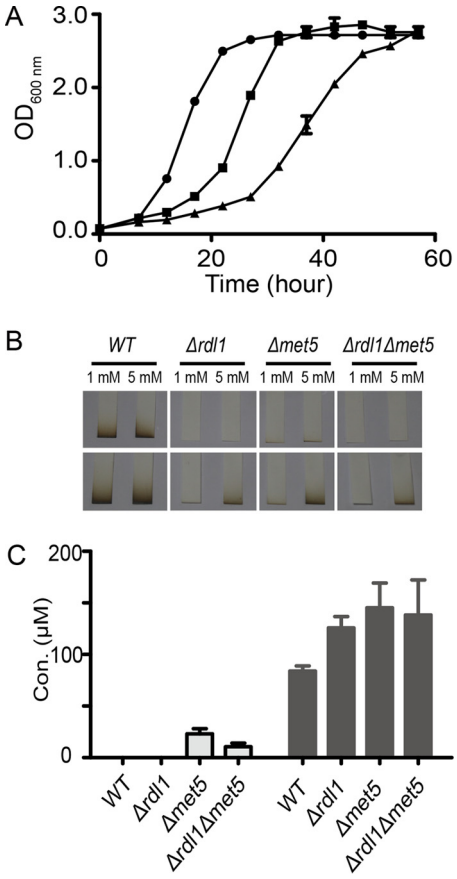

FIG 5 Yeast used both sulfur atoms of thiosulfate. (A) Growth of the parental and different mutant strains on 100 μM thiosulfate. Wild-type (●), Δ*met5* (■), and Δ*rdl1* Δ*met5* (▲) strains were used. All data are averages with standard deviations (error bars) from at least three cultures. (B) The parental and different mutant strains were subjected to sulfur starvation for 2 days. A concentration of 1 mM or 5 mM thiosulfate was added to 3-ml cell cultures (OD_{600} of 1) and H_2S accumulation was detected with lead-acetate paper strips, with incubation for 2 h (top) or 24 h (bottom). (C) The production of sulfite by the *MET5* mutant. The parental and mutant cells were diluted to an OD_{600} of 10 in 50 mM potassium phosphate buffer (pH 6), and 150 μM thiosulfate was added. After incubating for 60 min, the levels of sulfite (light gray bar) and thiosulfate (dark gray bar) remaining in the medium were determined.

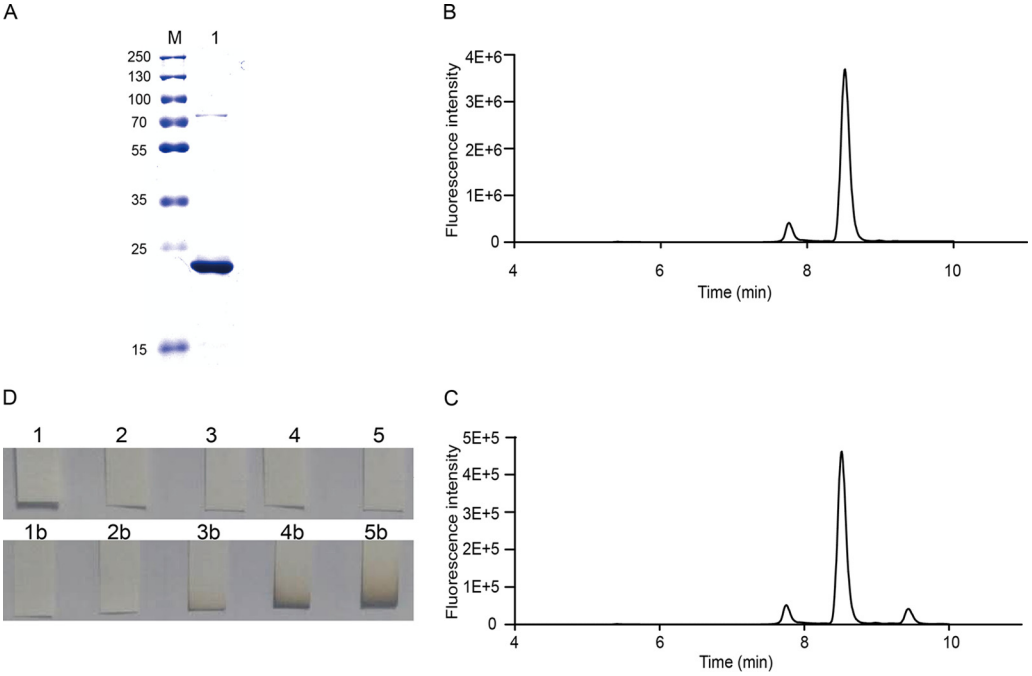

FIG 6 Rdl2p catalyzes the reaction of thiosulfate and GSH to produce sulfite. (A) SDS-PAGE analysis of purified Rdl2p. The SDS–12% polyacrylamide gel was stained with Coomassie blue staining solution. Lane M, molecular markers; lane 1, 2 μl of purified Rdl2p. Measurements are in kDa. (B) HPLC analysis of sulfite production. The reaction mixture (0.5 ml) contained 50 mM potassium phosphate buffer (pH 8), 1 mM thiosulfate, 2 mM GSH, and 3 μg/ml Rdl1p, and it was incubated at 30°C for 15 min. One hundred microliters of sample was derivatized with monobromobimane and analyzed via HPLC, and the retention time of sulfite was 8.51 min. (C) HPLC experiment with 30 μM sulfite as the control. (D) Three milliliters of the enzyme reaction mixture was incubated at 30°C for 60 min, and H_2S accumulation was detected with lead-acetate paper strips. (Top) Control with different concentrations of GSH in the absence of Rdl2p. (Bottom) Reaction mixture contained Rd12p at 3 μg/ml. Gels: 1 and 1b, 2 mM GSH; 2 and 2b, 1 mM thiosulfate; 3 and 3b, 1 mM thiosulfate plus 2 mM GSH; 4 and 4b, 1 mM thiosulfate plus 4 mM GSH; 5 and 5b, 1 mM thiosulfate plus 6 mM GSH.

medium without added sulfur and incubated for 2 days. When the sulfur-starved cells were tested to release H_2S in the modified SD medium with 1 or 5 mM thiosulfate, the wild type released about 100 μM H_2S, the Δ*met5* mutant released about 10 μM H_2S, and the Δ*rdl1* and Δ*rdl1* Δ*met5* strains did not release any H_2S after a 2-h incubation (Fig. 5B). After a 24-h incubation, the Δ*rdl1* and Δ*rdl1* Δ*met5* strains also released some H_2S in the medium with 5 mM thiosulfate (Fig. 5B). Further, the *MET5* deletion strain accumulated sulfite in the medium when thiosulfate was added to the cell suspension, while the wild type did not (Fig. 5C). These results suggest that both sulfur atoms of thiosulfate are used by the wild type.

