Me₂SO is a polar solvent that is widely used in biochemical, pharmacology, and industry. Although there are several reports in the literature concerning the biological effects of Me₂SO, the total cellular response remains unclear. In this paper, DNA microarray technology combined with the hierarchical clustering bioinformatics tool was used to assess the effects of Me₂SO on yeast cells. We found that yeast exposed to Me₂SO increased phospholipid biosynthesis through up-regulated gene expression. It was confirmed by Northern blotting that the level of INO1 and OPI3 gene transcripts, encoding key enzymes in phospholipid biosynthesis, were significantly elevated following treatment with Me₂SO. Furthermore, the phospholipid content of the cells increased during exposure to Me₂SO as shown by conspicuous incorporation of a lipophilic fluorescent dye (3,3′-dihexyloxacarbocyanine iodide) into the cell membranes. From these results we propose that Me₂SO treatment induces membrane proliferation in yeast cells to alleviate the adverse effects of this chemical on membrane integrity.

Dimethyl sulfoxide (Me₂SO)¹ is widely used as a solvent in the chemical industry and as a cryoprotectant in biotechnology. It is present in the environment as a waste product of the paper industry and from the production of dimethyl sulfoxide (DMS) and also arises from the degradation of sulfur-containing pesticides (1). In addition, Me₂SO forms naturally from photooxidation of DMS in the atmosphere and from degradation of DMS by phytoplankton in the marine environment (2). Because Me₂SO has low volatility and is highly hygroscopic, it is rapidly scavenged from the atmosphere by rain and returned to earth and thereby plays a role in the global sulfur cycle (1, 3).

Dimethyl sulfoxide reductase from *Rhodobacter sphaeroides* catalyzes the reduction of Me₂SO to DMS (7–9). This enzyme, which contains a molybdenum cofactor at the active center, is well characterized at the biochemical, biophysical, and molecular level and provides an excellent model system for investigating the structure and mechanism of electron transfer chain complexes (1, 10). *Saccharomyces cerevisiae* has a number of NADPH-dependent enzymes that, in conjunction with methionine sulfoxide reductase (MXR1), can reduce Me₂SO to DMS (11).

In *E. coli*, the combination of divalent cations as Ca²⁺ and Me₂SO has been shown to stimulate the efficiency of DNA transfer into the cell (12). In the case of mammalian cells, it is reported that the transfection efficiency is increased by Me₂SO treatment after electroporation (13). The exact mechanism of how Me₂SO increases membrane permeability leading to DNA uptake is unclear.

In mammalian cells Me₂SO (2% (v/v)) can induce morphological changes, for example in mouse erythroleukemic cells (14), or cause differentiation, for example in neuroblastoma cells (15). Conversely, Me₂SO blocks the differentiation of myoblasts, adipocytes, and antibody-producing plasma cells (16–18). Thus, the effect of Me₂SO varies depending on the cell line and cell type. At the molecular level Me₂SO is known to affect RNA splicing and causes a shift toward the proximal pair of splice sites on pre-mRNAs carrying competing 5′-splice sites or competing 3′-splice sites (19).

In this paper we have examined the molecular response to Me₂SO exposure in yeast cells by studying global gene expression profiles using DNA microarrays. The expression of genes involved in phospholipid biosynthesis and cell wall organization was affected by Me₂SO treatment, and a comparison with other stress-inducing chemicals was performed using the bioinformatics tool, hierarchical cluster analysis. We carried out Northern blot analysis and phospholipid component analysis, and we quantified the amount of cellular membrane to assess the influence of Me₂SO on the yeast cell. We found that Me₂SO induces the expression of genes involved in phospholipid biosynthesis, and we demonstrate elevated phospholipid biosynthesis and cellular membrane proliferation. From these studies we conclude that Me₂SO damages cell membrane integrity, but the effects of this are mitigated in part by increased phospholipid biosynthesis.
Preparation of Total RNA and mRNA—Total RNA was extracted from Me$_2$SO-treated or non-treated cells by the hot phenol method (21). Poly(A)$^+$ RNA was purified from total RNA with an Oligotex-T30 mRNA purification kit (Takara Otsu, Shiga, Japan).

Preparation of Labeled cDNA—Fluorescently labeled cDNA probes were prepared from the mRNA pool by direct incorporation of fluorescent nucleotide analogues during the first-strand reverse transcriptase reaction. Each 20 μl of labeling solution consisted of 3–5 μg of poly(A)$^+$ RNA, 0.5 μg of oligo(dT) primer, 0.5 mM each of dATP, dCTP, and dGTP, 0.2 mM dTTP, 40 units of RNase inhibitor, 10 mM dithiothreitol, 4 μl of 5X reaction buffer provided with SuperScript TM II (Invitrogen). For estimation of the induced genes, cDNA made from poly(A)$^+$ RNA of the control culture was fluorescently labeled with Cy3-UTP and that of the Me$_2$SO-treated sample was labeled with Cy5-UTP (Amersham Biosciences) (normal method). For estimation of the repressed genes, cDNA made from poly(A)$^+$ RNA of the control culture was fluorescently labeled with Cy5-UTP and that of the Me$_2$SO-treated sample was labeled with Cy3-UTP (reverse method). Because the fluorescence of Cy5 is quenched more than that of Cy3, labeling was reversed in the control and Me$_2$SO-treated samples for a more precise estimation of gene repression. The solution was incubated at 70 °C for 5 min and 42 °C for several minutes, and 200 units of SuperScript II reverse transcriptase (Invitrogen) was added, and reverse transcription was performed at 42 °C for 2 h. Another 200 units of SuperScript II reverse transcriptase was added 1 h after initiating reverse transcription. The reaction was stopped by addition of EDTA. Any remaining RNA was dissolved by addition of 0.5 N NaOH for 1 h at 65 °C. After neutralization with 1 M Tris-HCl, pH 8.0, Cy3- and Cy5-labeled cDNA were mixed. The mixed cDNA probes were purified using Microcon YM-30 (Amicon Millipore).

