Cystathionine β-Synthase as a Carbon Monoxide–Sensitive Regulator of Bile Excretion

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Carbon monoxide (CO) is a stress-inducible gas generated by heme oxygenase (HO) eliciting adaptive responses against toxicants; however, mechanisms for its reception remain unknown. Serendipitous observation in metabolome analysis in CO-overproducing livers suggested roles of cystathionine β-synthase (CBS) that rate-limits transsulfuration pathway and H_2S generation, for the gas-responsive receptor. Studies using recombinant CBS indicated that CO binds to the prosthetic heme, stabilizing 6-coordinated CO-Fe(II)-histidine complex to block the activity, whereas nitric oxide (NO) forms 5-coordinated structure without inhibiting it. The CO-overproducing livers down-regulated H_2S to stimulate HCO_3^--dependent choleresis: these responses were attenuated by blocking HO or by donating H_2S. Livers of heterozygous CBS knockout mice neither down-regulated H_2S nor exhibited the choleresis while overproducing CO. In the mouse model of estradiol-induced cholestasis, CO overproduction by inducing HO-1 significantly improved the bile output through stimulating HCO_3^- excretion; such a choleretic response did not occur in the knockout mice. *Conclusion:* Results collected from metabolome analyses suggested that CBS serves as a CO-sensitive modulator of H_2S to support biliary excretion, shedding light on a putative role of the enzyme for stress-elicited adaptive response against bile-dependent detoxification processes. (HEPATOLOGY 2009;49:141-150.)

Carbon monoxide (CO) is generated from inducible heme oxygenase 1 (HO-1) and constitutive heme oxygenase 2 (HO-2), respectively, and has the ability to regulate neurovascular functions,[1,2] apoptotic responses,[3,4] and metabolism of xenobiotics and toxicants.[5,6] This gas is overproduced through increased delivery of heme as a substrate and the HO-1 induction on exposure to stressors such as hypoxia and oxidative stress. Mechanisms by which CO regulates cell functions appear to involve an activation of soluble guanylate cyclase (sGC), the enzyme that allows the gas to bind to the prosthetic heme to synthesize cyclic guanosine monophosphate as a second messenger.[1] Distinct from nitric oxide (NO) that forms 5-coordinated NO-Fe(II) complex to trigger full activation of the enzyme, CO activates this enzyme only modestly because the gas binding stabilizes 6-coordinated CO-Fe(II)-histidine complex.[7] Mitogen-activated protein kinase has also been shown to serve as a CO-responsive signal transducer.[8] Gene disruption of HO-1 increases sensitivity to overproduction of reactive oxygen species, inflammatory mediators or xenobiotic metabolism, whereas the gene transfer or CO inhalation under these circumstances suppresses such pathogenic responses.[7-9] However, direct mechanisms for the CO reception to trigger these adaptive responses of metabolism remain unknown.

Because this gas has the ability to inhibit ferrous form of the prosthetic heme of enzymes, tryptophan 2,3-dioxygenase or cytochromes P450 have been considered puta-

Abbreviations: CBS, cystathionine β-synthase; CE-MS, capillary electrophoresis equipped with mass spectrometry; CO, carbon monoxide; CORM, CO-releasing metal carbonyl tricarbonyldichlororuthenium (II); ES, 17α-ethinylestradiol; GSH, glutathione; GSNO, S-nitrosyl glutathione; H12, liver exposed to 12-hour hemin treatment; NO, nitric oxide; $RuCl_3$, CO-free ruthenium (III) chloride; SAM, S-adenosyl methionine; SE, standard error; sGC, soluble guanylate cyclase.

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Received July 3, 2008; accepted August 25, 2008.

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T.H. is a postdoctoral research fellow supported by Grant-in-Aid for Creative Scientific Research 17GS0419 from JSPS in Japan. Development of the methodology for differential metabolomic analyses using contrast-enhanced time of flight/mass spectrometry was supported by Leading Project for Biosimulation from MEXT. T.I. and T.Y. are research associates of Global COE Program for Metabolomic Systems Biology from MEXT and MHLW.

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.22604

Potential conflict of interest: Nothing to report.

tive CO-sensitive signal transducers regulating cell functions, including cell proliferation,[10] immune responses,[11] microvascular tone, xenobiotic detoxification, and biliary excretion in the liver.[5,6,12] However, ferrous heme of these enzymes is not only sensitive to CO but to NO. In this context, whether mechanisms by which CO regulates cell and organ functions is not shared by those for NO has not fully been studied yet.

This study aimed to mine novel CO-responsive regulators for stress-inducible adaptation of metabolism. To this end, we have used metabolome analyses based on capillary electrophoresis equipped with mass spectrometry (CE-MS) for systematic mining CO-responsive gaseous signal transducers. The current results suggest that cystathionine β-synthase (CBS), the enzyme rate-limiting transsulfuration pathway is such a novel CO-sensitive regulator of metabolism that plays an important role for quality control of bile excretion under disease conditions.

Materials and Methods

Preparation of Mice. The experimental protocols herein described were approved by our institutional guidelines provided by the Animal Care Committee of Keio University School of Medicine. Mice heterozygous for disruption in the CBS gene were purchased from Jackson Labs (Bar Harbor, MI) and bred at our institution. Male heterozygous CBS-deficient mice ($CBS^{+/-}$) and their littermates ($CBS^{+/+}$), and wild-type B6J mice, which were purchased from Clea Japan, Inc (Kawasaki City, Japan), were used at 8 to 12 weeks of age. Mice were allowed free access to laboratory chow and tap water, and were fasted for 18 hours before experiments. Mice were anesthetized with an intraperitoneal injection of ketamine at 120 mg/kg, and xylidine at 6 mg/kg. Their common bile ducts were ligated in proximity to the duodenum, and the gallbladder was nicked and cannulated with a polyethylene P-10 tube to collect bile for 20 minutes after a 10-minute stabilization period.[6,13] Biliary constituents such as total bile salts, bilirubin-IXα, pH values, and bicarbonate (HCO_3^-) were measured according to previous methods described elsewhere.[13] When necessary, biliary samples were collected into tubes containing 10% trichloroacetate to measure glutathione through high-performance liquid chromatography.[14] Determination of bilirubin-IXα in bile serves as an indicator of HO-mediated heme degradation in the liver that occurs in parallel with endogenous CO generation. Hepatic CO contents were also measured by gas chromatography as described previously,[15] except that the flame ionization detector equipped with a methanizer was used in this study instead of a reduction gas detector. Combination of these methods to determine CO allowed us to distinguish endogenous CO generation from the same gas exogenously administered as an intervention as described in the following session.

Administration of Reagents Studied. Protoheme IX (hemin) was administered at 40 μmol/kg intraperitoneally at 12 hours before surgical preparation for bile collection. This protocol was denoted as liver exposed to 12-hour hemin treatment (H12) treatment in the text. After collecting bile, livers were excised immediately to be snap-frozen in cold methanol, and the lysates served as samples for contrast-enhanced time of flight/mass spectrometry analyses as described later. In separate sets of experiments, liver samples were minced with 10% trichloroacetic acid at 4°C to measure cysteine and glutathione (GSH) through high-performance liquid chromatography to confirm the data collected from contrast-enhanced time of flight/mass spectrometry, when necessary.

A series of protocols were employed to examine roles of HO-derived CO in regulation of H_2S-modulated choleresis in the H12-treated mice. First, zinc protoporphyrin, a potent HO inhibitor, was administered intravenously at 12.5 μmol/hour/kg at 30 minutes before the bile collection; this dose was sufficient to block endogenous CO in the liver. When necessary, tricarbonyldichlororuthenium (II) dimer, the CO-releasing metal carbonyl [tricarbonyldichlororuthenium (II): CORM, Sigma-Aldorich][16] was administered intraportally at 30 minutes before the start of bile collection. When necessary, CO-free ruthenium (III) chloride ($RuCl_3$) was used as a negative control reagent. To examine whether the elevation of H_2S in the liver could alter biliary HCO_3^- excretion, sodium hydrosulfide (NaHS) was administered at 20 μmol/hour/kg through the portal vein at 30 minutes before the bile collection; as seen later in Results, this protocol restored the H12-induced decrease in the hepatic H_2S contents without altering a reduction of systemic blood pressure that was induced by a systemic bolus of the NaHS injection. S-nitrosyl glutathione (GSNO) was used as an NO donor. The reagent was injected intraportally with a dose of 7 μmol/kg at 30 minutes before the collection of bile; this protocol did not induce a reduction of systemic blood pressure, whereas greater doses caused hypotension and subsequent decrease in the bile output. In these experiments, administration of the reagent was performed through a 30-gauge miniature needle that was inserted into the portal vein to be fixed at the site of puncture. Finally, to examine therapeutic effects of CO, we examined effects of H12 treatment or administration of CORM in the mice exposed to drug-induced cholestasis. To this end, cholestasis was induced by a subcutane-

ous injection of 17α-ethinylestradiol (ES) at 5 mg/kg daily for 5 consecutive days before the experiments.[17]

Metabolome Analysis. We performed metabolome analyses of tissue lysates collected from snap-frozen livers of mice using contrast-enhanced time of flight/mass spectrometry according to our previous methods.[18,19] Measurements of hepatic H_2S contents were based on gas chromatography described in our previous method.[14] Biliary flux of bilirubin-IXα (BR-IXα) in bile samples were determined by enzyme-linked immunosorbent assay using the anti–BR-IXα monoclonal antibody as described previously.[6,20] Because BR-IXα is an end product of the HO-mediated degradation of protoheme IX, its measurements in bile serves as an index of endogenous CO generation in the liver.[20] The conversion of ^{15}N-methionine to its downstream metabolites was determined by CE-MS to examine different rates of the metabolic flux through CBS in the liver. In these experiments, ^{15}N-methionine was intraperitoneally injected at 150 μmol/100 g body weight, and ^{15}N-homocysteine and ^{15}N-cystathionine were measured by CE-MS using the lysates of liver tissues at 30, 60, and 120 minutes after the methionine challenge. Data were expressed as percentages of the mass-labeled metabolites versus total amounts of metabolites in remethylation cycle [ΣRM: methionine + S-adenosyl methionine (SAM) + S-adenosyl homocysteine (SAH) + homocysteine]. In a separate set of experiments, effects of application of CO on contents of methionine and cystathionine in HepG2 cells were determined in culture. In these experiments, the cells were maintained in Roswell Park Memorial Institute 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum; the mixture was supplemented with 1× penicillin/streptomycin and maintained at 37°C in an atmosphere of 5% CO_2/95% air. The cells were treated with either 50 μmol/L CORM or $RuCl_3$ as a negative control for 16 hours. To measure the metabolites, a frozen pellet of the 1×10^6 cells was homogenized in 10% trichloroacetic acid with 10 mM diethylene triamine pentaacetic acid following brief centrifugation, and the supernatant was used as a sample.

Western Blot Analysis. Western blot analysis was carried out to examine an induction of heme oxygenase (HO)-1 using the polyclonal antibody SPA896 (Stressgen, Ann Arbor, MI). In these experiments, the blotting against α-tubulin was carried out using the polyclonal antibody (Cell Signaling, Danvers, MA) as an internal control.

Recombinant Full-Length Rat CBS. The complementary DNA of the full-length rat CBS was a gift from Professor Masao Ikeda-Saito in Tohoku University. Stopped-flow equipment was purchased from Unisoku, Inc. (Tokyo) and used to examine binding of CO or NO to the CBS protein according to previous methods.[21] Electron paramagnetic resonance spectrometry to determine 5-coordinated structure of the nitrosylheme complex of CBS was carried out according to previous methods.[21,22]

Statistical Analyses. The statistical significance of data among different experimental groups was determined by one-way analysis of variance and Fischer's multiple comparison test. $P < 0.05$ was considered significant.