Recombinant Rdl2p exhibited thiosulfate sulfurtransferase activity. The *RDL2* gene was cloned into pET30a, and the recombinant protein with an N-terminal His tag was produced in *E. coli* and purified (Fig. 6A). Rdl2 had apparent thiosulfate sulfurtransferase activity, producing 231 ± 30 μM sulfite (Fig. 6B and C) and releasing about 50 μM H_2S (Fig. 6D) in 50 mM potassium phosphate buffer (pH 8) with 1 mM thiosulfate and 4 mM GSH at 30°C after 1 h of incubation. Rdl2 likely catalyzed the reaction of thiosulfate with GSH to produce GSSH (equation 1), which is further reduced by GSH to produce H_2S (equation 2). We tested the possibility of GSSH formation with a bacterial persulfide dioxygenase, *Cupriavidus pinatubonensis* JMP134 Pdo2 (WP_011299714), that oxidizes GSSH to sulfite (23). When CpPdo2 was added to the reaction mixture, H_2S was not produced and 966 ± 70 μM sulfite was formed, confirming the production of GSSH as an intermediate during the conversion of thiosulfate to H_2S in the reaction mixture containing Rdl2.

$$SO_3\,S^- + GS^- \rightleftharpoons SO_3^- + GSS^- \quad (1)$$

$$2H^+ + GSS^- + GS^- \rightleftharpoons GSSG + H_2S \quad (2)$$

TABLE 1 Doubling times of different strains[a]

Strain	Doubling time (h)
WT	1.4 ± 0.1
4k	2.4 ± 0.2
4k::*PSPE*	1.2 ± 0.1
4k::*GLPE*	1.1 ± 0.1
4k::*RDHA*	1.1 ± 0.1
4k::*SSEA*	1.3 ± 0.2
4k::*DUF442*	1.1 ± 0.1

[a]The doubling time was derived from the length of the exponential growth phase. The data are averages with standard deviations from three cultures. The *P* values (by *t* test) for the 4k strain versus others were all <0.05.

GSH could be replaced by other small thiols, such as cysteine, dithiothreitol, and coenzyme A, in the enzymatic conversion of thiosulfate to H_2S and sulfite (Fig. S8A). Thus, most small organic thiols, either natural or nonnatural, can serve as sulfane sulfur acceptors. Dithiothreitol was the best acceptor, as the reaction with it accumulated the most H_2S. Cys106 in Rdl2 was predicted to be the catalytic residue via sequence comparison, and the purified Rdl2 C106A or Rdl2 C106S was inactive (Fig. S8B). Further, iodoacetamide, a thiol blocking agent, inactivated Rdl2 (data not shown). In conclusion, both Rdl1 and Rdl2 have thiosulfate sulfurtransferase activities, and they share similar predicted three-dimensional structures (Fig. S9).

Rdl1, Tum1, Uba4, and Ych1 were also purified. Rdl1 had strong rhodanese activity, as reported previously (15). Tum1 and Uba4 showed low activity for releasing H_2S from thiosulfate and GSH, but Ych1 displayed no activity (Fig. S10A). Although the Δ*rdl1* Δ*rdl2* strain and the 4k mutant grew poorly at similar rates in the SD medium modified with thiosulfate, the expression of *TUM1* or *UBA4* in the 4k mutant allowed the mutant to growth in the SD medium modified with thiosulfate (Fig. S10B and C). Perhaps the physiological levels of Tum1 and Uba4 were not sufficient to support thiosulfate utilization in its natural state. The expression of Ych1 in the 4k mutant did not help with growth on thiosulfate (Fig. S10D).

Rhodaneses from other organisms also participate in thiosulfate utilization. Since rhodaneses are common in most organisms (23), their potential role in helping other organisms to use thiosulfate was tested. Several bacterial rhodaneses were selected. They were PspE, GlpE, and SseA from *E. coli* MG1655, RdhA from *Pseudomonas aeruginosa*, and Duf442 from *Cupriavidus pinatubonensis* JMP134 (24–27). When they were cloned and expressed in the 4k strain, all restored the mutant's growth on thiosulfate (Table 1). They were also cloned with a His tag, produced in *E. coli*, and purified. All of them catalyzed the conversion of thiosulfate to H_2S in the presence of GSH (Fig. S11). PspE, GlpE, RdhA, and Duf442 produced about 30 μM H_2S, and SseA generated about 10 μM H_2S under the same conditions (see the legend to Fig. S11). Thus, rhodanese involvement in thiosulfate utilization is likely a common phenomenon.

DISCUSSION

We deciphered the pathway for thiosulfate utilization in *S. cerevisiae* (Fig. 7A). It extensively overlaps that of sulfate utilization. The two pathways share the same substrate transporters, Sul1 and Sul2, sulfite reductase, cysteine synthase, and cystathionine-γ-lyase. *S. cerevisiae* uses cysteine synthase to produce homocysteine, which is then converted to cysteine via cystathionine (20). Sul1 and Sul2 are high-affinity H^+-dependent symporters for sulfate uptake (5), and we showed that they also transport thiosulfate. Soa1, another H^+-dependent symporter for organic sulfonate uptake, also transports both sulfate and thiosulfate (28). Here, we showed that Sul1 and Sul2 are high-affinity thiosulfate transporters and Soa1 is a low-affinity thiosulfate transporter (Fig. 1; see also Fig. S3 in the supplemental material). These findings are in agreement with an ABC-type transporter that transports both thiosulfate and sulfate in *E. coli* (9). Therefore, sulfate and thiosulfate are often transported into the cell by the same transporters.