Hybridization and Image Scanning of the DNA Microarray—Competitive hybridization was performed on a DNA microarray of yeast. The fluorescent intensity of the resulting image data was quantified using Genepix (version 3.2.12). The calculation and normalization of the data were performed using GeneSpring (Silicon Genetics, Redwood City, CA). To eliminate background noise, the median value of some genes (TE) included as negative controls were subtracted from the row values for each gene. The intensity of each gene was divided by its control channel value in each sample. If the control channel value was below 0.01, the data point was deleted. The 50th percentile of all measurements was used as a positive control for each sample; the measurement for each gene was divided by this synthetic positive control value. The bottom 10th percentile was used as a test for correct background subtraction. This was never less than the negative value of the synthetic positive control. The fluorescent intensity of each spot on the images was subtracted from each background, and the ratios of intensity Cy5/Cy3 were calculated and normalized with a median value.

To assess the variation of DNA microarray experiments, we labeled the mRNA samples eluted from the same culture with the fluorescent dyes, Cy3 or Cy5, respectively, and determined the expression ratio after competitive hybridization on a DNA chip. The expected level of experimental variation was estimated to be within ~2-fold, because the hybridization ratio varied between 2.0 and 0.5 (data not shown). We identified genes that had more than a 2.0-fold hybridization ratio for up-regulation and genes that had less than 0.5-fold hybridization ratio for the down-regulation. The experiments were independently repeated six times, and the genes with hybridization value ratios of more than 2.0-fold in at least five of the six experiments were considered to be up-regulated by Me$_2$SO. The experiments for down-regulation were independently repeated three times, and the genes with hybridization value ratios of less than 0.5-fold in at least two of the three experiments were considered to be down-regulated by Me$_2$SO treatment. The values for up-regulated genes were the average ratio from six independent culture experiments, and the ratio values averaged from three independent culture experiments were adopted in the case of down-regulated genes.

Cluster Analysis—To extract meaningful information from the large amount of data generated by the DNA microarrays, we applied a bioinformatics tool called cluster analysis to recognize changes in the transcription patterns following exposure to Me$_2$SO. The transcriptional data of herbicides, detergents, and food additives together with circumstance changes, such as temperature, pressure, and freezing/thawing, for cluster analysis are shown in kasumi.nibh.jp/~eganomiX/. The various stress factors are outlined in the legend of Fig. 3. The gene groups that gave a significant variation in expression were selected from the genome-wide gene expression profile. 3,875 open reading frames, which induced commonly under different stress conditions, were extracted from the gene groups. We performed the hierarchical cluster analysis using GeneSpring. The settings for the calculations were as follows: similarity was measured by standard correlation; the separation ratio was 1.0; and the minimum distance was 0.001. The hierarchical clustering was constructed using the 3,875 open reading frames. The transcriptional profiles for exposure to Me$_2$SO were compared with the expression profiles of other stress conditions. As shown in Fig. 3, the data are graphically displayed so that each row of colored bars represents the variation of transcript levels for every gene in a given mRNA sample, as detected on the DNA microarray. The variations in abundance of transcripts for each gene are depicted by means of a color scale.
Northern Blot Analysis—Northern blotting was performed by the method of Ausubel et al. (22). Total RNA (20 μg), isolated from control or Me2SO-treated cells for 2 h, was subjected to electrophoresis through a 1.0% formaldehyde denaturing agarose gel (4–5 h at 100 V). RNA was transferred to a nylon membrane (Roche Diagnostics) and fixed using a UV cross-linker (120 mJ). For Northern blot analysis, the double-strand DNA probes of the genes implicated in metabolism of phospholipid and methionine were amplified from chromosomal DNA using the following primers: INO1, 5'-TCTGCAACACGCTTGAAGGCG-3' as the forward sequence and 5'-AGCCATTACCCGGTGTAATCC-3' as the reverse sequence; OPI3, 5'-ATGAAGGAGTCAGTCCAAGAGATCA-3' as the forward sequence and 5'-CATATTCTTTTTGGCCTTATCACGG-3' as the reverse sequence; MET6, 5'-AGACCACTCAATTTAAGTTGAACGG-3' as the forward sequence and 5'-TCTCTCAGTACCTTCTCTCAATGG-3' as the reverse sequence; MET17, 5'-ATCCAGAGCTGTACC-3' as the forward sequence and 5'-ACCAACATTGGCAAGTTAGAGGC-3' as reverse sequence; and ACT1, 5'-TAACGGTTCTGGTATGTGTAAAGCC-3' as forward sequence and 5'-TGTAAGTAGTTTGGTCAATACCGGC-3' as reverse sequence. Denatured probe was hybridized to the membrane-bound RNA and detected with anti-digoxigenin antibody according to the manufacturer's instructions (Roche Diagnostics).