Results

CO Overproduction Inhibits Transsulfuration and H_2S and Stimulates HCO_3^- Choleresis. Metabolome analyses based on CE-MS allowed us to pinpoint metabolic pathways responding to disease conditions. In mouse liver, we detected more than 1800 metabolites, and compared differences between the control and acetaminophen-treated livers.[18] This method was used to determine differences in metabolic responses between mouse livers and those overloaded with heme, the stressor inducing oxidative stress and subsequent CO overproduction through increasing the substrate and inducing HO-1 (Fig. 1A). The hepatic CO flux peaked at 6 hours, becoming threefold to fourfold greater during the 6 to 12 hours after challenging with hemin, as judged by BR-IXα, an end product of HO-mediated heme degradation (Fig. 1B).[13] Under these conditions, bile output was modestly but significantly increased at 12 to 18 hours after the treatment (Fig. 1C) in parallel with significant elevation of HCO_3^- to make bile more alkaline (Fig. 1D-F), enhancing solubility of organic anions during the detoxification processes. These results suggest that the heme-elicited choleretic response is not correlated with vasodilatory mechanisms by the gas. Based on these results, we used CE-MS analyses to examine metabolomics in the liver exposed to 12-hour hemin treatment (H12), in which phenotypes of bile remodeling became evident.

Among known metabolites (Table 1), most prominent differences between the control and H12 groups occurred in global decreases in amino acids concurrent with increases in Krebs cycle substrates such as acetyl CoA: the fact that these changes coincided with sustained glutamate, significant increases in glutamine, and high-energy adenosine phosphates appeared to suggest utilization of the amino acid pool for energy substrates. By contrast, several essential amino acids such as methionine, tryptophan and histidine, and serine were maintained. Another important alteration was a global decrease in transsulfuration metabolites such as cystathionine, cysteine, and

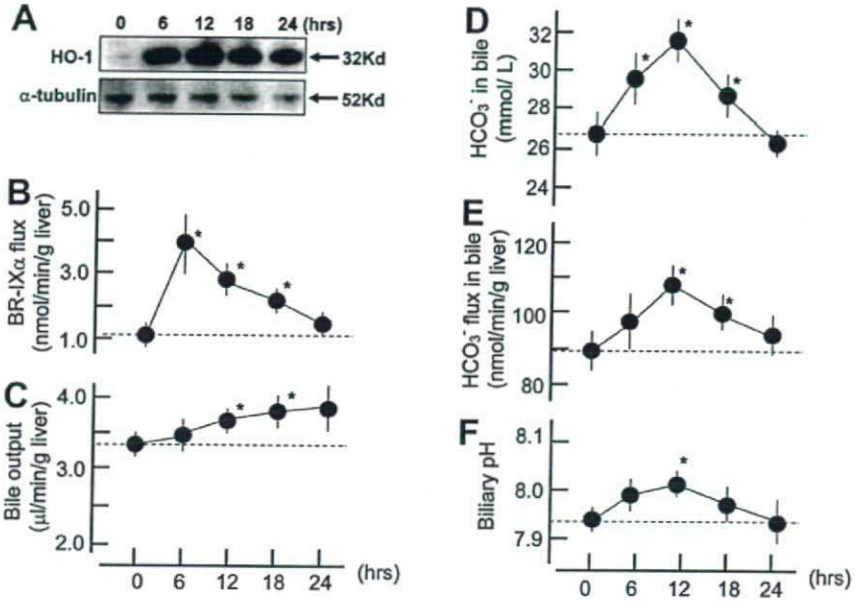

Fig. 1. Temporal alterations in hepatic generation of CO and biliary function after overloading heme. (A) Western blots indicating the induction of heme oxygenase (HO)-1. Alpha-tubulin is an internal control. (B) Biliary excretion of bilirubin-IXα (BR-IXα), a terminal metabolite of HO-dependent heme degradation, as an index of endogenous CO generation through heme oxygenase in the liver. (C) Bile output. (D) Biliary concentration of HCO_3^-. (E) Biliary flux of HCO_3^-. (F) pH values of bile. $^*P < 0.05$ versus the value measured at time 0, which is before the intraperitoneal hemin administration at 40 μmol/kg.

hypotaurine. These results led us to determine tissue contents of H_2S, the terminal product derived from CBS or cystathionine γ-lyase that constitute transsulfuration pathway; this gaseous compound turned out to be suppressed in the H12 group. Based on these measurements, we hypothesized that the H12 treatment limits the activity of CBS so far as judged from maintenance of methionine pool (ΣRM) and serine, a substrate of the enzyme, with suppression of the transsulfuration metabolites residing in the downstream (Fig. 2A). This hypothesis was confirmed by in vivo pulse-chase analysis showing accumulation of ^{15}N-homocysteine and suppression of ^{15}N-cystathionine after the ^{15}N-methionine challenge in the H12 group (Fig. 2B).

Such an inhibitory action of the H12 treatment on the transsulfuration pathway was reproducible when HepG2 cells was treated with CO in culture; contents of cystathionine were significantly suppressed by the application of 50 μmol/L CORM (9.3 ± 1.3 versus 15.9 ± 1.4 nmol/g protein for the vehicle treatment with $RuCl_3$. Mean ± standard error (SE) of three separate experiments, $P < 0.03$), whereas methionine exhibited no difference (66.3 ± 3.7 versus 80.3 ± 12.2 nmol/g protein for CORM and $RuCl_3$, respectively. Mean ± SE of three separate experiments), suggesting inhibitory action of the gas on CBS.

CO But Not NO Inhibits CBS. H12-induced metabolomic changes indicating dissociation between remethylation cycle and transsulfuration pathway led us to hypothesize that CBS, a heme-containing enzyme that rate-limits the transsulfuration pathway, is a sensor of the H12-elicited CO overproduction. Rat full-length recombinant CBS were purified (Fig. 3A) to examine whether CO or NO could inhibit the enzyme activities. CO, but not NO, specifically inhibited the enzyme (Fig. 3B). Previous crystallographic studies using a truncated form of CBS showed that the axial ligands for the prosthetic heme were cysteine and histidine, indicating a large peak of absorbance at 448 nm.[23] On CO application, the heme formed a 6-coordinated CO-Fe(II)–histidine complex, as judged by a decrease in the absorbance at 448 nm and a reciprocal elevation at 422 nm (Fig. 3C). These results were consistent with previous works using the truncated form of human recombinant CBS.[24] Such an inhibitory effect of CO on CBS activity occurred even when sufficient amounts of SAM were present as an allosteric activator,[25] whereas the CO concentrations necessary to suppress CBS became greater in the presence of SAM (Fig. 3D). Conversely, NO was able to bind to the heme but with a distinct structure of 5-coordinated nitrosyl-heme as judged by electron paramagnetic resonance spectrometry (Fig. 3E), suggesting that the enzyme responds specifically to the binding of CO but not that of NO.

CO-Induced HCO_3^- Choleresis Is Sensitive to H_2S and Disappears in CBS$^{+/-}$ Mice. Recent studies indicated that H_2S derived from cystathionine γ-lyase, an enzyme using cysteine to generate the gas, modulates biliary HCO_3^- excretion via mechanisms involving glibenclamide-sensitive channels, a putative H_2S target.[14,26] We hypothesized that the stress-induced CO stimulates the HCO_3^- excretion to increase pH in bile through its inhibitory action on CBS-derived H_2S. To examine this hypothesis, we chose the dose of the CO-releasing molecule (CORM) that was able to increase hepatic contents

Table 1. Comparison of Metabolome Analysis by CE-MS in Liver Extracts Between Control and the Hemin-Treated (H12) Mice

	Control	H12
Carbohydrates (nmol/g liver)		
Glucose 1-P	20 ± 4	31 ± 5
Glucose 6-P	24 ± 1	22 ± 6
Ribulose 5-P	206 ± 60	115 ± 17
Fructose 6-P	25 ± 1	21 ± 6
Glycerol 3-P	1800 ± 250	1663 ± 218
Lactate	3490 ± 633	2920 ± 385
Acetyl CoA	3.4 ± 0.5	6.2 ± 1.1*
Malonyl CoA	37 ± 6	83 ± 15*
Citrate	70 ± 13	88 ± 20
Fumarate	120 ± 22	167 ± 52
Malate	343 ± 91	479 ± 90
CoA	132 ± 21	111 ± 20
Nucleotides (nmol/g liver)		
ATP	208 ± 35	480 ± 90*
GTP	33 ± 4	79 ± 14*
ADP	577 ± 104	1060 ± 154*
AMP	1866 ± 277	1863 ± 70
IMP	501 ± 82	660 ± 99
Adenosine	203 ± 18	151 ± 11
Adenine	12 ± 1	12 ± 2
Hypoxanthine	58 ± 8	43 ± 15
	Control	**H12**
Amino acids (μmol/g liver)		
Gly	3.16 ± 0.11	2.20 ± 0.05*
Ala	3.12 ± 0.48	1.47 ± 0.40*
Ser	0.38 ± 0.07	0.31 ± 0.05
Pro	0.37 ± 0.03	0.27 ± 0.04*
Val	0.41 ± 0.01	0.23 ± 0.05*
Thr	0.31 ± 0.03	0.20 ± 0.04*
Lys	0.69 ± 0.13	0.46 ± 0.05*
Cys	0.20 ± 0.04	0.07 ± 0.03*
Leu	0.36 ± 0.02	0.25 ± 0.05†
Asp	0.76 ± 0.13	0.59 ± 0.12†
Glu	2.90 ± 0.16	2.75 ± 0.28
Gln	3.39 ± 0.58	6.48 ± 0.54*
His	0.43 ± 0.05	0.48 ± 0.02
Amino acids and derivatives (nmol/g liver)		
Met	49 ± 5	39 ± 10
GABA	29 ± 2	25 ± 4
Ornithine	420 ± 95	226 ± 22*
Asn	77 ± 7	59 ± 3*
Ile	175 ± 12	94 ± 17*
Arg	8.8 ± 1.2	4.8 ± 0.6*
Citrulline	64 ± 10	35 ± 3*
Trp	34 ± 2	31 ± 3
Tyr	111 ± 15	52 ± 8*
Glu-2 aminobutyrate	6.3 ± 2.3	5.7 ± 1.2
Ophthalmate	67 ± 7	83 ± 6

Data indicate mean ± SE of six separate experiments.

Data of metabolites in remethylation cycle and transsulfuration pathway were indicated in Fig. 2A.

*$P < 0.05$ and †$P < 0.1$ versus controls.

of CO comparably to those measured in the H12 treatment: As seen (Fig. 4A), the intraportal administration of CORM at 20 μmol/kg significantly increased hepatic CO contents comparable to those induced by H12 treatment in the intact mice. This dose of CORM suppressed hepatic H_2S and stimulated biliary HCO_3^- flux. Stimulatory effects of CO administration on biliary HCO_3^- excretion in intact mice were not shared by NO, as judged by observation in the mice administered with GSNO, an NO donor (Fig. 4B): These results were consistent with observation that CBS is sensitive to CO but not to NO in vitro (Fig. 3).