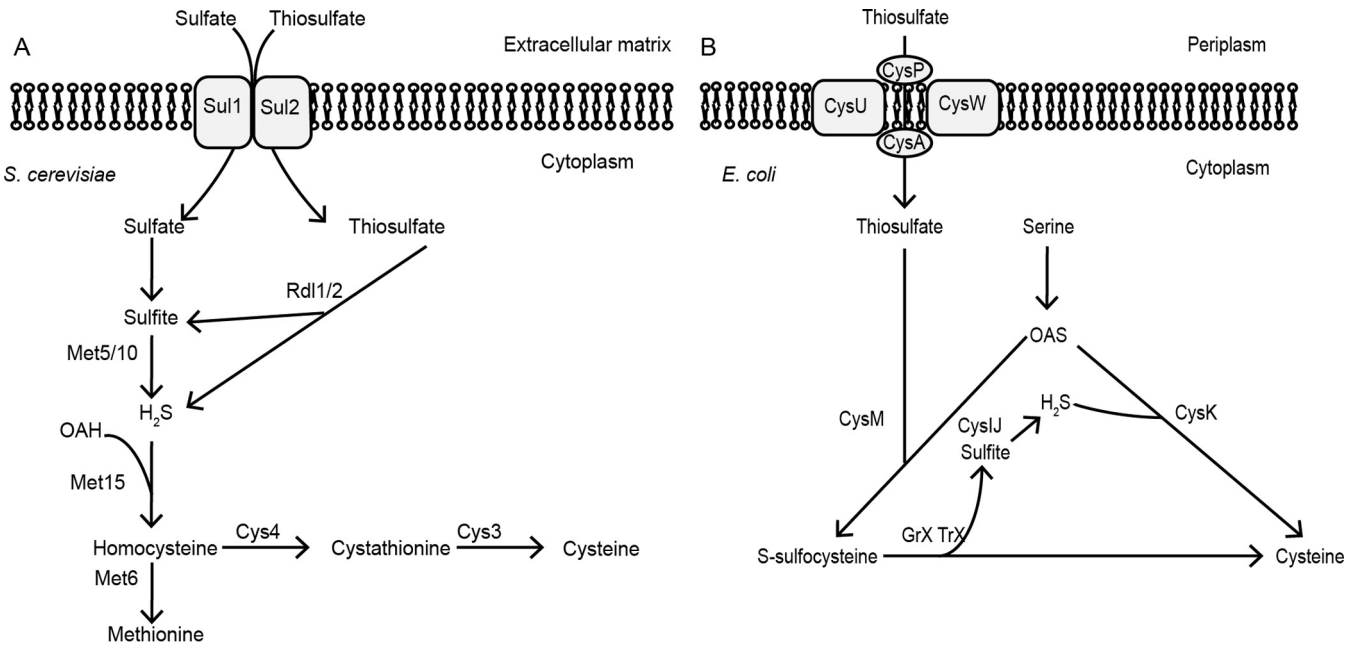

FIG 7 Thiosulfate assimilation pathways. (A) A proposed thiosulfate assimilation pathway in *S. cerevisiae*. (B) The thiosulfate assimilation pathway in *E. coli* (11).

The difference between sulfate and thiosulfate assimilation in yeast occurs in the initial steps. For sulfate, it is first reduced to sulfite by three enzymes. For thiosulfate, a rhodanese is required. It may be a common phenomenon that a rhodanese takes part in thiosulfate utilization; even *E. coli* 3-mercaptopyruvate sulfurtransferase (SseA) and yeast Uba4p with a rhodanese domain and low rhodanese activity (27, 29) replaced Rdl1 or Rdl2 in yeast for thiosulfate utilization (Fig. S10E and F). It appears that any fungi, bacteria, or plants with the sulfate utilization pathway also are able to assimilate thiosulfate; however, the physiological level of rhodanese in some organisms may not be sufficient to support thiosulfate utilization; for instance, the native level of Tum1 or Uba4 was insufficient to support the Δ*rdl1* Δ*rdl2* mutant to properly use thiosulfate (Fig. S7). The physiological level of rhodanese activities is also not sufficient to support thiosulfate utilization in an *E. coli cysM* mutant that resumed growth with the overexpression of the *E. coli* rhodanese GlpE (30). Thus, any organisms with the sulfate assimilation system may use thiosulfate as a sulfur source with the help of a rhodanese. For example, tobacco cells have been shown to use thiosulfate as a sulfur source (31), and rhodanese may play a role in the utilization.

Rhodaneses are widely present in all domains of life (32). They share evolutionary relationships with a conserved catalytic Cys residue and extensive heterogeneity at different levels, including the sequence, active-site loop length, and domain arrangement (33). They play a number of important biological roles, such as cyanide detoxification, tRNA thiolation, and the synthesis of iron-sulfur centers (24, 25, 32). They may also be involved in managing stress and maintaining redox homeostasis (34–37). Further, they participate in sulfur oxidation, catalyzing the conversion of GSSH and sulfite to thiosulfate, which is a step during sulfide oxidation by heterotrophic bacteria and by human mitochondria (26, 38, 39). The reversible reaction has been reported with yeast Rdl1 and the rhodanese domain of a bacterial persulfide dioxygenase (15, 40), which show thiosulfate sulfurtransferase activity, implying Rdl1 participates in thiosulfate metabolism *in vivo*. Rdl1 transfers the sulfane sulfur from thiosulfate to other thiols, such as GSH, coenzyme A, cysteine, and dithiothreitol (Fig. S8A), to form persulfides (15); however, GSH is likely the physiological sulfane sulfur acceptor due to high concentrations, up to 10 mM, in yeast cells (41). In yeast, Rdl1 and Rdl2 were the primary rhodaneses for thiosulfate utilization, and they can be replaced by rhodaneses

from various sources. Thus, another common function of rhodaneses is to catalyze the reaction of thiosulfate with GSH to produce GSSH.

SUL1 and *SUL2* are overexpressed when yeast is under sulfur starvation (42), and sulfur starvation causes an ~10,000-fold increase in the sulfate influx rate mediated by Sul1p and Sul2p (43). The addition of sulfate causes a rapid decrease in sulfate transport without mRNA degradation (43). The sulfur-starved cells also rapidly accumulated thiosulfate, and the addition of thiosulfate quickly decreased the rate of thiosulfate uptake. Further, thiosulfate induced rapid degradation of *SUL1* and *SUL2* mRNA. Sul1 and Sul2 are transceptors that not only transport but also sense extracellular sulfate and signal via the protein kinase A pathway to regulate sulfur-utilizing genes (44). Further research is required to understand their sensing and response to thiosulfate.