Phospholipid Analysis and Inorganic Phosphate (P<sub>i</sub>) Quantitative Analysis in Total Lipid—The extraction of total lipid from yeast cells was performed according to the method of Bligh and Dyer (23). Yeast cells were grown in YPD medium to exponential phase (OD<sub>660</sub> = 1.0), and 10% (v/v) Me<sub>2</sub>SO was added. Me<sub>2</sub>SO-treated or non-treated yeast cells were harvested after 0, 2, 4, and 6 h and frozen immediately in liquid nitrogen. Colony-forming units was counted at each time point. The frozen pellet was resuspended in a small volume of saturated NaCl solution, and total lipids were extracted with chloroform/methanol (1:2, v/v in the presence of 0.08% butylated hydroxytoluene, which acted as an antioxidant. After extraction of total lipid, chloroform and saturated NaCl solution were added, and the ratio of chloroform/methanol/water was adjusted to 1:1:1 (v/v). The organic and aqueous phase were separated by centrifugation. The chloroform layer was recovered, evaporated to dryness under nitrogen gas and then resuspended in chloroform to give the total lipid sample. Aliquots corresponding to 5.0 x 10<sup>8</sup> cells were analyzed by TLC using Silica Gel 60 (Merck; 20 x 20 cm). The silica gel plates were developed with a chloroform/methanol/acetic acid/formic acid (100:60:9:13, v/v) solvent system for ~60 min at room temperature and air-dried for about 15 min. The plates were then sprayed with 12% (v/v) H<sub>2</sub>SO<sub>4</sub>, 2% CuSO<sub>4</sub> and placed in an oven at 180°C for 15 min.

The P<sub>i</sub> in total lipid was determined by the method of Rouser et al. (24). Aliquots of total lipid (5, 10, and 20 μl) were resuspended in chloroform and placed in a test tube (18 x 180 mm). The chloroform solvent was evaporated to dryness under nitrogen gas. 70% perchloric
**MeSO Treatment and Induction of Phospholipid Synthesis**

acid (1 ml) was added to the dried lipid and then heated to 200 °C for 1.5 h. The test tube was cooled and rinsed with 5.0 ml of distilled water, and 1.0 ml of 25% ammonium molybdate solution was added. After thorough mixing with a vortex mixer, 1.0 ml of 10% ascorbic acid solution was added, followed by 2.0 ml of distilled water. The solution was heated in boiling water for 10 min, and after cooling the absorbance reading at 820 nm was determined. The absorbance values were then converted to micromoles of phosphorus by means of a factor derived from a standard curve (prepared using KH₂PO₄). The phosphorus content (micrograms) in the total lipid corresponding to 2.5 × 10⁵ cells was determined.

**DiOC₆ Staining**—Yeast cells were grown under MeSO-treated and non-treated conditions in YPD liquid culture. For 3,3'-dihexyloxacarboxylic acid (DiOC₆) (Molecular Probes, Eugene, OR) staining, cells were harvested at different time points (0, 0.5, 1, 4, 6, and 7 h), and washed with TE buffer. After washing, the pellet was resuspended in TE buffer. DiOC₆ was resuspended to 1 mg ml⁻¹ in ethanol (as DiOC₆ stock solution). 1.0 μl of DiOC₆ stock solution was added to 1 ml of the yeast cell suspension. If the cell density was OD₆₀₀ > 1.0 at harvesting, the cell suspension was diluted with TE buffer to an OD₆₀₀ of ~1.0. Cells stained with DiOC₆ were analyzed immediately. Staining was carried out as described by Block-Alper et al. (25).

**Flow Cytometry**—About 100,000–500,000 yeast cells were acquired per sample using EPICS XL system II (Beckman Coulter). The detection threshold was set in the channel just below the lowest detectable level of the yeast suspension with the lowest intensity. DiOC₆-stained cells were detected in channel FL1. Cell size and intracellular structure were detected by forward scatter and side scatter. Analysis of the results was carried out using the Exo 32 software (Beckman Coulter) and each mean value of FL1, forward scatter, and side scatter was calculated.

**Fluorescence Microscopy**—DiOC₆-stained cells were visualized by confocal laser scanning microscopy (TCS-SP2, Leica). DiOC₆ was excited using a 488-nm argon laser that was reflected by a dichroic mirror to the yeast cells through a ×100 objective oil immersion lens (PL APO; NA 1.4; Leica). The emission signal was directed through a pinhole to a slit (505–530 nm) and then to a photomultiplier device. Images were collected using the Leica 2000 confocal software program.

