Sphingolipids are signaling molecules that influence diverse cellular functions from control of the cell cycle to degradation of plasma membrane proteins. The synthetic sphingolipid-like compound FTY720 is an immunomodulating agent in clinical trials for transplant graft maintenance. In this report, we compare the effects of the natural yeast sphingolipid phytosphingosine with FTY720 in Saccharomyces cerevisiae. We show that the multicopy suppressor genes that induce growth resistance to FTY720 also confer resistance to growth-inhibitory concentrations of phytosphingosine. In addition, mutants for ubiquitination pathway proteins are shown to be resistant to the growth-inhibiting effect of both FTY720 and phytosphingosine. We observe fewer similarities between sphingosine and FTY720 than between FTY720 and phytosphingosine as revealed by genetic studies. Yeast cells lacking the specific sphingosine kinase LCB4 are sensitive to phytosphingosine and FTY720 but resistant to sphingosine, suggesting that FTY720 and phytosphingosine have a more related mechanism of action. Gene expression profile comparisons of sensitive and resistant yeast cells exposed to FTY720 and phytosphingosine highlight a number of similarities. In response to treatment with these compounds, ~77% of the genes that are regulated >2-fold by FTY720 also respond to phytosphingosine in the same direction in the parent strain. In addition, a close inspection of TAT1 and TAT2 transporters following exposure to phytosphingosine indicates that TAT1 protein is degraded in a similar fashion upon treatment with FTY720 and phytosphingosine. There were differences, however, with respect to the TAT2 protein level and the expression profiles of a subset of genes. The genetic, transcriptional, and biochemical data together indicate that FTY720 and phytosphingosine influence similar pathways in yeast cells. These findings offer further insights into the physiological pathways influenced by these compounds in all eukaryotic cells and help us to understand the therapeutic consequences of FTY720 in humans.

FTY720 is a synthetic compound attracting significant interest in the field of immune suppression. It is derived by chemical synthesis from myriocin, a natural metabolite isolated from the fungus Isaria sinclairii. Myriocin was shown previously to interfere with a key step of the sphingolipid biosynthesis pathway (1), whereas FTY720 does not share this mechanism of action (2). In synergy with several conventional immunosuppressive drugs, FTY720 has shown strong graft protection in several animal models of transplantation (3–5). FTY720 treatment causes sequestration of circulating lymphocytes to the secondary lymph nodes and Peyer’s patches (6). It does not affect T and B cell proliferation or maturation (3, 7) and does not impair the humoral response to systemic viral infection (6). Specific cellular mechanisms by which FTY720 may be mediating its effects are beginning to be elucidated. It was shown that the active in vivo metabolite of FTY720 is FTY720-phosphate (8). FTY720-phosphate shares some structural homologies with mammalian sphingosine 1-phosphate (S1P) and binds with high affinity to four of the five mammalian sphingosine 1-phosphate receptors (8, 9). These receptors belong to the family of seven transmembrane G protein-coupled receptors. S1P is derived from the parent compound sphingosine through the action of sphingosine kinases in vivo. The precise mechanisms by which FTY720 or its metabolite FTY720-P mediates immunomodulation or associated effects are not known. The yeast Saccharomyces cerevisiae produces a homolog to mammalian sphingosine called phytosphingosine (PHS), which is derived from the precursor dihydrosphingosine (DHS). Both PHS and DHS can be phosphorylated in S. cerevisiae by the phytosphingosine kinases LCB4 and LCB5 (10). The sphingoid base PHS has initially been demonstrated to be a specific inhibitor of yeast growth (11) and the transport of several amino acids such as tryptophan, leucine, histidine, and uracil (12). A full prototrophic strain was more resistant to PHS than a tryptophan auxotrophic strain (11). PHS was shown later to be involved in heat stress signaling via a mechanism involving a ubiquitin-dependent proteolysis in which the target for degradation was the uracil permease FUR4, and the ubiquitin enzymes were RSP5 and DOA4 (13). PHS was also demonstrated to be crucial for the internalization step of endocytosis in yeast, as indicated indirectly by a reduced uptake of radio-labeled α factor due to the internalization of the corresponding receptor after ubiquitination (14).

We have shown previously that auxotrophic yeast strains are more sensitive to FTY720-mediated growth inhibition as compared with prototrophic strains. S. cerevisiae auxotrophic

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1 The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

2 The on-line version of this article (available at http://www.jbc.org) contains four supplemental tables concerning the effect of FTY720 treatment on genes in the parent and the bul1 mutant as well as the effect of PHS on genes in the parent and the bul1 mutant.