In *E. coli* the CysM pathway for thiosulfate assimilation is preferred, probably due to the lack of free sulfide during cysteine biosynthesis (Fig. 7B). However, Met15 did not use thiosulfate. Sequence analysis showed that CysM homologues are found mainly in bacteria and are not found in sequenced fungal genomes, in which Met15 homologues are more common and are closely related to CysK. This suggests that fungi use the yeast pathway for thiosulfate utilization. For organisms that do not have a CysM-type cysteine synthase, the yeast system can be used for thiosulfate assimilation. As presented here, yeast can efficiently use thiosulfate (Fig. S2), which is in agreement with a previous report that *S. cerevisiae* uses thiosulfate more effectively than sulfate during the production of useful materials (8). Thiosulfate contains a sulfane sulfur that requires one NADPH equivalent for sulfide production, while the conversion of sulfate to sulfide consumes 2 ATPs and 4 NADPHs (45). Therefore, microorganisms may preferentially use thiosulfate as a sulfur source for growth in nature. Thiosulfate is common in the environment, as heterotrophic bacteria can actively produce sulfide and then oxidize it to thiosulfate during growth on organic compounds (26, 46). Further, thiosulfate has the potential to become the choice of sulfur source in optimized media for industrial fermentation because less energy is required for its reduction than sulfate reduction.

MATERIALS AND METHODS

Materials and media. Thiosulfate, sulfate, *O*-acetyl-serine (OAS), *O*-acetyl-homoserine (OAH), and pyridoxal-5-phosphate (PLP) were purchased from Sigma Chemical (St. Louis, MO). The enzymes used in the DNA manipulations were from Thermo Fisher (Waltham, MA). Enzymes for PCR were from Toyobo (Osaka, Japan).

A modified SD medium (per liter: 20 g glucose, 10 g NH_4Cl, 1.42 g KH_2PO_4, 0.25 g $MgCl_2$, 142 mg NaCl, 142 mg $CaCl_2$, 567 mg niacin, 567 mg pyridoxine, 567 mg thiamine-HCl, 2.8 mg folic acid, 2.8 mg biotin, 283 mg *p*-aminobenzoic acid, 567 mg calcium pantothenate, 283 mg riboflavin, 2.8 mg inositol, 708 mg boric acid, 567 mg $MnCl_2$, 567 mg $ZnCl_2$, 57 mg $CuCl_2$, 283 mg $FeCl_3$, 283 mg Na_2MoO_4, and 142 mg KI, pH 6) had no added sulfur but might contain trace amounts of Na_2SO_4 or $Na_2S_2O_3$ as impurities from the added chemicals, which did not support normal growth of the yeast.

Strains and plasmids. General cloning procedures, sequencing, and PCR were carried out according to standard procedures (47). The yeast strains used in this study were derived from *S. cerevisiae* BY4742 (*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*), which was kindly provided by W. J. Guan (Zhejiang University, China). *Escherichia coli* strain DH5α served as the host strain for all plasmid constructions and was grown in lysogeny both (LB) medium with 50 μg/ml ampicillin or kanamycin if necessary. *S. cerevisiae* strains were grown at 30°C in yeast extract-peptone-dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% glucose) or in modified SD medium left unsupplemented or supplemented with a sulfur compound and with compounds necessary to meet auxotrophic requirements. Gene disruptions were carried out by one-step PCR-mediated gene disruption in BY4742 (48).

The *TEF1* promoter, a strong constitutive promoter, was amplified from yeast genomic DNA by PCR, and the PCR product was cloned into YEplac195 (digested with HindIII and BamHI) to obtain YEplac195-TEF1p. The *CYC1* terminator was amplified from the pYES2 plasmid, and the PCR product was cloned into YEplac195-*TEF1p* (digested with SacI and EcoRI) to obtain YEplac195-*TEF1p-CYC1t*. The DNA fragment (*MET15*) encoding bifunctional cysteine synthase was amplified from yeast genomic DNA by PCR, and the PCR product was cloned into YEplac195-*TEF1p-CYC1t* (digested by BamHI and KpnI) to obtain YEplac195-*TEF1p-MET15-CYC1*. The construct was sequenced and confirmed to be correct. The rhodanese gene from other organisms was incorporated into the YEplac195-*TEF1p-CYC1t* plasmid. The plasmids were transformed into yeast strains by using the LiAc/SS carrier DNA/polyethylene glycol method as reported previously (49).

The strains and plasmids used in this study are listed in Table 2. All primers are given in Table 3.

Measurement of *S. cerevisiae* growth curve with thiosulfate or sulfate as the sulfur source. Fresh cells of *S. cerevisiae* strains were inoculated in 5 ml of YPD and grown overnight at 30°C. Cells were collected by centrifugation (11,000 rpm, 5 min), washed twice with sterile water, and suspended in the