As already seen, H12 treatment increased CO generation (biliary BR-IXα flux), decreased hepatic H_2S contents, and stimulated biliary HCO_3^- flux (Fig. 1). HO blockade by zinc protoporphyrin-IX cancelled these changes elicited by H12 treatment. On the other hand, an administration of NaHS, an H_2S donor, abolished the H12-induced suppression of hepatic H_2S contents, and significantly attenuated the stimulatory response of biliary HCO_3^- flux (Fig. 5A), suggesting that H12-inducible CO stimulates biliary HCO_3^- excretion through modulation of CBS-derived H_2S. As previously reported, homozygous CBS knockout mice died of severe hepatic steatosis, whereas heterozygous knockout ($CBS^{+/-}$) mice survive through compensation without apparent phenotypes.[27] In these mice, indeed, the baseline H_2S content in livers of $CBS^{+/-}$ mice was comparable to that of $CBS^{+/+}$ mice, presumably because of compensation of the gas generation through cystathionine γ-lyase. On H12 treatment, $CBS^{+/-}$ mice exhibited an increase in the hepatic CO generation comparably to $CBS^{+/+}$ mice, but neither decreased H_2S contents nor up-regulated biliary HCO_3^- flux (Fig. 5B), indicating phenotypes distinct from those in $CBS^{+/+}$ littermates.

CO Protects Against Drug-Induced Cholestasis Through Mechanisms Involving CBS. We further attempted to investigate whether the administration of CO could improve biliary dysfunction occurring in disease models. To examine this, the mice were treated with ES, a cholestatic reagent suppressing three major osmolites such as HCO_3^-, glutathione, and bile salts in bile.[17] H12 treatment or the administration of CORM significantly increased bile output concurrently with a recovery of HCO_3^- excretion into bile (Fig. 6A). The anti-cholestatic effects of H12 treatment through stimulation of HCO_3^- excretion disappeared in the $CBS^{+/-}$ mice (Fig. 6B), suggesting again a pivotal role of CBS for triggering the CO-induced choleresis.

Discussion

CO administration or HO-1 induction has been shown to protect against tissue injury and considered a potentially useful therapeutic stratagem.[8,16] Serendipitous observation in the liver indicating effects of overproduced CO on metabolism of sulfur-containing amino

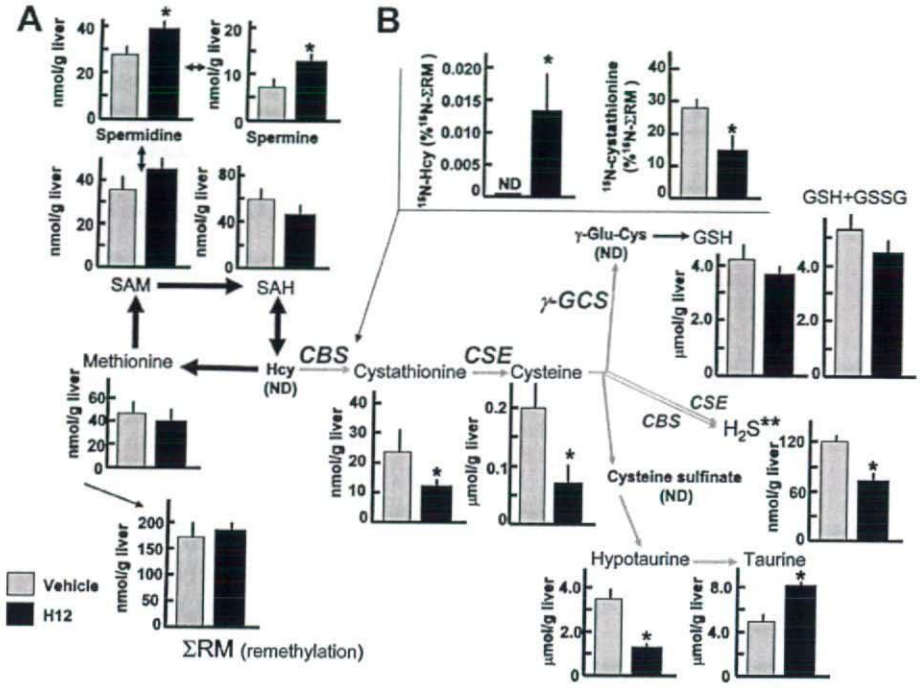

Fig. 2. Metabolomic comparison of sulfur-containing amino acids and their derivatives between the heme-overloaded and vehicle-treated livers of mice. (A) Differences in hepatic contents of the metabolites between the control and hemin-treated mice. H12: treatment with hemin at 12 hours before sampling the liver. Note decreases in transsulfuration metabolites. (B) *In vivo* pulse-chase analysis indicating conversion rates of ^{15}N-methionine into ^{15}N-homocysteine (Hcy) and ^{15}N-cystathionine in livers between the groups. The amounts of the downstream metabolites were measured at 30 minutes after the methionine administration. The data in B were normalized by total amounts of metabolites in remethylation cycle (^{15}N-methionine + ^{15}N-SAM + ^{15}N-SAH + ^{15}N-Hcy = ΣRM) at 30 minutes. ND, not detected. Data indicate mean ± SE of six to eight separate experiments for each group. $*P < 0.05$ versus the vehicle-treated group.

acids led us to reveal unique physiological actions of this gas on CBS *in vivo* that are not shared with NO. The current study suggested that stress-inducible CO targets CBS and thereby reduces H_2S significantly to stimulate biliary HCO_3^- excretion that could benefit detoxification processes. Conversely, such a property of stress-inducible CO might jeopardize anti-oxidative defense systems through an overflow of homocysteine or through a shortage of GSH. Under current experimental conditions, however, such a risk seemed little, if any, so far as judged from maintenance of GSH and adenosine triphosphate so far. This appears to result from large difference in amino acid pools between methionine (nmol/g) and thiols including cysteine and GSH (μmol/g). Furthermore, cysteine could be supplied through its uptake from extracellular space by mechanisms involving Nrf2, the transcriptional factor activated in response to oxidative stress or electrophiles such as heme.[28,29] By contrast, the amounts of sulfur-containing amino acids consumed to generate H_2S seems relatively smaller than that for synthesizing GSH or hypotaurine, as judged from quantitative information collected by metabolome analysis. Because CBS not only limits synthesis of cystathionine from homocysteine but also directly suppresses H_2S generation from cysteine, the inhibitory effects of CO on the enzyme could dictate largely on the action of H_2S in the liver, causing a stimulatory effect on bile excretion. Considering recent studies suggesting vasodilatory effects of H_2S,[26,30] suppression of CBS-derived H_2S by stress-inducible CO might trigger vasoconstriction, but such vasoactive responses did not occur so far as judged from choleretic response of the basal bile flow that is highly dictated by microvascular perfusion. This might result from the fact that stress-inducible CO itself has the ability to maintain the basal microvascular perfusion through multiple vasodilatory mechanisms involving activation of cyclic guanosine monophosphate and modulation of cytochrome P450–derived vasoconstrictors.[6,20,31]

Although the inhibitory action of stress-inducible CO on the transsulfuration pathway has first been shown in the heme-overloading detoxification model of mice in the current study, a similar event occurred in acetaminophen-induced acute liver injury model of mice in which CO was overproduced through degradation of cytochrome P450–derived heme.[5,18] Our previous study in rats suggested that another HO-derived product bilirubin but not CO

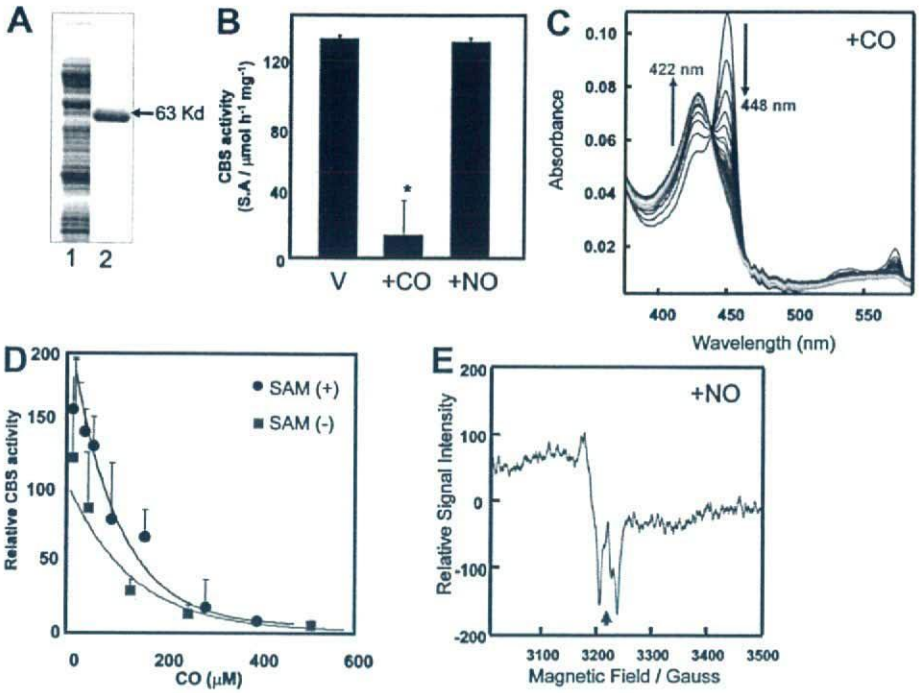

Fig. 3. Effects of CO and NO on the activity and structure of the prosthetic heme of rat recombinant full-length CBS. (A) Sodium dodecyl sulfate polyacrylamide gel electrophoresis for purification of rat recombinant CBS. Lane 1, crude extract; lane 2, purified CBS. (B) Effects of CO and NO on the Fe(II)-CBS activity under optimal substrate conditions at pH 7.4. CO but not NO (100 μM) significantly attenuated the activities of the ferrous enzyme. Data indicate mean ± SE of four experiments. The activities were measured by determining conversion of homocysteine and serine to cystathionine. *$P < 0.05$ versus the group treated with vehicle (V). The concentration of CBS-heme was 10 μM. (C) Stopped-flow visible spectrophotometry for Fe(II)-CBS to examine temporal transitional changes after mixing with CO. Data exhibited a drop at 449 nm and a reciprocal elevation at 422 nm, demonstrating stabilization of the 6-coordinated CO-Fe(II)-histidine complex. K_{obs} = 0.638/second. (D) Effects of CO on the CBS activities in the presence or absence of S-adenosyl methionine (SAM), the allosteric activator of the enzyme. (E) Electron spin resonance spectrometry indicating 5-coordinated NO-Fe(II) complex of the CBS-heme. Arrow: g-value = 2.008.

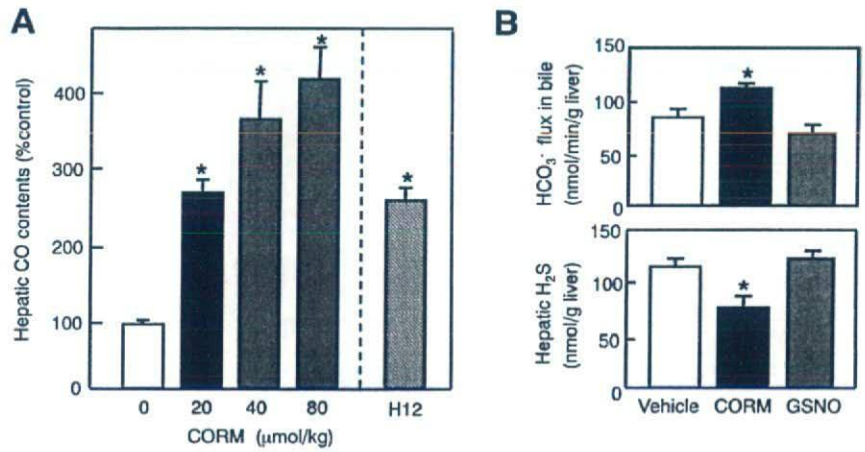

Fig. 4. Effects of the administration of CORM on hepatic CO delivery and biliary function, and their comparison with GSNO, an NO donor. (A) Effects of administration of CORM on hepatic CO contents. H12: the CO contents measured at 12 hours after an intraperitoneal injection of hemin at 40 μmol/kg. Data indicate mean ± SE of five separate experiments for each group. *$P < 0.05$ versus the controls. Note that 20 μmol/kg CORM caused an increase comparable to that induced by H12. (B) Effects of an intraportal administration of CORM on hepatic H_2S contents and biliary HCO_3^- flux. GSNO, S-nitrosyl glutathione, an NO donor. *$P < 0.05$ versus the values in the vehicle-treated controls. Data indicate mean ± SE of seven to eight separate experiments for each group.