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4 The abbreviations used are: S1P, sphingosine 1-phosphate; DHS, dihydrosphingosine; DMS, N,N-dimethylsphingosine; PHS, phytosphingosine.
strains expressing multiple copies of tryptophan transporter genes such as TAT1 and TAT2 and the genes UBP5 and UBP11 (which specify proteases in the protein ubiquitination cascade) as well as the heat shock protein homolog gene CAJ1 are resistant to yeast growth inhibition mediated by FTY720 (15). In addition, loss-of-function mutations in the ubiquitin structural protein UBI4, a reduction of ubiquitin ligase protein RSP5, the deletion of RSP5-binding protein BUL1, and a mutation of the glutamine transporter gene GNP1 also confer resistance to FTY720-mediated growth inhibition. We propose a model in which FTY720 influences the protein ubiquitination pathway and enhances specific protein degradation. Our observations indicate that FTY720 and PHS share multiple common properties and that amino acid transporters and the ubiquitin pathway play a significant role in reversing the growth inhibition mediated by these compounds.

Because effectors of resistance to FTY720 also mediate resistance to PHS, we compared the gene expression profiles of yeast in response to these two compounds. We report that up to 535 of 705 (75%) open reading frames up-regulated by FTY720 are also up-regulated by PHS, and 415 of 521 (80%) down-regulated open reading frames are also in common. This indicates that PHS and FTY720 similarly affect the transcription of a broad set of genes.

We have also measured the effects of N,N-dimethylphosphogingosine (DMS) and sphingosine on yeast strains and compared these with the effects mediated by PHS and FTY720. We show that, although the cellular responses to FTY720 and PHS are remarkably similar, there are significant differences between the effects of sphingosine and DMS. Overall, these results shed light onto the mechanism of action of FTY720 and highlight the differences and similarities between FTY720 and the endogenous mammalian and yeast sphingolipid molecules.

**Experimental Procedures**

**Strains and Plasmids**—The strains used in this study (listed in Table 1) are isogenic to JK9-3d. All plasmids are as described previously (15). Strains were maintained according to standard laboratory procedure (16). Cultivation and maintenance of cells were carried out as described previously (15). Strains were maintained according to standard laboratory procedure (16). Cultivation and maintenance of cells were carried out as described previously (15). Strains were maintained according to standard laboratory procedure (16). Cultivation and maintenance of cells were carried out as described previously (15).

**FTY720 and Phytosphingosine**

**List of the Saccharomyces cerevisiae strains that were used in this study and their genotype**

| Strain        | Genotype                                      | Source/Ref. |
|---------------|-----------------------------------------------|-------------|
| JK9-3da       | his4 leu2 trp1 ura3 MATa                       | 15          |
| MH338-2a-m    | Complete protophot (random integration of URA3) MATa | 15          |
| JK9-3da b1b1  | his4 leu2 trp1 ura3 ura::MATX MATa            | This study  |
| JK9-3da a1b3  | his4 leu2 trp1 ura3 ura::MATX MATa            | This study  |
| JK9-3da b1b4  | his4 leu2 trp1 ura3 ubi::LEU2 MATa            | This study  |
| JK9-3da b1b5  | his4 leu2 trp1 ura3 ubi::kanMX4 MATa          | This study  |
| JK9-3da a1b5  | his4 leu2 trp1 ura3 ubi::kanMX4 MATa          | This study  |
| JK9-3da d15   | his4 leu2 trp1 ura3 lcb5::phmXX MATa          | This study  |
| JK9-3d Tat1   | his4 leu2 trp1 ura3 tat::MATa MATa            | This study  |

**RNA Preparation and DNA Microarrays**—Cells were grown overnight and adjusted to an A600 of 0.1 in 50 ml of YPD medium. They were then grown at 30 °C to an A600 of 0.4–0.5 and split into 7-ml aliquots. Each sample was performed in duplicate. Samples were incubated with either 15 μM FTY720 or 25 μM PHS for 1 h. In the control cultures, the compound was replaced by an equivalent volume of MeSO. Total RNA was extracted from four independent cultures of each strain and hybridized onto a high-density oligonucleotide microarray. 