TABLE 2 Strains and plasmids used this study

Strain or plasmid	Relevant characteristic(s)	Source
S. cerevisiae		
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Laboratory stock
E. coli		Laboratory stock
DH5α	*supE44* Δ*lacU169*(ϕ80d*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	
BL21(DE3)	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal*(λ*1857 ind1 sam7 nin5 lacUV5* T7 gene 1) *dcm*	Laboratory stock
BY4742 Δ*sul1*	Δ*sul1*::*BLE*	This study
BY4742 Δ*sul2*	Δ*sul2*::*LEU2*	This study
BY4742 Δ*sul1* Δ*sul2*	Δ*sul1*::*BLE* Δ*sul2*::*LEU2*	This study
BY4742 Δ*met5*	Δ*met5*::*loxP* Δ*rdl1*::*BLE*	This study
BY4742 Δ*met5* Δ*rdl1*	Δ*met5*::*BLE*	This study
BY4742 Δ*cys3*	Δ*cys3*::*BLE*	This study
Δ*cys3*::*CYS3*	Δ*cys3*::YEplac195-*CYS3*	This study
Δ*met15*	Δ*met15*::*BLE*	This study
Δ*met15*::*MET15*	Δ*met15*::YEplac195-*MET15*	This study
Δ*rdl1*	Δ*rdl1*::*BLE*	This study
Δ*rdl2*	Δ*rdl2*::*BLE*	This study
Δ*tum1*	Δ*tum1*::*HIS5*	This study
Δ*ych1*	Δ*met15*::*LEU2*	This study
Δ*uba4*	Δ*uba4*::*kanMX*	This study
Δ*rdl1* Δ*rdl2*	Δ*rdl1*::*loxP* Δ*rdl2*::*BLE*	This study
Δ*rdl1* Δ*rdl2* Δ*tum1*	Δ*rdl1*::*loxP* Δ*rdl2*::*BLE* Δ*tum1*::*LEU2*	This study
4k	Δ*rdl1*::*loxP* Δ*rdl2*::*BLE* Δ*tum1*::*LEU2* Δ*ych1*::*HIS3*	This study
4k::*RDL1*	Δ*rdl1*::*loxP* Δ*rdl2*::*BLE* Δ*tum1*::*LEU2* Δ*ych1*::*HIS3* YEplac195-*RDL1*	This study
4k:: *RDL2*	Δ*rdl1*::*loxP* Δ*rdl2*::*BLE* Δ*tum1*::*LEU2* Δ*ych1*::*HIS3* YEplac195-*RDL2*	This study
4k::*TUM1*	Δ*rdl1*::*loxP* Δ*rdl2*::*BLE* Δ*tum1*::*LEU2* Δ*ych1*::*HIS3* YEplac195-*TUM1*	This study
4k::*YCH1*	Δ*rdl1*::*loxP* Δ*rdl2*::*BLE* Δ*tum1*::*LEU2* Δ*ych1*::*HIS3* YEplac195-*YCH1*	This study
4k::*PSPE*	Δ*rdl1*::*loxP* Δ*rdl2*::*BLE* Δ*tum1*::*LEU2* Δ*ych1*::*HIS3* YEplac195-*PSPE*	This study
4k::*GLPE*	Δ*rdl1*::*loxP* Δ*rdl2*::*BLE* Δ*tum1*::*LEU2* Δ*ych1*::*HIS3* YEplac195-*GLPE*	This study
4k::*RDHA*	Δ*rdl1*::*loxP* Δ*rdl2*::*BLE* Δ*tum1*::*LEU2* Δ*ych1*::*HIS3* YEplac195-*RDHA*	This study
4k::*DUF442*	Δ*rdl1*::*loxP* Δ*rdl2*::*BLE* Δ*tum1*::*LEU2* Δ*ych1*::*HIS3* YEplac195-*DUF442*	This study
4k::*SSEA*	Δ*rdl1*::*loxP* Δ*rdl2*::*BLE* Δ*tum1*::*LEU2* Δ*ych1*::*HIS3* YEplac195-*SSEA*	This study
Plasmid		
YEplac195	Multicopy plasmid for expression in yeast	Laboratory stock
pSH47	Cre expression plasmid	Laboratory stock
pUG66	*BLE* template plasmid[a]	Laboratory stock
pUG6	*kanMX* template plasmid[b]	
pFA6a-GFP(S65T)-His3MX6	*HIS5* template plasmid[c]	Laboratory stock
pYE242ws-fapr-fapo17	*LEU2* template plasmid[d]	Laboratory stock
YEplac195-*MET15*	*MET15* in YEplac195, control by *TEF1* promoter	This study
YEplac195-*CYS3*	*CYS3* in YEplac195, control by own promoter	This study
YEplac195-*RDL1*	*RDL1* in YEplac195, control by own promoter	This study
YEplac195-*RDL2*	*RDL2* in YEplac195, control by own promoter	This study
YEplac195-*TUM1*	*TUM1* in YEplac195, control by own promoter	This study
YEplac195-*YCH1*	*YCH1* in YEplac195, control by own promoter	This study
YEplac195-*PSPE*	*PSPE* in YEplac195, control by TEF1 promoter	This study
YEplac195-*GLPE*	*GLPE* in YEplac195, control by TEF1 promoter	This study
YEplac195-*RDHA*	*RDHA* in YEplac195, control by TEF1 promoter	This study
YEplac195-*DUF442*	*DUF442* in YEplac195, control by TEF1 promoter	This study
YEplac195-*SSEA*	*SSEA* in YEplac195, control by TEF1 promoter	This study
pET30a	Expression plasmid in *E. coli*	Lab stock
pET30a-*MET15*	*MET15* in pET30a, control by IPTG-induced *lac* promoter	This study
pET30a-*RDL2*	*RDL2* in pET30a, control by IPTG-induced *lac* promoter	This study
pET30a-*PDO2*	Cp*PDO2* in pET30a,control by IPTG-induced *lac* promoter	Laboratory stock
pET30a-*RDL2* 106A	*RDL2* in pET30a, 106 site was mutated to Ala, control by IPTG induced lac promoter	This study
pET30a-*RDL2* 106S	*RDL2* in pET30a, 106 site was mutated to Ser, control by IPTG-induced *lac* promoter	This study
pET30a-*PSPE*	*PSPE* in pET30a, control by IPTG-induced *lac* promoter	This study
pET30a-*GLPE*	*GLPE* in pET30a, control by IPTG-induced *lac* promoter	This study
pET30a-*RDHA*	*RDHA* in pET30a, control by IPTG-induced *lac* promoter	This study
pET30a-*DUF442*	Cp*DUF442* in pET30a, control by IPTG-induced *lac* promoter	This study
pET30a-*SSEA*	*SSEA* in pET30a, control by IPTG-induced *lac* promoter	This study

[a]*BLE*, Zeocin resistance gene.
[b]*kanMX*, G418 resistance gene.
[c]*HIS5* encodes a key enzyme for histone synthetase.
[d]*LEU2*, encoding a key enzyme for leucine synthetase.