**RESULTS**

**MeSO Concentration and Duration of Exposure**—S. cerevisiae can grow under various stress conditions such as temperature change, nutrient depletion, and the presence of noxious agents or toxic chemicals. However, for adaptation to drastic environmental growth conditions, complex changes in the pattern of gene expression are known to occur (26, 27). Genome-wide transcriptional analysis using a DNA microarray was employed to assess the effects of MeSO exposure. After treatment with MeSO, the growth inhibition and viability of yeast cells was examined (28). To find the optimum conditions, S. cerevisiae cells in the logarithmic phase of growth were exposed to various amounts of MeSO (Fig. 1A). 10% (v/v) MeSO gave approximately the half-maximal inhibitory concentration (IC₅₀) for growth inhibition. Yeast cells were allowed to survive and grow at this concentration of MeSO (Fig. 1B). Exposure to 10% (v/v) MeSO for 2 h was sufficient to induce large changes in the gene expression profile. Therefore RNA was extracted from these cells and analyzed using microarray technology.

**DNA Microarray Analysis**—The mRNA purified from non-treated or MeSO-treated cells was labeled with the fluorescence dye, Cy3 or Cy5, respectively. Generated cDNA probes were mixed and hybridized to a whole genome yeast DNA microarray. To evaluate differences in gene expression, we quantified the fluorescence intensity of each spot. The expression of each gene following MeSO treatment was quantified as the ratio of fluorescence intensity Cy5/Cy3. A total of 246 genes was down-regulated after 2 h of MeSO exposure (21), and we analyzed the distribution of these genes according to functional categories in Munich Information Center for Protein Se-

![Table 1](image)
meSO Treatment and Induction of Phospholipid Synthesis

... and part of the methionine synthesis pathway. The reactions indicated are catalyzed by the following enzymes: INO1, inositol-1-phosphate synthase; CDS1, CDP-diacylglycerol synthase; CHO1, phosphatidylinositol synthase 1; PSD1, phosphatidylserine decarboxylase 1; CHO2, phosphatidylethanolamine methyltransferase; OPI3, phosphatidyl ethanolamine methyltransferase; MET25, O-acetylhomoserine and O-acetylserine sulfhydrolase; MET6, homocysteine methyltransferase; SAM1, S-adenosylmethionine synthetase 1; SAM2, S-adenosylmethionine synthetase 2. The numbers in parentheses indicate the ratio of Cy5 (10% v/v MeSO) to Cy3 (no MeSO) in the DNA microarray experiment.

Fig. 4. Phospholipid biosynthesis and part of the methionine synthesis pathway. The reactions indicated are catalyzed by the following enzymes: INO1, inositol-1-phosphate synthase; CDS1, CDP-diacylglycerol synthase; CHO1, phosphatidylinositol synthase 1; PSD1, phosphatidylserine decarboxylase 1; CHO2, phosphatidylethanolamine methyltransferase; OPI3, phosphatidyl ethanolamine methyltransferase; MET25, O-acetylhomoserine and O-acetylserine sulfhydrolase; MET6, homocysteine methyltransferase; SAM1, S-adenosylmethionine synthetase 1; SAM2, S-adenosylmethionine synthetase 2. The numbers in parentheses indicate the ratio of Cy5 (10% v/v MeSO) to Cy3 (no MeSO) in the DNA microarray experiment.

Cluster Analysis of the Transcriptional Genes Responding to MeSO Treatment—We used cluster analysis to explore how MeSO affects the transcription of genes in S. cerevisiae. We exploited the various gene expression data for heavy metals, detergents, food additives, and herbicides that have been performed in our laboratory for the cluster analysis, and we aggregated these gene expression profiles with those of MeSO. The gene expression images in Fig. 3 indicate the characteristic patterns for various stress-inducing growth conditions. Genes co-expressed across the various conditions are likely to be involved in a common cellular process. We examined MeSO as an inducer of stress by comparing the gene expression pattern following exposure to this chemical with the patterns generated from other stress-inducing growth conditions. As shown in Fig. 3, the gene expression profile for MeSO was grouped in a cluster that included the detergents sodium n-dodecyl benzenesulfonate (LAS) and SDS, the food additives, capsaicin and gingerol, and 2,4,5-TCP. The detergents, LAS and SDS, are known to affect the structure and fluidity of membranes. Furthermore, the genes involved in membrane and cell wall construction, such as OPI3, CHS1, and GSC2, were induced by LAS and SDS (data not shown). Because genes involved in phospholipid synthesis, such as INO1 and OPI3, were also induced by both capsaicin and gingerol treatment, it was proposed that these food additives cause damage to the membrane structure and require the biosynthesis of PI and PC (29). The membrane organization group of genes were also induced by 2,4,5-TCP, Roundup, and growth under high (30 MPa) pressure conditions (data not shown). Because genes, such as INO1 and OPI3, involved in maintaining cell membrane and cell wall integrity were also induced by exposure to MeSO, it was thought that this treatment may adversely affect the structure of the cell membrane. To determine whether lipid synthesis was affected by MeSO treatment, we performed Northern blot analysis on key genes involved in lipid biosynthesis (e.g., INO1 and OPI3) as shown in Table I.