**Sensitivity Assay of Yeast Strains**—To test the sensitivity of our strains to the different compounds, a small amount of cells from a single fresh colony was streaked using different toothpicks. Plates were incubated for 2–3 days at 30 °C. The resistance of the strain was measured by the formation of colonies in the presence of the compound. The growth inhibitory concentration of FTY720 had to be readjusted from 30 to 15 μM when we changed the batch of YPD medium (Difco). The concentration of 15 μM corresponds, as in our previous work (15), to the concentration at which the growth of JK9-3d parent strain is inhibited and the bul1Δ mutant is growth-resistant.

**Construction of Mutant Strains**—The mutants were constructed using the PCR-based method as described (17) with the pFA6a (G418) (17), pAG25 (nourseothricin), and pAG2 (hygromycin) (18) resistance-confering plasmids. The oligonucleotides used for the PCRs were chosen to generate nucleotides upstream of the start codon and ~100 nucleotides beyond the stop codon of the different genes.

**Immunoblotting**—The parent strain JK9-3d and the mutants JK9-3d bul1Δ, JK9-3d 4Δ, and JK9-3d tat1Δ were transformed with the vector pRS426 containing the HA-tagged TAT1 (pCAW15) or TAT2 (pS555) gene. Transformed cells were grown to an early logarithmic phase and treated with 25 μg PHS for 1 h. Total proteins were extracted and quantified using a protein microarray (Pierce). 15 μg of total protein was loaded per well, and immunoblotting was performed as described previously (15). The protein loading was checked by Coomassie Blue staining followed by quantitation. Tagged proteins were detected using a mouse anti-hemagglutinin antibody (clone 16B12; Covance).

**DNA Microarrays**—Cells were grown overnight and adjusted to an A600 of 0.1 in 50 ml of YPD medium. They were then grown at 30 °C to an A600 of 0.4–0.5 and split into 7-ml aliquots. Each sample was performed in duplicate. Samples were incubated with either 15 μM FTY720 or 25 μM PHS for 1 h. In the control cultures, the compound was replaced by an equivalent volume of MeSO. Total RNA was extracted from four independent cultures of each strain and hybridized onto a high-density oligonucleotide microarray. 

**cDNA**—cDNA was synthesized from mRNA using Superscript II reverse transcriptase (Invitrogen) and a T7 oligo/dT24 primer. Second strand cDNA synthesis was performed by incubating the cDNA product at 16 °C for 2 h with Escherichia coli DNA polymerase and an E. coli DNA ligase in the presence of E. coli RNase H (Invitrogen). cDNA was extracted with phenol/chloroform/isoamyl alcohol and precipitated with ammonium acetate. cRNA was synthesized from the double strand purified cDNA in the presence of a mixture of unlabeled ATP, GTP, CTP, UTP, and biotin-labeled UTP and CTP using the Enzo Biosarray™ high yield RNA transcript-labeling kit and T7 RNA polymerase. Samples were purified using the RNeasy Mini kit from Qiagen following the manufacturer’s instructions for the enzymatic lysis protocol. This procedure was repeated independently on two different days, and purified RNA was pooled. RNA quality was analyzed using an Agilent 2100 Bioanalyzer.
RESULTS AND DISCUSSION

PHS- and FTY720-mediated Growth Inhibition Is Reversed by the Same Ubiquitin Pathway Proteins—In our previous work (15) we showed that S. cerevisiae cells transfected to overexpress the ubiquitin-specific protease UB5 and UB11 (mutants lacking the RSP5 ligand BUL1, which is purported to be involved in ubiquitination target specificity, or lacking the ubiquitin structural gene UB4) are resistant to FTY720. Here we show that each of these overexpressing strains and mutants are also resistant to a 25 μM concentration of PHS (Figs. 1 and 2). In addition, we have constructed novel strains carrying a deletion of BUL2, a BUL1 homolog, and UB11 and UB12, ubiquitin structural genes from the same family as UB4, and have observed that only the bul2 mutant is resistant to both FTY720 and PHS, whereas ub1 and ub12 mutants stayed sensitive to these compounds (Fig. 2 and Table IV).

Specific Amino Acid Transporters Confer Resistance to Growth Inhibition by PHS and FTY720—Skrzypek et al. (11) have shown previously that PHS inhibits the growth of S. cerevisiae. It is further shown that sphingolipids can affect amino acid transport. Upon treatment with PHS, the uptake of radiolabeled tryptophan, histidine, leucine, and uracil was strongly decreased (12). An identical effect with tryptophan and leucine was seen with exposure to FTY720 (15). We report that, as with FTY720, the overexpression of TAT1 and TAT2 confers resistance to PHS (Fig. 1). In further support for the role of amino acid transport in PHS toxicity, it was reported that full prototrophic strains are more resistant to PHS than is a tryptophan auxotrophic strain (11). We show here that, as for FTY720, a prototrophic strain needs roughly a 3-fold higher concentration of PHS than does the isogenic auxotrophic strain (Fig. 3). In addition, because none of the targets that can rescue the auxotrophic strain are able to rescue the prototroph growth inhibition, we hypothesize that other targets, besides the ones identified to date, are involved in the mechanisms of action of both FTY720 and PHS.