TABLE 3 Oligonucleotide primers used for plasmid construction

Name	Sequence
SUL1 ko F	ATGTCACGTAAGAGCTCGACTGAATATGTGCATAATCAGGAGGATGCTGA TGTTTAGCTTGCCTCGTCC
SUL1 ko R	CTAAACGTCCCATTTAGAAAAATCGGGTATATCGATATGAAAAAACGGTA GAATTCGAGCTCGTTTAAAC
SUL2 ko F	ATGTCCAGGGAAGGTTATCCAAACTTTGAAGAGGTAGAAATTCCTGACTT AACTGTGGGAATACTCAGGT
SUL2 ko R	CTAGATATCCCATTTAGCAAAATCTGGGATATCGATATGGAAGAAAGGTAATATCGACTACGTCGTTAAGG
MET5 ko F	ATGACTGCTTCTGACCTCTTGACGCTCCCACAATTGTTGGCGCAATATTCGCTTCGTACGCTGCAGGTC
MET5 ko R	TTAATAGGCATCTTCAGACACATCTTCATGGAAGTATTTACCCTCGGTGGCCAATACGCAAACCGCCTCT
CYS3 ko F	TAGACATTTGCACCTTATACATATACACACAAGACAAAACCAAAAAAAAT GCTTCGTACGCTGCAGGTC
CYS3 ko R	TTATCGTACTTAAAAAGGTCCGGTCGAAGGCAGAGACGTGGCACTGGCGACCAATACGCAAACCGCCTCT
MET15 ko F	ATGCCATCTCATTTCGATACTGTTCAACTACACGCCGGCCAAGAGAACCC GCTTCGTACGCTGCAGGTC
MET15 ko R	TCATGGTTTTTGGCCAGCGAAAACAGTTTCAAAAGATTGCTGGAAGTCTG CCAATACGCAAACCGCCTCT
MET15 ORF F	ATCTAAGTTTTAATTACAAAATGCCATCTCATTTCGATACTG
MET15 ORF R	CTAATTACATGAGAGCTCGGTCATGGTTTTTGGCCAGCGAA
MET15 his F	CGACGACGACAAGGCCATGGCTGATCCATCTCATTTCGATACTGTTCAACT
MET15 his R	CAGTGGTGGTGGTGGTGGTGCTCGATCATGGTTTTTGGCCAGCGAA
SUL1 in F	ACCGCACATATAGGGAATCTG
SUL1 in R	ATAGATTGGGGCACGAGTAC
SUL1 out F	TGCACATGGATCTTGTACTG
SUL1 out R	AAATAGGCTCAAGCTCAAGTTAAC
SUL2 out F	GATATGTCCAGGGAAGGTTATC
SUL2 out R	GGCACCATCCTACCTATTATTT
SUL2 in F	GATATGTCCAGGGAAGGTTATC
SUL2 in R	CCAGTTGATGATGGGAAAAAC
SOA1 ko F	ATGTCCGTACAAAAAGAAGAATACGATATTGTAGAAAAGGCCCAATTATCGCTTCGTACGCTGCAGGTC
SOA1 ko R	CTAGTGAATAAACCTATAATCAAGACGCTTCATTCCCTTATCTTTAGTTGCCAATACGCAAACCGCCTCT
SOA1 in F	ATTGTAGAAAAGGCCCAATTAT
SOA1 in R	CAAGTATAGAATATTGTCCGGG
SOA1 out F	ATTGTAGAAAAGGCCCAATTAT
SOA1 out R	GAATAAACCTATAATCAAGACGC
MET5 in F	ATGACTGCTTCTGACCTCTT
MET5 in R	TCTCTTCGTTAGCAATCTCC
MET5 out F	AAAGTAACAGTAGGGAACGG
MET5 out R	GATTTTATTCTTCACCTCGTT
CYS3 in F	CCACTCCGCTACAAAGTAC
CYS3 in R	CAGAGATTCTAACCAAGTCGTC
CYS3 out F	CGTGCCAGATTGAATTTTGAA
CYS3 out R	CATCTAACACGATTGATTGAGC
MET15 in F	GATCCAGAGCTGTACCAATTTAC
MET15 in R	ACAAATCTAGCCTCGATACC
MET15 out F	GAACACGCTCGATGAAAAAA
MET15 out R	CTTGTGAGAGAAAGTAGGTTTATAC
TEF1 F	CCAAGCTTGATCCCCCACACACCATA
TEF1 R	CGGGATCCTTTGTAATTAAAACTTAGATTAGATTGCT
CYC1 F	CTTGAGCTCTCATGTAATTAGTTATGTCACGCT
CYC1 R	CCGGAATTCGCAAATTAAAGCCTTCGAGC
SUL1 RT F	CACTGGGTTGGGTATACTGC
SUL1 RT R	ATGAGAGCCGGAATTTGACC
SUL2 RT F	CACGGTGAAAGGAGCATGTA
SUL2 RT R	CCAGTTGATGATGGGAAAAAC
ACT1 RT F	ATGGTCGGTATGGGTCAAAA
ACT1 RT R	TCCATATCGTCCCAGTTGGT
RDL1 ko F	AATTCTTTCTCGTTTATTTTCAGGGTTTGTGACTAAGAAACGATATTAAAGCTTCGTACGCTGCAGGTC
RDL1 ko R	GTTACTAGCTTACGAAAATACACAGGGTACATACCTAGAGTATACAAGGCCAATACGCAAACCGCCTCT
RDL1 in F	CCGTGATGAATGCTTGGAAT
RDL1 in R	TATCACCCCCATGAGAAACC
RDL1 out F	GAGAGTGGAGGCTTAATCAA
RDL1 out R	AACGGTGTGACATAAATGGC
RDL1 self F	TATGACCATGATTACGCCATTTTATTGGCGCATAGACAAG
RDL1 self R	GTCGACCTGCAGGCATGCATGGGGTGTTCGACTAGGTT
RDL1 his F	CGACGACGACAAGGCCATGGCTGATTGGAAGGCCGTGATGAATGC
RDL1 his R	CAGTGGTGGTGGTGGTGGTGCTCGATTATAAGTCAAGTTTATCACCCCCATGAG
RDL2 ko F	GCGATAACTCTCAACAAATGGAAGCGAGACAGAAGAAAAAGACCAACGCTTCGTACGCTGCAGGTC
RDL2 ko R	AAAGGTTGTCTATATACAGGATATATCGATTATACTTGTTTCTTTTTGGC CCAATACGCAAACCGCCTCT
RDL2 in F	CAAGATCGCCTACATTGGT
RDL2 in R	GATACCAGTGTTTTCGTACCC
RDL2 out F	TGGAAGCGAGACAGAAGAAA
RDL2 out R	CACAAGGAGAAAATCGAATGAATC

(Continued on next page)

TABLE 3 (Continued)