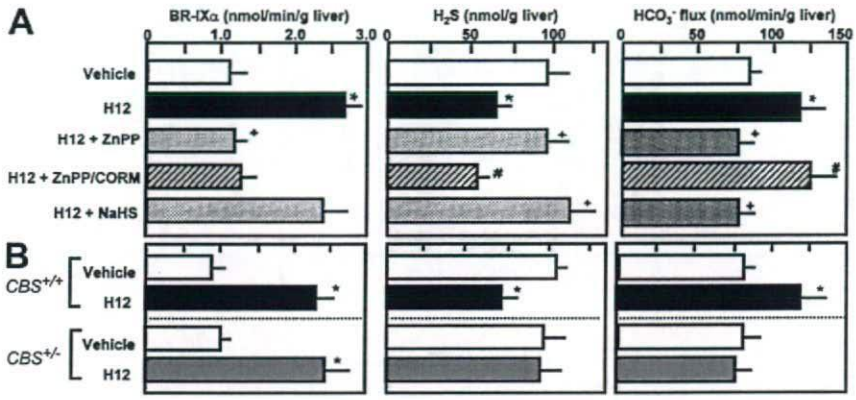

Fig. 5. Effects of HO blockade by zinc protoporphyrin and supplementation of NaHS, an H_2S donor, on biliary flux of BR-IXα, hepatic H_2S contents, and biliary HCO_3^- excretion in the 12-hour hemin-treated liver (H12). (A) Measurements in wild-type male B6 mice. Note that the hemin-induced suppression of H_2S generation and stimulation of biliary HCO_3^- excretion were sensitive to the HO inhibitor and reversed by supplementing CO (CORM). An injection of NaHS, an H_2S donor, restored hepatic H_2S contents and repressed the biliary HCO_3^- excretion in the H12-treated liver, suggesting that the biliary response is H_2S-dependent. (B) Disappearance of H12-induced reduction of H_2S and biliary HCO_3^- excretion in heterozygous CBS-knockout mice ($CBS^{+/-}$). Note that $CBS^{+/-}$ mice neither exhibit a reduction of H_2S nor up-regulate biliary HCO_3^- excretion, although overproducing CO (BR-IXα flux) comparably to the littermates ($CBS^{+/+}$). $*P < 0.05$ versus the vehicle-treated controls. $+P < 0.05$ versus the H12-treated groups. $\#P < 0.05$ versus the H12+ zinc protoporphyrin-treated groups.

has the ability to improve bile acid–dependent bile output of the post–cold ischemic liver grafts through its antioxidative action.[32] However, such an effect of bilirubin appears to be distinct from the stimulatory action of CO on biliary fluid excretion indicated in the current study. CO has been shown to exert diverse actions on biliary function through multiple mechanisms: First, stress-inducible levels of CO have the ability to elongate the intervals of bile canalicular contraction, which helps increase the stroke volume for promoting bile excretion; this process appears to involve mechanisms mediated by modulation of cytochrome P450 epoxygenases and intra-

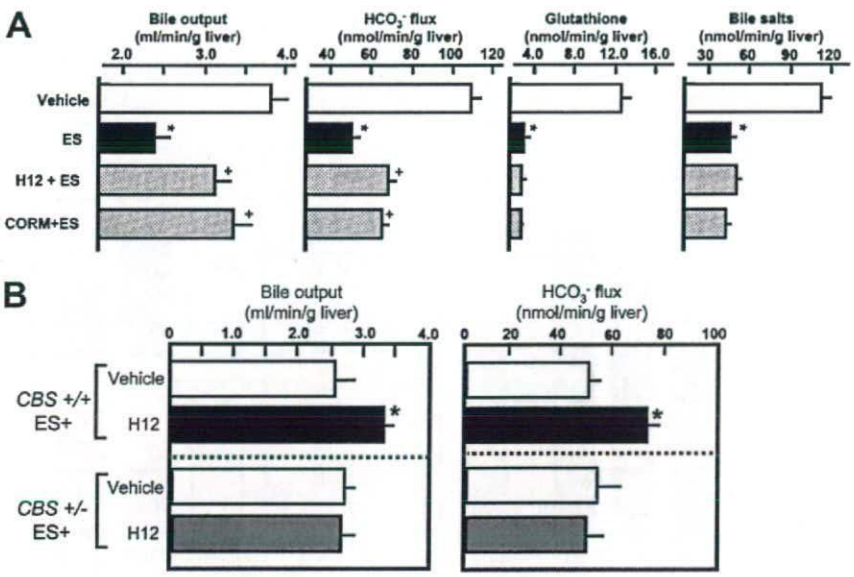

Fig. 6. Effects of H12 treatment or CORM administration on 17α-ethinylestradiol (ES)-induced cholestasis in male B6 mice. (A) Effects of H12 or CORM on ES-induced decreases in the bile output and bile constituents. ES elicited marked cholestasis, which coincided with decreases in HCO_3^-, glutathione, and bile salts in bile. Pretreatment with hemin at 12 hours before the administration of ES (H12 + ES) or the administration of CORM significantly attenuated ES-induced cholestasis through stimulation of HCO_3^- excretion into bile. (B) Effects of H12 treatment on ES-induced impairment of bile output and biliary HCO_3^- flux in $CBS^{+/+}$ and $CBS^{+/-}$ mice. $*P < 0.05$ versus the values in vehicle-treated controls. $+P < 0.05$ versus the values in ES-treated group. Data indicate mean ± SE of eight separate experiments for each group. Note disappearance of the improving effect of H12 treatment in the $CBS^{+/-}$ mice.

cellular Ca^{2+} mobilization.[12] Second, suppression of endogenous CO activates bile acid–dependent bile excretion through accelerated vesicular transport of taurocholate, while inducing no significant elevation of the bile acid–independent fraction.[33] Conversely, CO overproduction by the HO-1 induction or exogenous administration of CO stimulates bile acid–independent choleresis concurrently with increased mrp2-dependent excretion of bilirubin-IXα and glutathione, while suppressing biliary excretion of bile salts, indicating the effects of the gas for stimulating fluid excretion into bile.[34] Of interest is that glibenclamide, an inhibitor of K^+ channel that serves as a putative target for H_2S,[26] acts on Na^+-K^+-$2Cl^-$ cotransporter in bile duct epithelium to stimulate biliary HCO_3^- excretion in normal and cholestatic livers.[35] We showed that inhibition of cystathionine γ-lyase, another H_2S-generating enzyme, stimulates basal and glibenclamide-induced fluid output of bile through stimulating HCO_3^- excretion without altering the baseline vascular resistance of the liver.[14] Recent studies provided evidence that such a glibenclamide-responsive channel is present in rodent cholangiocytes[36] or in duodenum,[37] contributing to stimulation of the HCO_3^- excretion.[36] Based on these observations, it is not unreasonable to speculate that CO stimulates biliary fluid excretion through mechanisms involving H_2S-mediated modulation of glibenclamide-sensitive channels on biliary epithelium. Although further investigation is necessary to determine whether these mechanisms are sensitive to H_2S, the current results shed light on a possibility that the CO-CBS system serves as a putative mechanism for stimulating bile acid–independent fluid excretion, facilitating excretion of HCO_3^- and organic anions such as bilirubin to support heme detoxification. Both glibenclamide and CO help biliary fluid excretion in estrogen-induced hepatocellular cholestasis. Exploration of H_2S-sensitive molecular targets occurring on biliary epithelium deserves further studies for evidence that HO-1–derived CO serves as a therapeutic stratagem for protecting against cholestasis.

CO has been believed to share varied physiological effects on biological systems with NO. However, through extrapolation of studies *in vitro* indicating biochemical actions of CO to trigger structural changes in gas-responsive heme proteins (such as sGC, hemoglobin) distinct from those elicited by NO,[7,19,21,22,38] evidence that CO is a unique gaseous regulator distinct from NO has been emerging. In fact, CO itself modestly activates sGC, by which hepatic sinusoids are constitutively dilated.[2,20,39] By contrast, in vascular smooth muscle cells in which NO is sufficiently supplied from arteriolar endothelium (for example, brain microcirculation), the inducible CO inhibits NO-elicited sGC activation.[40,41] Besides these observations suggesting physiologic actions of CO occurring dependently of local NO levels, the current study provided evidence for a novel mechanism functioning irrespective of the NO effects. Furthermore, our results shed light on a metabolic link between CO and H_2S, suggesting that different gaseous mediators constitute an intriguing link for regulation of organ functions.

Acknowledgment: The authors thank Kayo Maruyama for technical support in measuring tissue H_2S contents.

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2008, p. 2787–2796 Vol. 74, No. 9
0099-2240/08/$08.00+0 doi:10.1128/AEM.01781-07

Development of Bottom-Fermenting *Saccharomyces* Strains That Produce High SO_2 Levels, Using Integrated Metabolome and Transcriptome Analysis[▽]

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Received 1 August 2007/Accepted 24 February 2008

Sulfite plays an important role in beer flavor stability. Although breeding of bottom-fermenting *Saccharomyces* strains that produce high levels of SO_2 is desirable, it is complicated by the fact that undesirable H_2S is produced as an intermediate in the same pathway. Here, we report the development of a high-level SO_2-producing bottom-fermenting yeast strain by integrated metabolome and transcriptome analysis. This analysis revealed that *O*-acetylhomoserine (OAH) is the rate-limiting factor for the production of SO_2 and H_2S. Appropriate genetic modifications were then introduced into a prototype strain to increase metabolic fluxes from aspartate to OAH and from sulfate to SO_2, resulting in high SO_2 and low H_2S production. Spontaneous mutants of an industrial strain that were resistant to both methionine and threonine analogs were then analyzed for similar metabolic fluxes. One promising mutant produced much higher levels of SO_2 than the parent but produced parental levels of H_2S.

The bottom-fermenting yeast *Saccharomyces pastorianus* is used to produce beer and has been proposed to be a natural hybrid between *Saccharomyces cerevisiae* and *Saccharomyces bayanus* (30). Bottom-fermenting yeasts have two types of genes, one set highly homologous (more than 90% identity) to those of *S. cerevisiae* and the other less so but highly homologous to *S. bayanus* (i.e., non-*S. cerevisiae* [Lg type]) (8, 14, 27, 33). One way in which *S. pastorianus* differs from baker's yeast (*S. cerevisiae*) is its tendency to produce higher levels of both sulfite (SO_2) and hydrogen sulfide (H_2S).