Northern Blot Analysis of Genes Involved in Phosphatidylcholine and Methionine Synthesis Pathway—Northern blot analysis revealed elevated levels of transcripts for the INO1 gene and OPI3 gene in MeSO-treated cells (Fig. 5). INO1 encodes soluble inositol-1-phosphate synthase, which catalyzes the first step in yeast phospholipid synthesis, and this gene product is involved in the pathway leading to PI. OPI3 encodes the phospholipid methylethyltransferase that catalyzes the last two methylation reactions in PC synthesis. The expression of INO1 and OPI3 had been shown previously to be regulated at the level of transcript abundance. The rate-limiting step in inositol phospholipid biosynthesis is mediated by the INO1 gene product (30, 31). Therefore, we propose that PC synthesis in yeast cells is promoted by MeSO exposure, because there was significant enhancement in the levels of mRNA for INO1 and OPI3 as shown in Table I and Fig. 4.

* Monsanto’s herbicide is composed of ammonium = N-phosphomethylglycinate and surfactant.
Microarray analysis also demonstrated transcriptional induction of some of the genes involved in methionine synthesis, such as MET17/25, MET6, SAM1, and SAM2 (Table I). As shown in Fig. 4, MET6 and MET17/25 genes encode homocysteine methyltransferase and O-acetylhomoserine sulfhydrylase, respectively, and mediate the conversion of O-acetylhomoserine and sulfide to methionine. SAM1 and SAM2 genes encode two distinct forms of AdoMet synthetase and catalyze the biosynthesis of AdoMet from methionine in the methionine synthesis pathway. In the biosynthetic pathway to PC, PE is methylated by PE methyltransferase (CHO2) and phospholipid methyltransferase (OP15), which both utilize AdoMet as the methyl donor (32, 33). AdoHcy resulting from the demethylation of AdoMet is converted to methionine or AdoMet in the methionine synthesis pathway (Fig. 4). Because both DNA microarray and Northern blot analysis showed increased levels of MET17/25, MET6, SAM1, and SAM2 gene transcripts in Me2SO-treated cells (Fig. 5), we propose that the net effect is to increase phospholipid synthesis.

DNA microarray analysis revealed that in Me2SO-treated cells, the expression of genes involved in cell wall organization increased relative to the non-treated cells (Table II). These gene products are either involved in the synthesis of the cell wall components (i.e. glucan, mannoproteins, and chitin) or in maintaining cell wall organization (34–37). Therefore, exposure to Me2SO appears to promote the synthesis and maintenance of the cell wall.

**Analysis and Quantification of Phospholipid in Me2SO-Treated Cells**—An up-regulation in the level of mRNA expression is an indication of increased biological activity of a particular gene product but is not sufficient on its own for a quantitative description of a biological system (38). We therefore analyzed the phospholipid components (PC, PI, PS, and PE) in Me2SO-treated and non-treated cells to determine whether phospholipid synthesis was affected. Total lipid was extracted from culture samples of Me2SO-treated and non-treated cells, and a comparison was made of the phospholipid components. As shown in Fig. 6A, during a 2-h period following Me2SO treatment, no significant changes could be detected, but after 4 and 6 h the level of phospholipid components, such as PC, PI, PS, and PE in the Me2SO-treated cells, was elevated by more than 3-fold compared with non-treated control cells. To quantify the level of phospholipid, we determined the phosphorus content within total lipid corresponding to $2.5 \times 10^8$ cells. The phosphorus content within total lipid increased especially at 4 and 6 h after Me2SO treatment, although those in non-treated cells did not increase (Fig. 6B). From these results, it has become apparent that phospholipid biosynthesis was stimulated by Me2SO exposure.

**Quantification of Me2SO-Induced Membrane Proliferation**—In addition to up-regulating genes involved in phospholipid biosynthesis, genes concerned with cell membrane and cell wall organization were also induced by Me2SO treatment. To demonstrate whether membrane proliferation resulted from enhanced phospholipid biosynthesis, total membrane quantification was carried out. The lipophilic fluorescent dye DiOC$_6$ has been used routinely to assess and quantify membrane proliferation arising from overexpression of an integral ER protein that catalyzes the rate-limiting step in sterol biosynthesis (39, 40). DiOC$_6$ was also used to quantify increases in intercellular membrane content in living cells upon expression of the phospholipid biosynthetic genes. Me2SO-treated and non-treated cells were incubated with DiOC$_6$, and fluorescence was quantified immediately by flow cytometry. From 0 to 1 h after Me2SO treatment, DiOC$_6$ uptake into Me2SO-treated cells was no different from that of non-treated cells (Fig. 7A). The fluorescence level of DiOC$_6$ incorporated into intracellular membranes increased gradually from 2 to 4 h after Me2SO treatment and was about 2-fold greater at 6–7 h compared with non-treated cells (Fig. 7A). To confirm that DiOC$_6$ absorption resulted from an increase in cellular membrane content, Me2SO-treated and non-treated cells stained with DiOC$_6$ were analyzed by fluorescence microscopy (Fig. 7B). Differences between the Me2SO-treated and non-treated yeast cells became apparent after 4–6 h of exposure to Me2SO. Bright rings emanating from the ER and nuclear membranes (Fig. 7B) could be visualized in the Me2SO-treated cells. In contrast, control cells gave dim images resulting from intracellular membrane staining (Fig. 7B). These observations indicated that cellular membrane proliferation was caused by Me2SO treatment.