We have generated a TAT1 deletion strain in an auxotrophic background and observed that it retains the same sensitivity to both FTY720 and PHS as the parent (Table IV). This suggests that TAT1 multicopy suppression is not due to drug export. A TAT2 deletion strain was not constructed, as this mutant was reported to grow poorly if made in an auxotrophic background (20).

During our previous work we also identified a mutation in the glutamine permease GNP1, which conferred resistance to FTY720. We therefore tested whether this mutation conferred resistance to phytosphingosine. As with FTY720, this mutant is resistant to PHS (Table IV).

Other evidence that supports the role of transporters in PHS resistance include the proposal that the uracil permease FUR4 is degraded in the presence of PHS (13). This degradation was also reported to require the presence of the ubiquitin ligase RSP5 and the deubiquitinating enzyme DOA4, potentially linking control of ubiquitination with amino acid transport in PHS-induced toxicity. Lastly, PHS was shown to induce polyubiquitination, although UB4 was not clearly shown to be involved in these processes (13).

Exposure to PHS Leads to a Decreased Level of the TAT1 Protein but Not of TAT2 in S. cerevisiae—Because over-expression of TAT1 and TAT2 can rescue growth inhibition by FTY720 and PHS and because tryptophan transport is reduced in the presence of both compounds, it appears logical that FTY720 and PHS might influence the protein expression level of these transporters. Our results here demonstrate that, after treatment with 25 μM PHS, the TAT1 protein level is decreased in the four isogenic strains, i.e. JK9-3d, bul1Δ, bul2Δ, and
### TABLE II

**Affymetrix microarray quality information**

The quality features for both replicates (1) and (2) of each sample as well as the average and S. D. (Stddev) of all the samples are presented. Each feature is described under “Experimental Procedures.”