It is well known that sulfur compounds in beer make significant contributions to flavor and aroma. SO_2, for example, acts as an antioxidant, which slows the development of oxidation haze and staling of flavors in beer. In contrast, H_2S has an aroma of rotten eggs and is also a precursor of other compounds with undesirable sensory characteristics. SO_2 and H_2S are produced by yeast during reductive sulfate assimilation (Fig. 1). Inorganic sulfate is taken up through a sulfate permease and reduced to SO_2 by enzymes encoded by *MET3*, *MET14*, and *MET16*. SO_2 is then reduced to H_2S by SO_2 reductase encoded by *MET5* and *MET10* (29). The next intermediate, homocysteine, which is synthesized from H_2S and *O*-acetylhomoserine (OAH) by OAH sulfhydrylase encoded by *MET17*, leads to the formation of cysteine, methionine, and *S*-adenosylmethionine (SAM). SAM transcriptionally represses all of the genes involved in sulfate assimilation. Park and Bakalinsky previously reported that *SSU1* encodes an SO_2 efflux pump that exports intracellular SO_2 through the plasma membrane (18).

In the postgenomic era, systematic and high-throughput analyses of mRNA and proteins have become central to recent functional genomics initiatives. Metabolomics entails the analysis of all cellular metabolites and has become a powerful new tool for gaining insight into functional biology. Measurement of numerous metabolites within a cell and tracking concentration changes as a function of growth or environmental conditions not only provides direct information on metabolic phenotypes but also complements gene expression and proteomic studies. Current large-scale methods for the analysis of metabolites are based on gas chromatography (GC)-mass spectrometry (MS) (7), liquid chromatography-MS (31), nuclear magnetic resonance (20), and Fourier transform ion cyclotron resonance-MS (1). Recently, capillary electrophoresis-electrospray ionization (CE-ESI)-MS has emerged as a powerful analytical tool, and a number of CE-ESI-MS methods have been developed for the analysis of charged species such as carboxylic acids, phenolic compounds, amino acids, metal species, tetramines, and herbicides (25). While metabolite-profiling analysis of the glutathione synthesis pathway in baker's yeast was recently reported (15), to our knowledge, the metabolomic analysis of bottom-fermenting yeast has not yet been reported.

The physiology of sulfur metabolism in *Saccharomyces* yeasts, particularly in regard to SO_2 and H_2S production, has received significant attention (11, 17, 28). Although the biosynthesis of these two compounds is interconnected, it would be desirable to increase SO_2 and to decrease H_2S in beer. Yeast strains that produce lowered levels of H_2S have been

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▽ Published ahead of print on 29 February 2008.

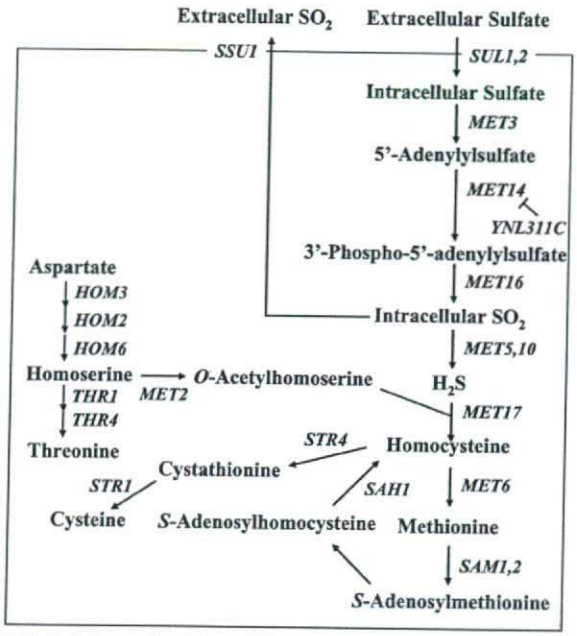

FIG. 1. Schematic of the reductive sulfate assimilation pathway in yeast. *YNL311C* encodes an F-box protein (13). Ynl311c negatively regulates Met14 and degrades it via a ubiquitin-related pathway.

constructed or isolated by gene disruption, conventional mutagenesis, and cell fusion techniques. However, those treatments have also resulted in undesirable phenotypes (11). Mutants that produce higher levels of SO_2 have been obtained by the overexpression of *SSU1* and/or *MET14* (4) and by the disruption of *MET10* (11). However, bottom-fermenting yeast strains in which *MET10* was disrupted were also found to have acquired undesirable phenotypes (11), possibly due to a reduction in intracellular methionine or slow growth. Furthermore, because these yeasts were obtained by the use of recombinant DNA technology, they cannot be used commercially.

In this paper, we report integrated metabolome and transcriptome analysis of reductive sulfate assimilation in baker's and bottom-fermenting yeasts. Based on these data, we proposed that OAH is one of the rate-limiting factors for SO_2 and H_2S production, and we constructed high-level SO_2-producing mutants without increasing H_2S levels by simultaneously increasing the flux from aspartate to OAH and increasing the flux from sulfate to SO_2. One candidate mutant that produced much higher levels of SO_2 than the parent, parental levels of H_2S, and no undesirable changes in fermentation properties was isolated.

MATERIALS AND METHODS

Strains and media. Bottom-fermenting yeast *S. pastorianus* strains used in this study were KBY011 (our laboratory stock), YMO106 (mutant selected from KBY011), and B43 (meiotic segregant of KBY011). Baker's yeast *S. cerevisiae* strains used were S288C (*MAT*α) and SYT001 (*MAT***a**; derived from DBY7286). Strains were grown in YPD (1% yeast extract, 2% peptone, and 2% glucose), YPD10 (1% yeast extract, 2% peptone, and 10% glucose), SD10 (0.4% yeast nitrogen base without amino acids and ammonium sulfate, 0.2% ammonium sulfate, and 10% glucose), SD10(2) (0.2% yeast nitrogen base without amino acids and ammonium sulfate, 0.1% ammonium sulfate, and 10% glucose), or Brewer's wort. Strains and media used in each experiment are shown in Table 1. YPDL plates (0.5% yeast extract, 0.3% peptone, 4% glucose, 0.02% ammonium sulfate, 0.1% lead nitrate, and 2% agar) were used to test for H_2S production (9). SDLE plates (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 2% glucose, 0.1% lead nitrate, 10 mg/ml DL-ethionine, and 2% agar) and SDH medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 2% glucose, and 100 mg/ml of DL-hydroxynorvaline) were used to isolate mutants. Geneticin (G418) at 200 μg/ml, blasticidin S at 50 μg/ml, or aureobasidin A at 1 μg/ml was added to YPD plates to select and screen transformants. Transformation of yeast strains was carried out by use of a modified gene pulser procedure (16).

Isolation of meiotic segregant from bottom-fermenting yeast. Meiotic segregants were obtained from KBY011 as described previously by Bilinski et al. (2). With respect to SO_2 and H_2S production, the meiotic segregant B43 is similar to KBY011.

Isolation of mutants resistant to amino acid analogs. Wild-type cells were incubated in 5 ml of YPD at 20°C for 3 days. After centrifugation, cells were resuspended in 5 ml of distilled water. One hundred fifty microliters of cells was spread onto SDLE plates and incubated at 20°C for 10 days. Ethionine-resistant mutants were incubated in 5 ml of YPD at 20°C for 3 days. After centrifugation, the cells were resuspended in 10 ml of distilled water. One hundred microliters of cells was added to SDH and incubated at 20°C for 5 days. One hundred microliters of culture was spread onto YPDL plates, which were then incubated at 20°C for 7 days.

Construction of overexpression plasmids. To construct pYES-G418GP, a 1.5-kb SalI fragment containing a *GPD* promoter and *PGK1* terminator cassette derived from pSY114 (32) was inserted at the SalI site of pYES-G418 (34). To construct plasmids overexpressing *S. cerevisiae MET14* (Sc*MET14*), Sc*HOM3*, Lg*HOM3*, Lg*MET17*, and Lg*THR1*, these genes were cloned by PCR using KBY011 genomic DNA and inserted at the BamHI site of pYES-G418GP. To construct the integration vector pAUR-HOM3, Sc*HOM3* was inserted into the BamHI site of pSY114, and a 3-kb *GPD* promoter-Sc*HOM3-PGK* terminator cassette was inserted at the SalI site of pAUR101 (Takara Shuzo) carrying an aureobasidin A resistance marker. Integration of the Sc*HOM3* gene was confirmed by Southern hybridization analysis.

Construction of disruption plasmids. To disrupt both Lg and *S. cerevisiae* types of *YNL311C* and *HOM3* genes in B43, the Lg*YNL311C* gene was cloned from a KBY011 cosmid library based on homology with the corresponding *S. cerevisiae* genes. To construct a vector harboring a blasticidin S resistance cassette, a 0.5-kb HindIII fragment carrying blasticidin S resistance was inserted at the HindIII site of pSY114 carrying a *GPD* promoter and a *PGK* terminator. This blasticidin S resistance expression cassette was then integrated into the open reading frames of the Lg*YNL311C* and Lg*HOM3* genes, respectively. The corresponding *S. cerevisiae*-type genes were disrupted by DNA fragments amplified using genomic DNA derived from the yeast knockout strains (Open Biosystems). Disruption of both types of genes was confirmed by PCR.

Growth conditions for comprehensive analyses. KBY011 and S288C were precultured with shaking at 20°C for 3 days in 500 ml of YPD10. Cells were harvested and diluted to a density of 0.5% (wt/vol) in 2 liters of fresh SD10 and grown with gentle stirring at 20°C for 3 days under anaerobic conditions produced by initial headspace exclusion and N_2 flushing. Cells were then harvested, diluted to a density of 0.5% (wt/vol) in 2 liters of the fresh SD10, and grown at 20°C under anaerobic conditions with gentle stirring (experiment 1). To test the effect of the addition of threonine, SYT001 cells were harvested and diluted to a density of 0.5% (wt/vol) in 2 liters of fresh SD10(2) with or without 1 g/liter of threonine and grown at 20°C under anaerobic conditions with gentle stirring. Cells were then collected at different time points (0, 6, 24, and 48 h) and harvested for metabolome analysis (experiment 2).

Growth conditions for small-scale fermentation. B43, KBY011, and YMO106 were precultured with shaking at 20°C for 3 days in 500 ml of YPD10 (200 mg/liter of G418 was added in experiment 3). Cells were harvested, diluted to a

TABLE 1. Strains and media used in this study

Expt	Strain(s)	Medium
1	S288C and KBY011	SD10
2	SYT001	SD10(2) with or without threonine
3	B43 and derivatives	YPD10 + G418
4	B43 and derivatives	YPD10
5	KBY011 and YMO106	Wort

density of 0.5% (wt/vol) in 200 ml of fresh YPD10 (experiments 3 and 4) or 500 ml of wort (experiment 5), and grown at 20°C for 4 days under anaerobic conditions produced by initial headspace exclusion and N_2 flushing. Cells were then harvested, diluted to a density of 0.5% (wt/vol) in 200 ml of fresh YPD10 or 500 ml of wort, and grown with gentle stirring at 20°C under anaerobic conditions.

DNA microarray analysis. DNA microarray experiments were carried out using bottom-fermenting yeast oligoarrays (Agilent DNA microarray system). Oligonucleotide probes (60 bp) were spotted onto the array, which carries 3,181 probes derived from Lg-type genes and 6,637 probes derived from *S. cerevisiae* genes. Total RNA was extracted using glass beads (21) and purified using an RNeasy column (Qiagen), and 0.2 μg of RNA was labeled using the Agilent linear amplification/labeling kit (Agilent Technologies) according to the manufacturer's instructions. Hybridization of labeled cRNA to the arrays was performed using the manufacturer's hybridization protocol. Microarrays were washed, dried, and scanned on a dual-laser DNA microarray scanner (model G2565BA; Agilent Technologies). Feature Extraction and Image Analysis software programs were used. Normalization was carried out by the Lowess method. For each experiment, the data presented are hybridization means for two arrays in two-dye swap experiments (i.e., Cy3 and Cy5 dye-swapping experiments).