**DISCUSSION**

We used DNA microarrays to perform a genome-wide transcriptional analysis to assess changes in the relative expression level of yeast mRNA during exposure to Me2SO. As shown in Fig. 2A, the distribution of up-regulated genes following Me2SO treatment provided our initial evidence concerning a potential molecular mechanism involving increased phospholipid synthesis (Table I and Fig. 4). In addition, the transcriptional profiles for Me2SO exposure were located in the cluster con-

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**Table II. The genes implicated in cell wall and plasma membrane organization**

| ORFs     | Cys/Cy3 ratio | Gene name       | Function                                      |
|----------|---------------|-----------------|----------------------------------------------|
| YGR189C  | 9.3           | CRH1            | Family of putative glycosidases might exert a common role in cell wall organization |
| YLR121C  | 8.9           | YPS3            | GPI-anchored aspartyl protease 3 (yapin 3)    |
| YDR077W  | 6.4           | SED1            | Abundant cell surface glycoprotein           |
| YDR055W  | 5.8           | PST1            | Strong similarity to SPFS2 protein           |
| YHR142W  | 5.7           | CHS7            | Control of protein export from the ER (like chitin synthase III) |
| YGR032W  | 5.1           | GSC2            | Catalytic component of 1,3-β-D-glucan synthase |
| YNL322C  | 4.5           | KRE1            | Cell wall β-glucan assembly                  |
| YGR282C  | 3.9           | BGL2            | Endo-β-1,3-glucanase of the cell wall hypothetical protein |
| YLR194C  | 3.3           |                 |                                               |
| YJR004C  | 3.1           | YAL023C         | 108 cells.                                  |
| YNL300W  | 3.1           | YAL023C         | -Acetylhomoserine sulfhydrylase, respectively, and mediate the conversion of O-acetylhomoserine and sulfide to methionine. SAM1 and SAM2 genes encode two distinct forms of AdoMet synthetase and catalyze the biosynthesis of AdoMet from methionine in the methionine synthesis pathway. In the biosynthetic pathway to PC, PE is methylated by PE methyltransferase (CHO2) and phospholipid methyltransferase (OP15), which both utilize AdoMet as the methyl donor (32, 33). AdoHcy resulting from the demethylation of AdoMet is converted to methionine or AdoMet in the methionine synthesis pathway (Fig. 4). Because both DNA microarray and Northern blot analysis showed increased levels of MET17/25, MET6, SAM1, and SAM2 gene transcripts in Me2SO-treated cells (Fig. 5), we propose that the net effect is to increase phospholipid synthesis. DNA microarray analysis revealed that in Me2SO-treated cells, the expression of genes involved in cell wall organization increased relative to the non-treated cells (Table II). These gene products are either involved in the synthesis of the cell wall components (i.e. glucan, mannoproteins, and chitin) or in maintaining cell wall organization (34–37). Therefore, exposure to Me2SO appears to promote the synthesis and maintenance of the cell wall.

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We used DNA microarrays to perform a genome-wide transcriptional analysis to assess changes in the relative expression level of yeast mRNA during exposure to Me2SO. As shown in Fig. 2A, the distribution of up-regulated genes following Me2SO treatment provided our initial evidence concerning a potential molecular mechanism involving increased phospholipid synthesis (Table I and Fig. 4). In addition, the transcriptional profiles for Me2SO exposure were located in the cluster con-
constructed for the herbicide, detergents, food additives, and circumstance stress factors. A common theme with all these stress factors is the damage caused to the cellular membrane structure and the tendency to induce expression of genes involved in limiting this potential damage (Fig. 3). We performed Northern blot analysis of genes involved in phospholipid biosynthesis, which demonstrated that $\text{INO1}$, $\text{OPI3}$, $\text{MET6}$, and $\text{MET17}$ are indeed induced by exposure to $\text{Me}_2\text{SO}$ (Fig. 5).

According to Gygi et al. (38), the correlation between the level of mRNA and that of the corresponding translation product is not always reliable. Therefore, to fully understand the cellular response to chemical stress, transcriptional data alone are not sufficient. We analyzed the phospholipid components (PC, PI, PS, and PE) in both Me$_2$SO-treated and non-treated cells from the mid-exponential to stationary phase to investigate possible differences in biosynthesis of these membrane components. If there was any defect or anomaly in gene expression in the phospholipid synthesis pathway, the relative proportions of phospholipid components, such as PC, PI, PS, and PE, should change (32). These studies demonstrated that the amounts of phospholipid increased greatly after 4–6 h of Me$_2$SO treatment (Fig. 6, A and B), but there was no alteration in the relative proportions of phospholipid components such as PC, PI, PS, and PE in comparison with the non-treated cells (Fig. 6A). When the cells were stained with the fluorescent lipophilic dye DiOC$_6$, the amount of fluorescence in the cellular membrane increased for the Me$_2$SO-treated cells (Fig. 7, A and B), and bright rings emanating from intracellular membranes probably localized to the ER were observed (Fig. 7B). Because the ER is the site of lipid biosynthesis in eukaryotes, this result supports the proposal that membrane proliferation was initiated in Me$_2$SO-treated cells. In both $\text{E. coli}$ and animal cells, it has been reported that transformation or transfection efficiency is increased by Me$_2$SO treatment, because Me$_2$SO affects the permeability of the cell membrane to facilitate uptake of DNA (12, 13). Our investigations show that Me$_2$SO destroys or damages the integrity of the membrane, and it seems likely that this increases membrane permeability.