| Microarray | JK9-3d (1) | JK9-3d (2) | JK9-3d + F (1) | JK9-3d + F (2) | JK9-3d + P (1) | JK9-3d + P (2) | bull1 (1) | bull1 (2) | bull1 + F (1) | bull1 + F (2) | bull1 + P (1) | bull1 + P (2) | Average | Stddev |
|------------|------------|------------|---------------|---------------|---------------|---------------|------------|------------|---------------|---------------|---------------|---------------|--------|-------|
| Noise      | 1.79       | 1.58       | 1.75          | 1.51          | 1.73          | 1.71          | 1.73       | 1.54       | 1.61          | 1.56          | 1.61          | 1.32          | 1.62    | 0.13  |
| Scaling factor | 0.81      | 0.84       | 0.46          | 0.56          | 0.6           | 0.47          | 0.42       | 0.53       | 0.52          | 0.49          | 0.48          | 0.48          | 0.58    | 0.15  |
| Background | 52.34      | 47.01      | 49.96         | 44.21         | 50.25         | 48.23         | 44.45      | 45.56      | 48.33         | 44.76         | 39.55         | 47.3          | 3.85    |
| Present calls (P) | 7608     | 7804       | 7826          | 7779          | 7615          | 7836          | 7874       | 7783       | 7898          | 7906          | 7906          | 7710          | 7790    | 99.41 |
| Marginal calls (M) | 134      | 130        | 96            | 127           | 134           | 97            | 123        | 123        | 100           | 112           | 114           | 113           | 116.92  | 13.81 |
| (P + M)/all calls (%) | 82.94    | 84.99      | 84.86         | 84.69         | 83.01         | 84.98         | 85.67      | 84.69      | 85.68         | 85.2          | 85.91         | 83.8          | 84.7    | 0.98  |
| BioB-3_at | 95.80 (P)  | 90.70 (P)  | 54.00 (P)     | 55.10 (P)     | 74.20 (P)     | 83.50 (P)     | 58.10 (P)  | 128.30 (P) | 85.90 (P)     | 114.70 (P)    | 67.60 (P)     | 94.60 (P)     | 83.54   | 23.3  |
| BioB-M_at | 166.00 (P) | 138.60 (P) | 77.90 (P)     | 67.40 (P)     | 89.90 (P)     | 112.60 (P)    | 97.00 (P)  | 178.60 (P) | 100.50 (P)    | 129.60 (P)    | 86.10 (P)     | 121.70 (P)    | 113.82  | 34.5  |
| BioB-5_at | 56.30 (P)  | 56.10 (P)  | 33.10 (P)     | 36.30 (P)     | 46.30 (P)     | 40.00 (P)     | 35.00 (P)  | 64.50 (P)  | 43.20 (P)     | 58.10 (P)     | 41.50 (P)     | 54.60 (P)     | 47.15   | 10.55 |
| Ratio: 3/5’ of BioB-X_at | 1.7      | 1.62       | 1.63          | 1.56          | 1.6           | 2.09          | 1.66       | 1.99       | 1.99          | 1.97          | 1.63          | 1.73          | 1.765   | 0.188 |
| Ratio: 3/M of BioB-X_at | 0.577    | 0.654      | 0.693         | 0.818         | 0.825         | 0.742         | 0.999      | 0.718      | 0.855         | 0.885         | 0.785         | 0.777         | 0.744   | 0.099 |
| BioC-3_at | 175.80 (P) | 178.50 (P) | 95.60 (P)     | 67.40 (P)     | 112.80 (P)    | 111.10 (P)    | 104.30      | 177.80 (P) | 123.40 (P)    | 130.90 (P)    | 103.30 (P)    | 124.10 (P)    | 125.42  | 35.28 |
| BioC-5_at | 203.60 (P) | 192.50 (P) | 96.10 (P)     | 78.70 (P)     | 115.50 (P)    | 114.20 (P)    | 104.20      | 211.80 (P) | 133.40 (P)    | 151.40 (P)    | 115.00 (P)    | 143.10 (P)    | 138.32  | 43.64 |
| Ratio: 3/5’ of BioC-X_at | 0.583    | 0.927      | 0.985         | 0.856         | 0.974         | 0.973         | 1          | 0.839      | 0.925         | 0.865         | 0.898         | 0.867         | 0.915   | 0.059 |
| BioDn-3_at | 1087.00 (P)| 1218.20 (P)| 685.20 (P)    | 607.80 (P)    | 918.50 (P)    | 807.60 (P)    | 794.50      | 1148.10 (P)| 1069.50 (P)   | 1138.20 (P)   | 809.70 (P)    | 989.20 (P)    | 939.46  | 198.22 |
| BioDn-5_at | 263.60 (P) | 284.70 (P) | 143.60 (P)    | 114.30 (P)    | 157.60 (P)    | 165.20 (P)    | 154.20      | 228.00 (P) | 185.80 (P)    | 209.90 (P)    | 153.00 (P)    | 189.60 (P)    | 187.68  | 50.78 |
| Ratio: 3/5’ of BioDn-X_at | 4.12     | 4.28       | 4.77          | 5.32          | 5.83          | 4.89          | 5.15       | 5.04       | 5.67          | 5.42          | 5.29          | 5.084         | 5.22    | 0.509 |
| CraX-3_at | 2937.40 (P)| 2826.80 (P)| 1472.10 (P)   | 1566.80 (P)   | 1937.80 (P)   | 1974.40 (P)   | 1907.40     | 2467.10 (P)| 1766.40 (P)   | 2424.90 (P)   | 1628.00 (P)   | 2182.50 (P)   | 2090.97 | 483.12 |
| CraX-5_at | 4411.70 (P)| 3664.90 (P)| 1645.10 (P)   | 1264.60 (P)   | 1595.30 (P)   | 1844.30 (P)   | 1712.90     | 2883.70 (P)| 1512.60 (P)   | 1926.40 (P)   | 1292.10 (P)   | 2033.00 (P)   | 2148.88 | 990.2  |
| Ratio: 3/5’ of CraX-X_at | 0.666    | 0.771      | 0.885         | 1.24          | 1.21          | 1.07          | 1.11       | 0.856      | 1.17          | 1.26          | 1.07          | 1.049         | 0.204   |
FTY720 and Phytosphingosine

We have used reverse (RT) PCR methods to validate the changes in expression levels of a subset of genes, including those described under “Results and Discussion,” and have found that the gene regulation (up, down, or unchanged) in response to the compound treatment could be confirmed, although the absolute values are not identical.