Extraction of intracellular metabolite. Metabolites were extracted using a modification of a previously described procedure (25). Cells were harvested from a culture medium (optical density at 600 nm of 30) by filtration through a 0.45-μm-pore-size filter. Methionine sulfone and 2-morpholinoethanesulfonic acid (MES) were used as internal cationic and anionic standards, respectively. Lyophilized samples were dissolved in 50 μl of Milli-Q water before CE-ESI-MS analysis.

CE-ESI-MS conditions for metabolite analysis. All CE-ESI-MS experiments were performed using an Agilent capillary electrophoresis system equipped with an air pressure pump, an Agilent 1100 series MSD mass spectrometer and an isocratic high-performance liquid chromatography (HPLC) pump, a G1603A Agilent CE-MS adapter kit, and a G1607A Agilent CE-ESI-MS sprayer kit (Agilent Technologies) as described previously (23, 24).

Other analyses. The supernatants from filtered samples were assayed for free SO_2 and organic acids by HPLC. H_2S was detected quantitatively using a headspace GC-sulfur chemiluminescence detector system. Higher alcohols, esters, acetaldehyde, and diacetyl were measured by GC.

Statistical analysis. Each metabolomic experiment (experiments 1 and 2) was done in triplicate in independent experiments, and data were analyzed by using mean values from triplicate samples, except for SO_2 and H_2S. The data below the detection limit were not included for statistical analysis. In experiments involving overproduction of Met14 and Hom3 in B43, significant differences in SO_2 and H_2S production levels between the two strains were compared using Student's *t* test (ystat2006.xls; S. Yamazaki, Igaku Tosho Press, Inc., Japan).

Microarray data accession number. The microarray data are registered in the ArrayExpress (EBI) database under accession number E-MEXP-1086.

RESULTS

Metabolite and gene expression profiling analyses of sulfur metabolism in bottom-fermenting and baker's yeasts. For unknown reasons, bottom-fermenting yeast produces high levels of SO_2 and H_2S under anaerobic conditions, while baker's yeast does not (26). To determine the basis for this difference, we carried out transcriptome and metabolome analyses. SD10 medium lacking amino acids was used to minimize complications resulting from amino acid uptake. Most intracellular metabolites related to sulfate assimilation were measured by CE-ESI-MS, while SO_2 was analyzed by HPLC, and H_2S was analyzed by GC. Figure 2 shows levels of sulfur metabolites in cell extracts and SO_2 and H_2S in media. Because a commercial 3′-phospho-5′-adenylylsulfate standard was not available, 3′-phospho-5′-adenylylsulfate was quantified relative to the internal standard MES. As reported above, the production of extracellular SO_2 and H_2S by bottom-fermenting strain KBY011 was much greater than that by baker's yeast *S. cerevisiae* S288C. In contrast, levels of intracellular OAH and homoserine were much lower in KBY011 than in S288C. Figure 3 shows the expression profiles of genes involved in sulfur metabolism in S288C and KBY011. We constructed the bottom-fermenting yeast microarray based on the expressed sequence tag data for KBY011 (33), because the whole-genome sequence of bottom-fermenting yeast was not available at the time of this writing. Graphs for which a KBY011 data set are missing indicate that the respective probes were unavailable in the expressed sequence tag data. The expression patterns of the *MET2*, *MET14*, and *MET16* genes were found to differ between S288C and KBY011. Furthermore, the expression levels of both the Lg- and *S. cerevisiae*-type *HOM3* genes were much lower in KBY011 than in S288C in the early stage of fermentation. Based on the metabolome and transcriptome data, we suggest that KBY011 has a lower level of OAH than S288C, probably due to the low level of expression of *HOM3* or related enzyme activities. We presume that KBY011 may produce higher levels of SO_2 and H_2S than S288C due to limiting amounts of OAH, which reacts with H_2S to form homocysteine.

OAH is rate limiting for H_2S production. To test the hypothesis that OAH is a rate-limiting factor for SO_2 and H_2S production, we performed the experiments described below. It was reported previously that in yeast, threonine inhibits aspartate kinase encoded by *HOM3* through feedback inhibition (6) and that the addition of threonine increases SO_2 production (10), suggesting that the addition of threonine would shift the pattern of sulfate assimilation in baker's yeast to that observed in bottom-fermenting yeast. Therefore, metabolome analysis of baker's yeast strain SYT001 grown in SD10(2) was performed with and without added threonine. As expected, the addition of threonine caused elevated levels of SO_2 and H_2S production, especially 24 h after the addition (Fig. 4). The metabolome data show that homoserine and OAH levels were lower in the presence of added threonine than in the absence of threonine (Fig. 4). These results are consistent with threonine feedback-inhibiting aspartate kinase leading to a decrease in OAH production and a resulting increase in SO_2 and H_2S levels.

Moreover, the effect of the addition of OAH on H_2S production was tested on YPDL plates using KBY011. Figure 5 shows that threonine increased H_2S production, while homoserine and OAH decreased H_2S production at a final concentration of 1 mM. These results strongly suggest that OAH is a rate-limiting factor for H_2S production.

Effect of overexpression and disruption of genes involved in sulfate assimilation. As noted above, OAH appears to be the rate-limiting factor for SO_2 and H_2S production. SO_2 and H_2S production are linked (5), as SO_2 is the immediate biochemical precursor of H_2S in the reductive sulfate assimilation pathway (Fig. 1), indicating that flux from sulfate to SO_2 is important for both SO_2 and H_2S production. Therefore, the effects of genetically altering the flux from aspartate to OAH and the flux from sulfate to SO_2 on SO_2 and H_2S production were investigated using relevant overexpressed and disrupted genes. As bottom-fermenting yeast is proposed to be a tetraploid, meiotic segregants expected to each have a set of *S. cerevisiae*-type and Lg-type genes from KBY011 were isolated to make the experiment more tractable. One of them, B43, exhibited sulfur metabolism and a fermentation profile similar to those of parent strain KBY011 (data not shown).

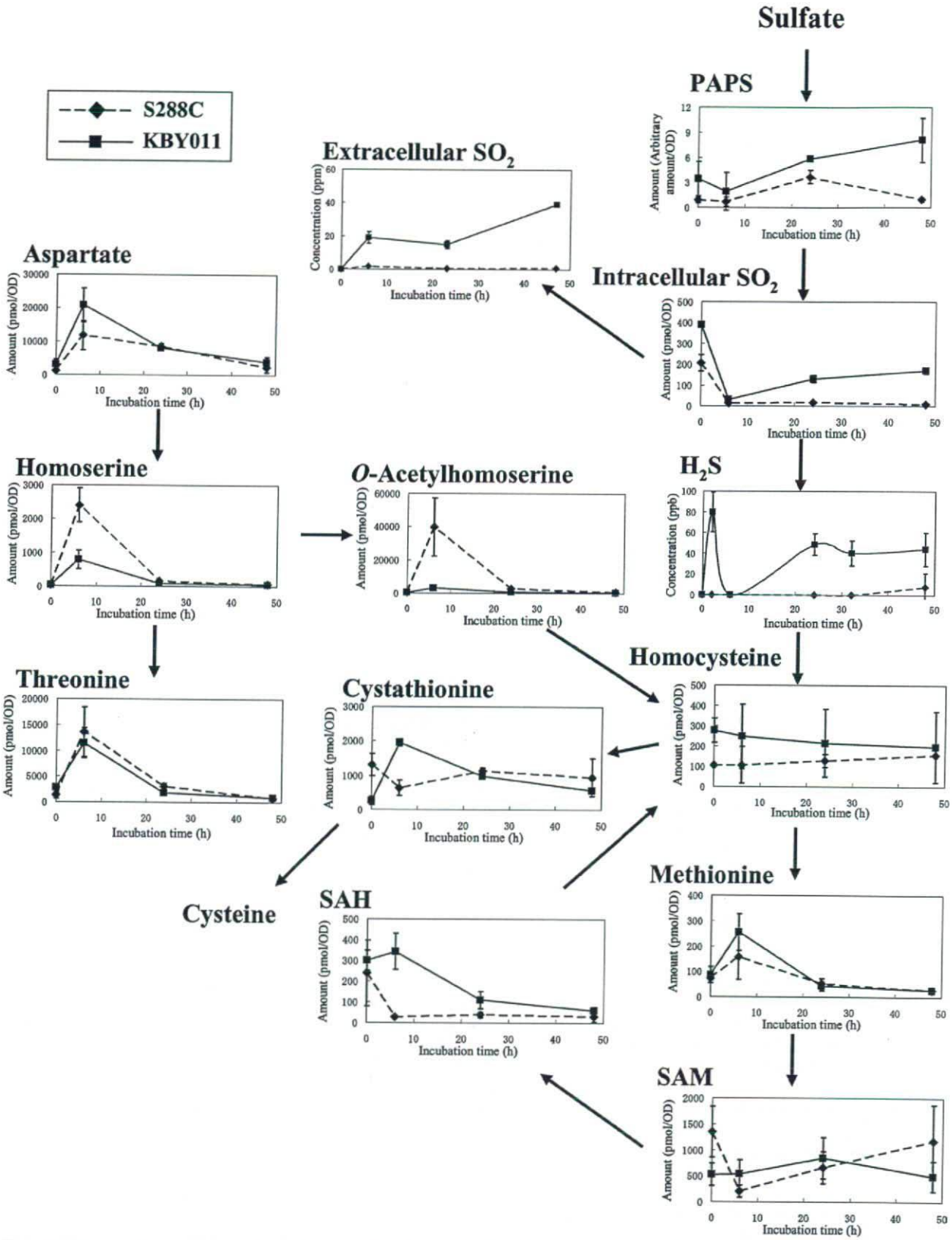

FIG. 2. Changes in metabolite pools related to reductive sulfate assimilation in bottom-fermenting and baker's yeasts. Extracellular SO_2 and H_2S levels are concentrations in the spent media. Concentrations of the other metabolites (pmol) are expressed per unit yeast biomass (an amount of cells equivalent to an optical density [OD] at 600 nm of 1). The data are means of three independent experiments, with the error bars indicating standard deviations. Diamonds and squares indicate baker's yeast (S288C) and bottom-fermenting yeast (KBY011), respectively (experiment 1). SAH, *S*-adenosylhomocysteine; PAPS, 3′-phospho-5′-adenylylsulfate.