Name	Sequence
RDL2 self F	TATGACCATGATTACGCCAGAACCATCTGAGTACTCGATT
RDL2 self R	GTCGACCTGCAGGCATGCAGAAAAAGTCTGAGAAACGTAAAGT
RDL2 his F	CGACGACGACAAGGCCATGGCTGATTTCAAGCATAGTACAGGTATTCT
RDL2 his R	CAGTGGTGGTGGTGGTGGTGCTCGATTATTTTTTGGGCTTAACGTCAG
TUM1 ko F	ATGCCATTATTTGATCTTATTTCTCCAAAAGCGTTTGTTAAGTTAGTGGC TGTTTAGCTTGCCTCGTCC
TUM1 ko R	TAATCTCTGTTTTCAGCAATCCACTCGGGCCCGGATTTCAAGACCCACTGAATTCGAGCTCGTTTAAAC
TUM1 in F	CCAGCTTTTCATACTTTCTACC
TUM1 in R	TCTGTACCTTCGAATCTACCG
TUM1 out F	CCAGCTTTTCATACTTTCTACC
TUM1 out R	GGTTTAATCGTGTTGGAATTTCG
TUM1 self F	TATGACCATGATTACGCCACTTGATCCCTCCTTTGTTGC
TUM1 self R	GTCGACCTGCAGGCATGCAACTTGTGCCATTCGTTATCA
TUM1 his F	CGACGACGACAAGGCCATGGCTGATCCATTATTTGATCTTATTTCTCCAAAAG
TUM1 his R	CAGTGGTGGTGGTGGTGGTGCTCGATTAATCTCTGTTTTCAGCAATCCA
YCH1 ko F	GGACTCGTACTCAATAACAAACGTAAAATACCTGGATCCGACTGAATTAACTGTGGGAATACTCAGGT
YCH1 ko R	TCAACGCCACAGATCGGGTAGGTAACCCGCCGTAACGCTCTCGTCGTCACATATCGACTACGTCGTTAAGG
YCH1 in F	CACTACTACGCTGAGGGAG
YCH1 in R	AAACCTGTCAACGCCACA
YCH1 out F	GTGTCTGCTGTGCTTTGTACTA
YCH1 out R	AAACCTGTCAACGCCACA
YCH1 self F	TATGACCATGATTACGCCAGTGTCTGCTGTGCTTTGTACTA
YCH1 self R	GTCGACCTGCAGGCATGCACATGCGTACATATGTGGCT
YCH1 his F	CGACGACGACAAGGCCATGGCTGATGACTCGTACTCAATAACAAACGTAAAATAC
YCH1 his R	CAGTGGTGGTGGTGGTGGTGCTCGA AAACCTGTCAACGCCACA
UBA4 ko F	ATGAATGACTACCATCTCGAGGATACCACGTCTGAACTTGAAGCATTAAG GCTTCGTACGCTGCAGGTC
UBA4 ko R	CTAATATTTAGGAATGGTTTGATCAATATCGTCTATGTATTTGAAGTATC CCAATACGCAAACCGCCTCT
UBA4 in F	CTGCAAAAGGAAGTAAATAGAAG
UBA4 in R	GGGTTCAGTTTCGTGATATA
UBA4 out F	CTGCAAAAGGAAGTAAATAGAAG
UBA4 out R	TATTTAGGAATGGTTTGATCAAT
UBA4 self F	TATGACCATGATTACGCCATTTTACAGCTTTAGACACAGG
UBA4 self R	GTCGACCTGCAGGCATGCAAAATATTTCCATCATGCGAC
UBA4 his F	CGACGACGACAAGGCCATGGCTGATAATGACTACCATCTCGAGG
UBA4 his R	CAGTGGTGGTGGTGGTGGTGCTCGACTAATATTTAGGAATGGTTTGATCAATA
RDL2 106A F	GAATTGATTTTTCTTgcTGCGAAAGGAGTAAGAGC
RDL2 106A R	GCTCTTACTCCTTTCGCagcAAGAAAAATCAATTC
RDL2 106S F	GAATTGATTTTTCTTtcTGCGAAAGGAGTAAGAGC
RDL2 106S R	GCTCTTACTCCTTTCGCagaAAGAAAAATCAATTC
PspE F	ATCTAAGTTTTAATTACAAAATGTTTAAAAAAGGCTTACTTGCTCT
PspE R	CTAATTACATGAGAGCTCGG TTAACCTTTGACCTTCGGCATTG
GlpE F	ATCTAAGTTTTAATTACAAAATGGATCAGTTCGAATGTATTAACGTTGCCGAC
GlpE R	CTAATTACATGAGAGCTCGG TTACGCGCCGTACGCCACC
RdhA F	ATCTAAGTTTTAATTACAAA ATGAGCGACACCTTCCAGCG
RdhA R	CTAATTACATGAGAGCTCGG CTACTCCGCTTCGCCGCTG
SseA F	ATCTAAGTTTTAATTACAAA ATGTCCGTTTTCTCCGACC
SseA R	CTAATTACATGAGAGCTCGG TCAAACCTCTACAGGGGTATCG

modified SD culture. Equal amounts of cells (OD_{600} of 0.05) were cultured in 400 μl of the modified SD medium containing sulfate or thiosulfate at 30°C with shaking for 24 h or longer, and growth was monitored at OD_{600} or by a Microplate Reader (BioTek, Synergy H1). Yeast inoculated into the modified SD medium without sulfur was used as the control. The relationship of plate data and OD_{600} is shown in Fig. S12.

Thiosulfate uptake assay. Fresh *S. cerevisiae* cells were inoculated in 30 ml of the YPD medium and grown overnight at 30°C. Cells were collected by centrifugation (11,000 rpm, 5 min), washed twice with sterile water, and suspended in the modified SD medium without sulfur to the same volume. The cells were incubated at 30°C with shaking for 1 day, and the cells were harvested, washed, and resuspended in the same medium and incubated for another day. The sulfur-starved cells were then harvested and resuspended in sterile 50 mM potassium phosphate (pH 6, 2% glucose) at an OD_{600} of 10. Thiosulfate was added to a final concentration of 200 μM to initiate the reaction. One milliliter of cell suspension was centrifuged (13,000 rpm, 5 min) at various time intervals, and thiosulfate in the supernatant was measured by using the cyanide method (26, 50).