The genes implicated in the methionine biosynthesis pathway were induced by Me$_2$SO exposure for 2 h (Table I and Fig. 5). AdoMet synthesized from $\text{O}$-acetylhomoserine and sulfide is a ubiquitous metabolite involved in almost as many reactions

**Fig. 6.** Me$_2$SO treatment increased the synthesis of phospholipid components, such as PC, PI, PS, and PE, in yeast cells. Yeast cells grown in YPD medium were treated with Me$_2$SO and harvested at different time points as described in Fig. 6. A, total lipids of either non-treated (–) or 10% (v/v) Me$_2$SO-treated (+) yeast cells were extracted with chloroform/methanol solution and analyzed by TLC. Phospholipids PC, PI, PS, and PE were used as standards. M1, PE; M2, PE and PC; M3, PS and PI; M4, PI. B, the phosphorus within total lipids was determined as indicated under “Experimental Procedures” for quantification of phospholipids. The inorganic phosphate contents (μg) corresponding to 2.5 × 10$^8$ cells were compared in both Me$_2$SO-treated cells and non-treated cells.

**Fig. 7.** Me$_2$SO treatment induced membrane proliferation in yeast cells. Membrane proliferation was quantified by the incorporation of the lipophilic dye DiOC$_6$. Yeast cells were incubated with 0 and 10% Me$_2$SO for different times. Cells were stained with DiOC$_6$ as described under “Experimental Procedures.” Cells harvested at each time point were subjected to flow cytometry analysis, and the mean fluorescence values were plotted in A. DiOC$_6$-stained cells viewed by fluorescence microscopy (B). The bar in the figures indicates the scale of 1.0 μm.
as ATP (41). In phospholipid biosynthesis (Fig. 4), AdoMet is needed in the last three methylation reactions and is transformed to AdoHcy by a demethylation reaction (32, 33). AdoHcy is converted to homocysteine in the methionine biosynthesis pathway, which is then remethylated to methionine by methionine synthase (MET17/25 and MET6). AdoMet is regenerated by AdoMet synthetase (SAM) (42, 43). Because AdoMet is the methyl donor in hundreds of transmethylation reactions of nucleic acids, proteins, and lipids (44), we propose that in Me$_2$SO-treated cells, integration of this pathway with the bio-synthesis of phospholipids helps maintain membrane integrity.

In _S. cerevisiae_ the SAM1 and SAM2 genes encode two distinct forms of AdoMet synthetase (45) that are highly homologous (83% similarity) (42). DNA microarray analysis indicates the transcriptional abundance of SAM1 and SAM2 genes increased by 2.7- and 6.9-fold, respectively, following treatment with Me$_2$SO (Table 1). Because these genes are closely related, we cannot rule out the possibility that this expression data was affected by cross-hybridization. The expression of the SAM1 gene is normally constant during growth but is repressed in media containing methionine. However, the expression of SAM2 is dependent on the phase of growth and increases during late exponential phase (46). The expression profiling of Me$_2$SO-treated cells was compared with the expression data for the various culture incubation periods. Gene expression in response to Me$_2$SO treatment was similar to the transcriptional profiles at the stationary phase (21). This suggests that in response to Me$_2$SO treatment, the increase in the transcriptional level of SAM2 was greater than that of SAM1.

In general, a number of genes involved in phospholipid biosynthesis are known to be repressed after addition of inositol and choline to the growth medium (inositol-choline regulation) (31, 32). The regulatory responses to inositol and choline are established by specific regulatory regions, conventionally referred to as upstream activation sequence, and localized in their upstream region (47, 48). Surprisingly, the expression of methionine related genes, such as MET6, MET17, SAM1, and SAM2, were also induced by Me$_2$SO exposure and together the genes involved in phospholipid biosynthesis (Fig. 4 and Table 1). We speculate that transcription of these genes involved in methionine biosynthesis was controlled by inositol-choline regulatory elements, because some of these genes contained at least an upstream activation sequence element within the non-coding region (data not shown).

Me$_2$SO is utilized as a terminal electron acceptor in a large number of microorganisms and is converted to DMS by Me$_2$SO reductase (Dms ABC) in _E. coli_ and _R. sphaeroides_ (1, 7, 10). Genome sequencing has revealed that _S. cerevisiae_ does not encode a gene with significant similarity to a bacterial Me$_2$SO reductase. However, this yeast has an NADPH-dependent enzyme activity that reduces Me$_2$SO to DMS (11). Recently, a gene encoding methionine sulfoxide reductase (msra) was cloned from yeast (49), and the corresponding enzyme, MXR1, has been shown to act as a methionine sulfoxide reductase as well as reducing Me$_2$SO to DMS (11). In our DNA microarray data, the transcriptional level of _mrx1_ was slightly elevated, and the expression levels of genes involved in mitochondrial respiration were repressed (Fig. 2B). A yeast strain with a disrupted _MXR1_ gene retained 66% of its reductase activity against free methionine sulfoxide (11, 49), indicating the presence of another enzyme and/or other routes to degrade Me$_2$SO.