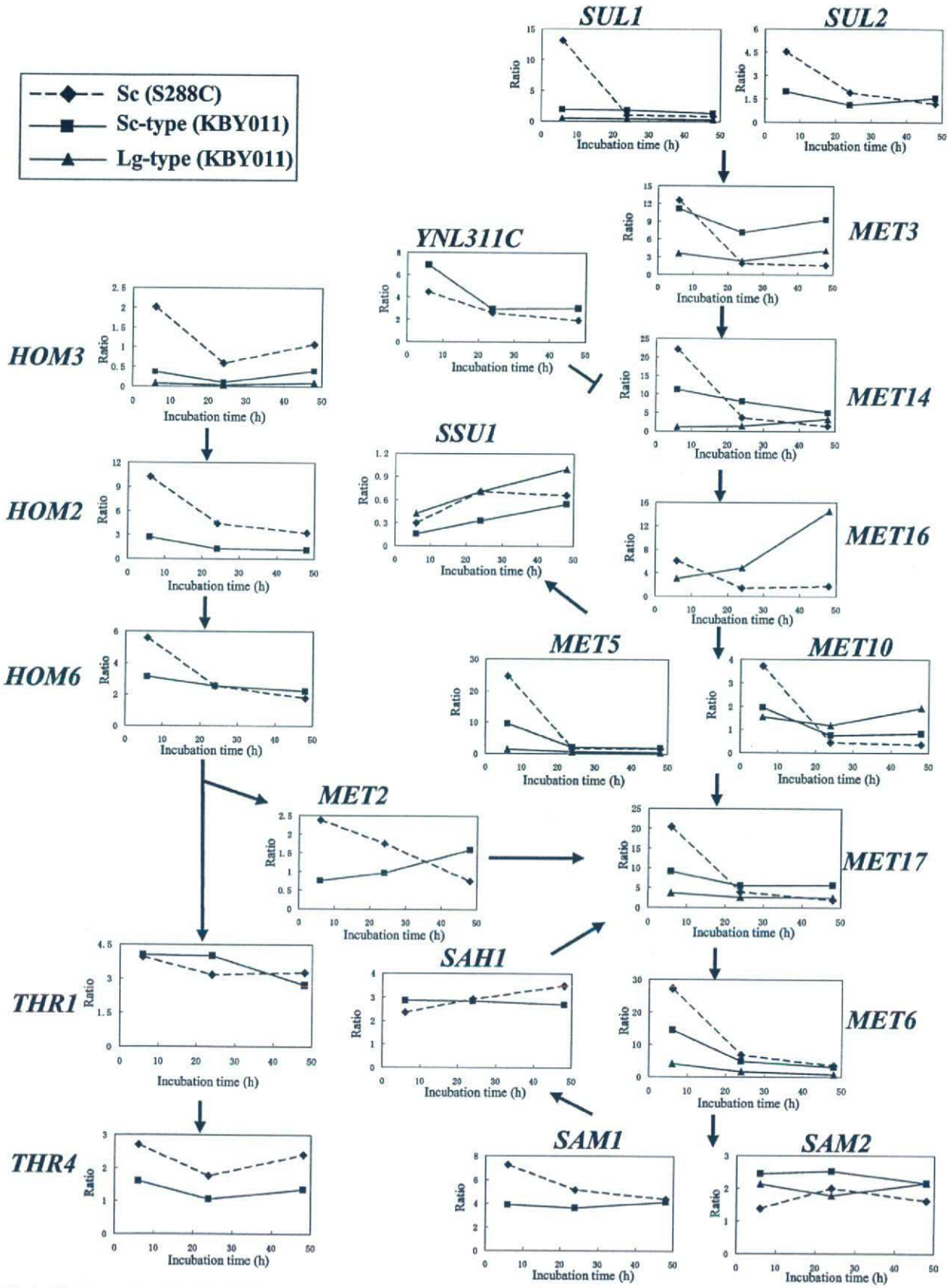

FIG. 3. Changes in expression levels of genes involved in reductive sulfate assimilation in bottom-fermenting and baker's yeasts. Diamonds, squares, and triangles indicate baker's yeast, bottom-fermenting yeast *S. cerevisiae* (Sc)-type, and bottom-fermenting yeast Lg-type genes, respectively (experiment 1). The *y* axis is the expression ratio relative to the zero time point. These microarray data are means of analyses taken from two independent fermentation experiments with very similar results (experiment 1).

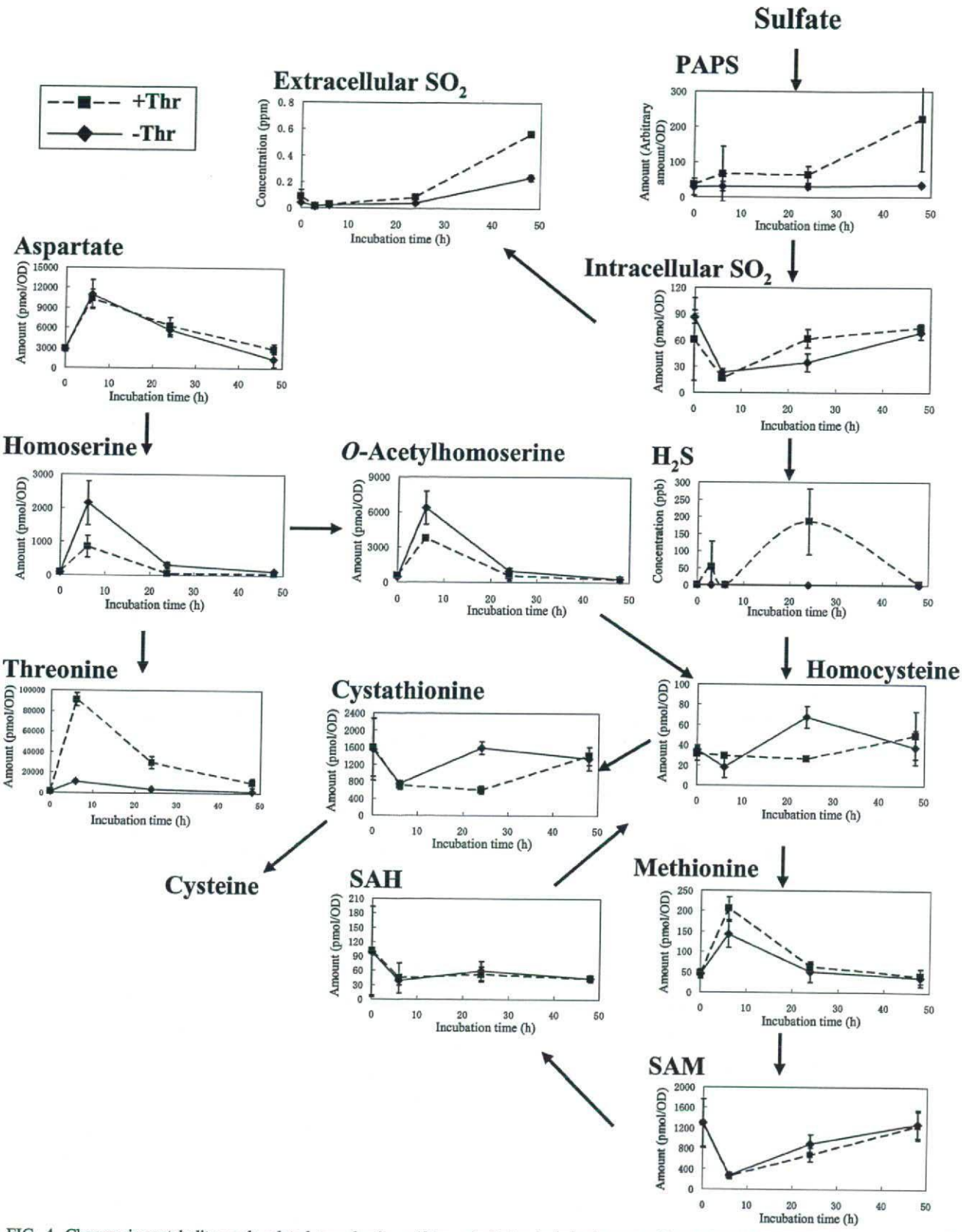

FIG. 4. Changes in metabolite pools related to reductive sulfate assimilation in baker's yeast with or without threonine. Concentrations of metabolites are indicated as described in the Fig. 2 legend. The data represent mean values from three independent experiments, with error bars showing standard deviations. Squares and diamonds indicate baker's yeast with and without the addition of threonine, respectively (experiment 2). SAH, *S*-adenosylhomocysteine; PAPS, 3′-phospho-5′-adenylylsulfate; OD, optical density.

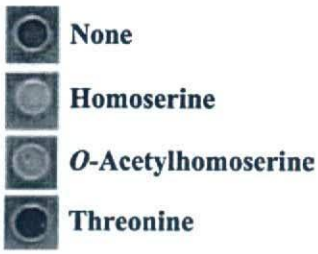

FIG. 5. Effect of OAH on H_2S production in bottom-fermenting yeast. All compounds shown were added to YPDL plates at a final concentration of 1 mM. Cells of bottom-fermenting yeast strain KBY011 were incubated at 25°C on YPDL plates for 4 days.

Initially, in order to regulate the flux from aspartate to OAH, a B43 strain overexpressing Lg*HOM3*, Lg*MET17*, or Lg*THR1* was constructed. The levels of SO_2 and H_2S of the Lg*HOM3*-overexpressing B43 strain were found to be 1.1- and 3.5-fold lower than those of the control, respectively (Table 2). A *hom3* disruptant was also constructed and evaluated. SO_2 and H_2S levels in the *hom3* disruptant were 2.6- and 2.8-fold higher than those of the parent, respectively (Table 3). These results indicate that the flux from aspartate to OAH has more significant effects on H_2S production than on SO_2 production.

To alter the flux from sulfate to SO_2, a Met14-overproducing strain was constructed by disrupting *YNL311C*. Ynl311c has an F-box motif, interacts with Met14 (13), and is involved in its proteolysis via the ubiquitin pathway (S. Yoshida et al., unpublished results). The levels of SO_2 and H_2S in the *ynl311c* disruptant were found to be 2.5- and 1.6-fold higher, respectively, than those in the controls (Table 3). This indicates that the flux from sulfate to SO_2 has a greater effect on SO_2 production than on H_2S production.

Based on these results, strains producing high SO_2 and low H_2S levels were sought by simultaneously increasing the flux from aspartate to OAH and increasing the flux from sulfate to SO_2.

Breeding of bottom-fermenting yeast by genetic modification and spontaneous mutants resistant to both methionine and threonine analogs. Strain B43, which overproduced both Hom3 and Met14, produced almost same amount of H_2S as the parent strain on YPDL plates (data not shown). The levels of SO_2 and H_2S after the overproduction of Met14 and Hom3 were 2.68 ± 0.22 ppm and 165.4 ± 11.8 ppb, respectively, for the parent strain and 3.86 ± 0.50 ppm ($P < 0.05$) and 131.8 ± 13.3 ppb ($P < 0.05$), respectively, for the Δ*ynl311c* Sc*HOM3* strain, as measured after 24 h (means and standard deviations from three independent experiments [experiment 3] are shown). Similarly, with respect to the simultaneous overexpression of both Sc*MET14* and Sc*HOM3*, the levels of SO_2 and H_2S of the control strain were 1.57 ± 0.56 ppm and 113.1 ± 19.2 ppb, respectively, and those of the strain overexpressing both Sc*MET14* and Sc*HOM3* were 2.55 ± 0.65 ppm ($P < 0.05$) and 88.4 ± 17.4 ppb ($P < 0.05$), respectively, as measured after 23 h (values are means and standard deviations from five independent experiments [experiment 3]). Significant differences between the parent and genetically modified strains were determined by a Student's *t* test. These results indicate that high SO_2 and low H_2S production can be achieved by simultaneously increasing the flux from aspartate to OAH and increasing the flux from sulfate to SO_2.

TABLE 2. Effect of gene overexpression on SO_2 and H_2S production in strain B43[a]

Gene	SO_2 level (ppm)	H_2S level (ppb)
Vector	2.51	118.3
Lg*HOM3*	2.27	33.5
Lg*MET17*	2.24	0
Lg*THR1*	2.58	143.5

[a] SO_2 and H_2S levels were measured after 23 h. The data represent means for two independent experiments (experiment 3).

TABLE 3. Effect of gene disruption on SO_2 and H_2S production in strain B43[a]

Genotype	SO_2 level (ppm)	H_2S level (ppb)
Parent	1.55	102
Δ*hom3*	3.97	290
Δ*ynl311c*	3.89	166

[a] SO_2 and H_2S levels were measured after 24 h (experiment 4).