Measurement of thiosulfate uptake by *S. cerevisiae* strains. Thiosulfate was added to a final concentration of 1 mM in the sulfur-starved cell suspensions. One milliliter of the cell suspension was centrifuged (12,000 $\times$ *g*, 2 min) at various time intervals with one wash in H_2O. The pellets were suspended in 50 mM potassium phosphate (pH 6, 2% glucose), and thiosulfate was added to 200 μM to initiate the reaction. After 1 h of incubation, the thiosulfate concentration in the supernatant was measured and the rate was calculated (51).

H_2S measurement. H_2S production by yeast cells was measured with 3 ml of the sulfur-starved yeast cells in 50 mM potassium phosphate (pH 6) at an OD_{600} of 1 in a 15-ml glass tube. The tube was sealed with a rubber stopper, and a lead-acetate paper strip was placed in the gas phase. The cells were incubated at 30°C. When H_2S is produced and evaporated into the gas phase, sulfide reacts with lead to produce a dark stain on the paper strip (52). H_2S production by enzymes (in 50 mM potassium phosphate, pH 8) was detected in the same way.

To obtain a standard curve, different concentrations of NaHS were added to 3 ml of a specific buffer and incubated at 30°C for 1 h; the paper strips then were taken out to measure the darkness via densitometer (Fig. S13).

Real-time PCR analysis. The sulfur-starved cells were harvested at specific time intervals before and after the addition of 1 mM thiosulfate. Total RNA was isolated by using the total RNA extract kit (R6834-01; Omega), and the *SUL1* and *SUL2* mRNAs were analyzed by real-time PCR normalized against the *ACT1* mRNA according to a reported method (53).

Recombinant protein production and purification. *E. coli* BL21(DE3) cells with the expression plasmid pET30a-*MET15* were incubated in the LB medium with 50 μg/ml kanamycin and cultured to an OD_{600} of 0.6 at 25°C; 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to induce the production of the recombinant proteins. The cultures were further incubated with shaking for 8 h. Cells were harvested by centrifugation and disrupted by a pressure cell homogenizer (SPCH-18; Stansted Fluid Power Ltd., UK) in ice-cold buffer I (20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, 0.2 mM PLP, pH 8). The lysate was centrifuged at 12,500 $\times$ *g* for 10 min to remove cell debris. The target protein was purified via nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Shanghai, China) according to the supplier's recommendations. The final buffer was exchanged to buffer II (20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, 0.2 mM PLP, pH 8), and then 50% glycerol was added to give a final concentration of 10% before storage at −80°C (10). The concentration of purified Met15p was estimated via its molar extinction coefficient (ε_{280} = 51,800 M^{-1} cm^{-1}), calculated with the ProParam tool (http://web.expasy.org/protparam/) (54, 55). Other recombinant proteins were produced and purified in the same way.

Analysis of Met15 activities. Met15p was incubated at 25°C for 10 min prior to the experiment. For reaction experiments, *O*-acetyl-homoserine (HCl form) was first dissolved in 50 mM potassium phosphate buffer (pH 8), and the pH of the mixture was then adjusted to 7.5 with 5 M NaOH. All experiments were carried out in 1.5-ml microcentrifuge tubes. In a typical experiment, a 0.1-ml portion of enzyme solution was added to 0.9 ml of a reaction mixture containing 0.5 to 5 mM OAH, 0.0125 to 0.2 mM $Na_2S_2O_3$ or NaHS, and 0.1 mM PLP in 50 mM potassium phosphate buffer (pH 7.4). The final Met15 concentration was 2 nM. At appropriate time points, 0.3 ml of the reaction mixture was withdrawn and added to 0.03 ml of 1.0 M HCl solution to stop the reaction, followed by measuring the concentrations of thiosulfate and cysteine by using a reported high-performance liquid chromatography (HPLC) method (26, 56, 57).

Thiosulfate sulfurtransferase assay. Thiosulfate sulfurtransferase activity was estimated by measuring the production of sulfite (58). Briefly, the reactions were initiated by adding the enzyme to 1 ml of the assay mixture containing 50 mM potassium phosphate buffer (pH 8), 20 mM thiosulfate, and 20 mM GSH at 30°C. The reactions were quenched after 2 min by heating at 100°C and centrifuged. The produced sulfite in the supernatant was assayed by using a monobromobimane-derived method (26).

Potential CysK, CysM, and CysO from sequenced fungal genomes. A fungal genomic protein sequence set from NCBI, updated through 10 January 2018, was downloaded as a preliminary database for sorting cysteine synthases consisting of three types: CysK, CysM, and CysO. This database contains all proteins from 17 completely annotated genomes and 176 chromosomes. The reported sequences belonged to cysteine synthases (CysK, CysM, and CysO), cystathionine beta-synthase (CBS; EC 4.2.1.22), threonine dehydratase (THDH; EC:4.2.1.16), and aminocyclopropane-1-carboxylate deaminase (ACCD; EC:3.5.99.7). They were used to establish a phylogenetic tree by using a neighbor-joining analysis with the MEGA version 7.0 program, running a pairwise deletion, p-distance distribution, and bootstrap analysis of 1,000 repeats. The reported CysK from *Helicobacter pylori* (UniProt identifiers P56067 and Q9ZMW6) was found to be in the same clade as CBS. Thus, these two sequences were removed. The rest of the CysKs were found to be in the same clade. The reported CysO and CysM were grouped in the same clade. The reported CBS, THDH, and ACCD were used as outgroups.

The grouped CysK, CysM, and CysO proteins were used as queries for BLAST searches of the total GenBank fungal genomes with conventional criteria (E value of $\leq$1e−10, coverage of $\geq$60%, identity of $\geq$35%) via standalone BLASTP algorithm, resulting in 460 candidates. Redundancy was removed from these candidates by using CD-HIT with >99% identity within each group. The remaining 196 candidates were further tested via phylogenetic tree analysis with known CysK, CysM, CysO, proteins and the outgroups mentioned above. One hundred twenty-eight proteins were found to be in the same clade with CysK. No candidates were found to be in the same clade with CysM and CysO. The rest of the proteins all were distributed in the CBS clade.

The annotated 128 proteins were grouped into 67 subgroups by using CD-hit with 80% identity, and 67 representative proteins were combined with the known CysK, CysM, and CysO proteins and the outgroups to generate a phylogenetic tree.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01241-18.

SUPPLEMENTAL FILE 1, PDF file, 10.1 MB.

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