_E. coli_ is capable of inducing specialized respiratory chains comprising a primary dehydrogenase when grown anaerobically with respiratory oxidants such as nitrate and Me$_2$SO (1). In our DNA microarray experiments, the genes having a high similarity to the AAD gene of the lignin-degrading fungus _Phanerochaete chrysosporium_ were induced by Me$_2$SO treatment (21). The function of AAD in yeast is unclear, but it may metabolize Me$_2$SO to generate DMS. We propose that Me$_2$SO or DMS is metabolized by AAD and is converted to AdoMet in the methionine pathway, where it might be utilized as a methyl group source for phospholipid biosynthesis in the PC biosynthetic pathway.

In the case of mammalian cells, Me$_2$SO can induce or block cell differentiation and apoptosis depending on the cell lines and cell types (14–18). It has been reported that Me$_2$SO affects the selection of splice sites and causes a shift toward the proximal pair of splice sites on pre-mRNAs carrying competing 5′-splice sites or competing 3′-splice sites (19). We did not observe any morphological changes when budding _S. cerevisiae_ cells were treated with 10% Me$_2$SO (Fig. 6B). The fission yeast, _Schizosaccharomyces pombe_ has 4,370 introns, distributed among 43% of total genes. In contrast, introns of the budding yeast _S. cerevisiae_ are much rarer and are found in only 5% of genes (50). Therefore, the lack of introns in _S. cerevisiae_ may explain why Me$_2$SO-induced interference in the alternative slicing of pre-mRNAs was not observed in this study.
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Cell 6, 1535–1547
40. Koning, A. J., Roberts, C. J., and Wright, R. L. (1996) Mol. Biol. Cell 7, 769–789
41. Thomas, D., Cherest, H., and Surdin-Kerjan, Y. (1989) Mol. Cell. Biol. 9, 3292–3298
42. Thomas, D., Rothstein, R., Rosenberg, N., and Surdin-Kerjan, Y. (1988) Mol. Cell. Biol. 8, 5132–5139
43. Mountain, H. A., Bystron, A. S., Larsen, J. T., and Korch, C. (1991) Yeast 7, 781–803
44. Thomas, D., Becker, A., and Surdin-Kerjan, Y. (2000) J. Biol. Chem. 275, 40718–40724
45. Thomas, D., and Surdin-Kerjan, Y. (1987) J. Biol. Chem. 262, 16704–16709
46. Thomas, D., and Surdin-Kerjan, Y. (1991) Mol. Gen. Genet. 226, 224–232
47. Kodaki, T., Nikawa, J.-I., Hosaka, K., and Yamashita, S. (1991) J. Bacteriol. 173, 7992–7995
48. Schuller, H.-J., Hahn, A., Troster, F., Schutz, A., and Schweizer, E. (1992) EMBO J. 11, 107–114
49. Moskovitz, J., Berlett, B. S., Poston, J. M., and Stadtman, E. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9585–9589
50. Wood, V., Gwilliam, R., Rajandream, M. A., Lyne, M., Lyne, R., Stewart, A., Sgouros, J., Peat, N., Hayles, J., Baker, S., Banham, D., Bowman, S., Brooks, K., Brown, D., Brown, S., Chillingworth, T., Churcher, C., Collins, M., Connor, R., Cronin, A., Davis, P., Feltwell, T., Fraser, A., Gentles, S., Goble, A., Hamlin, N., Harris, D., Hidalgo, J., Hodgson, G., Holroyd, S., Hornby, T., Howarth, S., Huckle, E. J., Hunt, S., Jagels, K., James, K., Jones, L., Jones, M., Leather, S., McDonald, S., McLean, J., Mooney, P., Moule, S., Mungall, K., Murphy, L., Niblett, D., Odell, C., Oliver, K., O’Neil, S., Pearson, D., Quail, M. A., Rabbinowitsch, E., Rutherford, K., Rutter, S., Saunders, D., Seeger, K., Sharp, S., Skeith, J., Simmonds, M., Squares, R., Squares, S., Stevens, K., Taylor, K., Taylor, R. G., Trivey, A., Walsh, S., Warren, T., Whitehead, S., Woodward, J., Volckaert, G., Aert, R., Robben, J., Grymonprez, B., Weltjens, I., Vanstreels, E., Rieger, M., Schafer, M., Muller-Auer, S., Gabel, C., Fuchs, M., Fritze, C., Holzer, E., Moestl, D., Hilbert, H., Borzym, K., Langer, L., Beck, A., Lehrach, H., Reinhardt, R., Pohl, T. M., Reger, P., Zimmermann, W., Wedler, H., Wambutt, R., Purnelle, B., Goffeau, A., Cadieu, E., Dreano, S., Gloux, S., Lelaure, V., Mottier, S., Galibert, F., Avez, S. J., Xiang, Z., Hunt, C., Moore, K., Hurst, S. M., Lucas, M., Roche, M., Guiard, C., Tallada, V. A., Garzon, A., Thode, G., Daga, R. R., Cruzado, L., Jimenez, J., Sanchez, M., del Rey, F., Benito, J., Domiguez, A., Revuelta, J. L., Moreno, S., Armstrong, J., Forsburg, S. L., Cerrutti, L., Lowe, T., McCombie, W. R., Paulsen, I., Potashkin, J., Shpakovski, G. V., Ussery, D., Barrett, B. G., and Nurse, P. (2002) Nature 415, 871–880