In order to isolate a spontaneous mutant exhibiting similar metabolic fluxes for commercial use, strains resistant to both methionine and threonine analogs were selected. Initially, we isolated ethionine-resistant mutants from KBY011, whose flux from sulfate to H_2S would be expected to increase. From among 1.4×10^8 cells, 14 ethionine-resistant candidates that formed black colonies on YPDL plates were isolated (Fig. 6A). SO_2 and H_2S productivities were analyzed in three of them. One, YMO2, produced 2.2-fold more SO_2 and 2.4-fold more H_2S than did the parent strain in YPD10 medium. Hydroxynorvaline-resistant mutants of YMO2 were then selected in order to increase the flux from aspartate to OAH. From among 10^8 cells, 10 candidates that formed thin brown colonies were isolated (Fig. 6A). One candidate, YMO106, produced 2.7-fold more SO_2 than did parent strain KBY011 but produced the same amount of H_2S (1.04-fold more) in wort after 24 h of incubation (Fig. 6B). No differences in H_2S accumulation were observed between KBY011 and YMO106 using YPDL plates (Fig. 6A). These results suggest that mutants that produce much higher SO_2 levels but without a significant increase in H_2S levels can be isolated by simultaneously increasing the flux from aspartate to OAH and increasing the flux from sulfate to SO_2. An independently isolated ethionine- and hydroxynorvaline-resistant mutant from a different industrial strain was also found to produce a high level of SO_2 and a low level of H_2S (data not shown). This mutant exhibited almost the same SO_2 and H_2S productivities as the *HOM3*- and *MET14*-overexpressing strain, demonstrating that mutants that produce higher SO_2 and lower H_2S levels can be isolated by the selection of mutants that are resistant to the two-amino-acid analogs.

Lager fermentations using strains KBY011 and YMO106 in 200-liter batches of wort were performed. Chemical analysis of the resultant beers is summarized in Table 4. With respect to SO_2, the beer produced by YMO106 (YMO106 beer) contained much higher levels of SO_2 than that produced by KBY011 (KBY011 beer) at all sampling times. At the end of

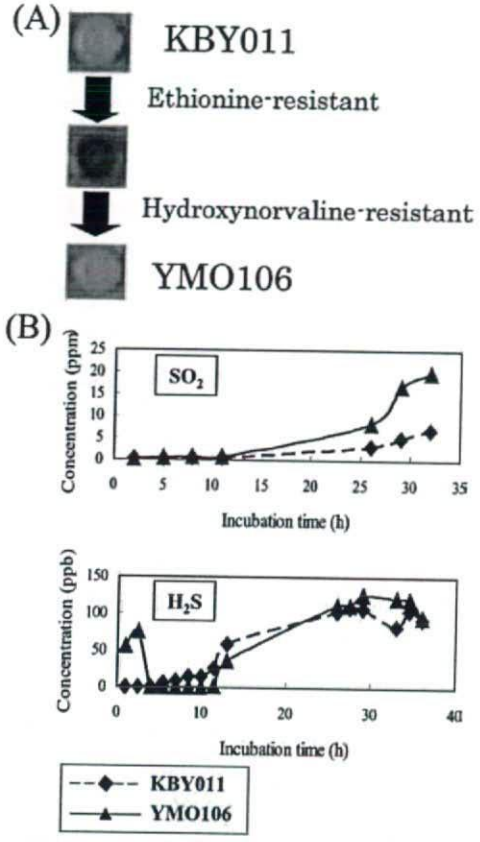

FIG. 6. SO_2 and H_2S production in the spontaneous mutant. (A) Strategy for isolating mutant strain YMO106 from parental strain KBY011. Yeast cells were incubated at 20°C on YPDL plates for 5 days. (B) Extracellular SO_2 and H_2S concentrations in media are shown. Diamonds and triangles indicate parental strain KBY011 and mutant strain YMO106, respectively (experiment 5).

the main fermentation, the YMO106 beer contained less H_2S than did the KBY011 beer, while the opposite was true after a period of maturation. It is more important to decrease H_2S during the main fermentation than during beer maturation, because undesirable compounds derived from H_2S are produced during the main fermentation. The increase in H_2S in the YMO106 beer would be expected to disappear upon longer maturation. The YMO beer contained about the same amount of organic acids and volatile compounds as did the KBY011 beer (diacetyl, acetaldehyde, esters, and fusel alcohols), suggesting that the introduced mutations did not cause undesirable changes in the concentrations of compounds having a major sensory impact in beer (Table 4).

DISCUSSION

Metabolomics, the global analysis of cellular metabolites, is becoming a powerful new tool for gaining insight into biological functions. Analysis of a number of metabolites and tracking concentration changes under various physiological and genetic conditions should provide direct information on metabolic phenotypes complementary to gene expression. Metabolome and transcriptome approaches in this work revealed three main insights. First, OAH is the rate-limiting factor for the production of H_2S. Second, the flux from aspartate to OAH has a greater impact on H_2S production than on SO_2 production, while flux from sulfate to SO_2 has a greater impact on SO_2 production than on H_2S production. Third, the simultaneous increase in the flux from aspartate to OAH and the flux from sulfate to SO_2 resulted in high levels of SO_2 production with no increase in H_2S formation relative to that of the parent.

In order to increase SO_2 and to lower H_2S production, we compared the formations of these metabolites in bottom-fermenting and baker's yeasts. Metabolome data indicated that the bottom-fermenting species produced less OAH, a homocysteine precursor, than did *S. cerevisiae*. One reason for this reduced amount of OAH is possibly due to lower expression levels of *HOM3* in bottom-fermenting yeast, which causes higher SO_2 and lower H_2S productivity, suggesting that OAH is the rate-limiting factor in SO_2 and H_2S production.

Alternative approaches to developing yeast strains that produce higher SO_2 and lower H_2S levels have been proposed. One is the disruption of genes encoding SO_2 reductase subunits or other proteins required for SO_2 reductase function, including *MET5*, *MET10*, *MET1*, and *MET8*. Hansen and Kielland-Brandt reported previously that a *MET10* disruption resulted in high levels of SO_2 production (11). Considering the function of *MET10*, one would have expected lower H_2S levels as well. The overexpression of *SSU1*, encoding a plasma membrane SO_2 efflux pump, has also been proposed to be a means of reducing H_2S levels. The use of a sulfite reductase mutant or a mutant that exports a significant amount of SO_2 in raw

TABLE 4. Levels of SO_2, H_2S, and selected aroma compounds in beer after the main fermentation and in bottled beer after maturation produced by strain KBY011 (parent) and derivative strain YMO106[a]

Compound	Concn														
	SO_2 (fermentation tank) (ppm)	SO_2 (bottled beer) (ppm)	H_2S (fermentation tank) (ppb)	H_2S (bottled beer) (ppb)	Total diacetyl (ppm)	Acetaldehyde (ppm)	Ethyl acetate (ppm)	Amyl alcohol (ppm)	Isoamyl acetate (ppm)	Sulfate (ppm)	Maleic acid (ppm)	Citric acid (ppm)	Succinic acid (ppm)	Lactic acid (ppm)	Acetic acid (ppm)
KBY-1	1.5	1.3	49.0	2.6		7.0	23.8	70.4	2.0	89	96	204	119	97	89
YMO-1	2.2	3.7	30.3	2.0		4.1	18.3	97.4	1.8	100	109	199	116	106	57
KBY-2	2.5	1.6	27.1	2.9	0.04	4.0	21.3	85.0	1.8	110	104	190	85	92	112
YMO-2	7.1	4.2	25.7	3.3	0.04	5.0	23.9	78.2	2.2	99	92	207	96	107	133

[a] KBY-1 and YMO-1 indicate beer produced in the first fermentation by KBY011 and YMO106, respectively. KBY-2 and YMO-2 indicate beer produced in the second fermentation by KBY011 and YMO106, respectively.

materials such as wort that has a limited amino acid content is likely to lead to limited growth due to the depletion of methionine and cysteine. In contrast, in the *HOM3*- and *MET14*-overexpressing strain, intracellular methionine or cysteine was sufficient, as evidenced by the ability of this strain to grow in unsupplemented minimal SD medium. It is possible that the combined overexpression of *SSU1* and *MET14* would work as well as overexpression of *HOM3* and *MET14*. Nonetheless, all the strains described above have been genetically manipulated and therefore are inappropriate for commercial use.

With commercial use in mind, we selected spontaneous mutants that were resistant to both ethionine (methionine analog) and hydroxynorvaline (threonine analog) to obtain a high-level SO_2-producing strain that did not produce increased levels of H_2S. Strain YMO106, which produced higher SO_2 levels but parental levels of H_2S, was selected in this screen. The bottom-fermenting yeast strain in which all *MET10* genes were disrupted produced 13-fold more SO_2 but 5-fold more acetaldehyde, 3-fold more 1-propanol, and 1.7-fold more dimethyl sulfide (DMS) than the parental strain (11). As shown in Table 4, strain YOM106 obtained in this study produced 2.8- and 2.6-fold more SO_2 (fermentation tank and bottled beer produced in the second fermentation, respectively), 1.3-fold more acetaldehyde, and 0.9- and 1.1-fold more H_2S than the parental strain (fermentation tank and bottled beer produced in the second fermentation, respectively). Beer produced by YMO106 was very similar to that produced by the parental strain except for the higher level of SO_2. These results indicate that selection for resistance to amino acid analogs, based on the integration of the metabolome and transcriptome data, was a successful approach for obtaining a high-level SO_2-producing strain. To our knowledge, this is the first report to integrate metabolome and transcriptome data for the development of improved industrial yeast strains.

It is possible that the mutation responsible for ethionine resistance in YMO106 is in *SAM1* or *SAM2*, both of which are involved in the transcriptional regulation of methionine biosynthesis (3, 22), in the methionine permease *MUP1* (12), or in *STR4*, which encodes cystathionine β-synthase (Fig. 1). Previous screening of the yeast gene knockout collection for H_2S-overproducing mutants led to the identification of *mup1* and *str4* deletion mutants (Yoshida et al., unpublished). Mutations in *SAM1* or *SAM2* would also be expected to result in a loss of function because SAM represses the transcription of the methionine biosynthetic genes including *MET3* and *MET14*. However, the *sam1* and *sam2* mutants were not found to overproduce H_2S, possibly because wild-type *SAM1* and *SAM2* complemented *sam2* and *sam1*, respectively, in the single mutants. Nonetheless, it is possible that *SAM1* and *SAM2* might be repressed simultaneously in YMO106. On the other hand, a mutation in *HOM3* might be responsible for hydroxynorvaline resistance, because *HOM3-R2* dominant mutants have been reported to exhibit hydroxynorvaline resistance in *S. cerevisiae* (19). However, as the hydroxynorvaline resistance of YMO106 is weak, it is possible that the mutation is not in *HOM3*. It is possible that the mutation responsible for hydroxynorvaline resistance is in genes involved in threonine uptake or in other genes including *HOM2*, *HOM6*, *MET2*, *MET17*, *THR1*, and *THR4*.

DMS, dimethyl disulfide, methional, and methionol were measured in bottled beers. Relative to KBY011, YMO106 was found to produce 1.6-fold more DMS, 0.6-fold less dimethyl disulfide, 0.6-fold less methional, and 1.5-fold more methionol, indicating minor consequences of the introduced mutation on the methionol synthesis and methionine degradation pathway. Significantly, these values are below taste threshold levels.

Finally, we report herein a method to identify rate-limiting factors in a metabolic pathway by the integration of transcriptome and metabolome data, followed by genetic and nongenetic metabolic engineering to increase desirable end products in yeast. This method should have general application to the problem of increasing the production of useful metabolites while minimizing the simultaneous production of undesirable but related compounds derived from a linked pathway.

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