| Genes | Samples | Expression tendency | Fold change gene chips | Fold change RT-PCR |
|-------|---------|---------------------|------------------------|-------------------|
| APG2  | JK9–3d FTY720/PHS | Up-regulated | +2.5 | +4.6 |
| AMS1  | Bul1 ± PHS | Up-regulated | +6.0 | +4.9 |
| BEM2  | JK9–3d FTY720/PHS | Down-regulated | −1.3 | −2.6 |
| BRX1  | JK9–3d ± PHS | Down-regulated | −3.3 | −2.1 |
| CCT2  | Bul ± FTY720 | Down-regulated | −2.3 | −23.8 |
| CHA1  | JK9–3d ± FTY720 | Unchanged | +1E-01 | +6E-02 |
| COX5B | JK9–3d ± PHS | Up-regulated | +7.1 | +13.5 |
| ERF25 | JK9–3d FTY720/PHS | Down-regulated | −1.3 | −4.0 |
| GCD7  | JK9–3d ± FTY720 | Unchanged | −8E-02 | −2E-03 |
| GDH3  | JK9–3d ± PHS | Up-regulated | +2.1 | +25.0 |
| GIC2  | JK9–3d FTY720/PHS | Down-regulated | −2.1 | −3.8 |
| HOC1  | Bul1 ± PHS | Down-regulated | −3.3 | −1.9 |
| IMD3  | JK9–3d ± FTY720 | Down-regulated | −2.8 | −4.3 |
| LCB1  | JK9–3d ± FTY720 | Down-regulated | −2.3 | −23.9 |
| RME1  | JK9–3d ± FTY720 | Up-regulated | +4.5 | +11.0 |
| RSA3  | JK9–3d ± FTY720 | Down-regulated | −4.2 | −2.5 |
| SHR3  | Bul1 ± FTY720/PHS | Unchanged | −8E-02 | +6E-02 |
| SIP18 | Bul ± FTY720 | Up-regulated | +13.6 | +44.0 |
| UBP11 | Bul ± FTY720 | Down-regulated | −3.4 | −1.4 |
| URA7  | JK9–3d ± FTY720 | Down-regulated | −4.7 | −3.8 |
| VSP33 | JK9–3d FTY720/PHS | Down-regulated | −1.3 | −3.0 |

ub1A (Fig. 4A). The TAT2 protein level stays stable in the bul1A, bul2A, and ub1A mutants and is only slightly increased in the parent strain JK9-3d (Fig. 4B). This finding is similar to what we observed after treatment with FTY720 (15), except that FTY720 appeared to have a more dramatic effect in up-regulating the expression of TAT2 in the parent strain. These results are supported by previous data wherein the inhibition of tryptophan import and, hence, the growth inhibition caused by PHS were proposed to be more the consequences of the decrease of the GAP1 protein than of TAT2. This is presumed because the TAT2 protein level stayed unchanged upon treatment (12). The possibility was raised that PHS could inhibit other specific amino acid transporters besides TAT2 (11). We propose that one of these additional transporters could be TAT1.

The Heat Shock Protein Homolog CAJ1 Rescues FTY720-induced but Not PHS-induced Growth Inhibition—We identified CAJ1, a yeast homologue of the E. coli DnaJ heat shock protein, from the multicopy suppressor screen of FTY720-induced growth inhibition. CAJ1 gene overexpression, however, could not rescue PHS-induced growth inhibition and is therefore specific for FTY720 resistance (Fig. 1).

Thus FTY720-mediated growth sensitivity and the yeast natural sphingolipid PHS-mediated growth inhibition have in common their effects on the ubiquitin pathway and amino acid transport. CAJ1 seems to belong to a third functional group that is not shared by both compounds.

Sphingosine Kinase Mutants Are Resistant to Sphingosine but Not to FTY720 or PHS—Sphingosine is the mammalian equivalent of yeast phytosphingosine. Phytosphingosine is produced through the metabolism of the intermediate DHS. In tissue culture and in vivo, sphingosine and FTY720 are phosphorylated by the same families of sphingolipid kinases and dephosphorylated by the same family of sphingolipid phosphatases (8, 9). At a high concentration there is evidence that the non-phosphorylated form may act as an intracellular second messenger influencing apoptosis mechanisms (8, 21). The phosphorylated sphingosine S1P binds with nanomolar affinity to the S1P family of G protein-coupled receptors (21). FTY720-phosphatase binds and activates four of the five S1P receptors (8, 9).

The major intracellular consequences of S1P binding in many cell types are intracellular calcium release, inhibition of cAMP production, and activation of the kinases involved in regulating actin cytoskeletal dynamics (21). S. cerevisiae has many sphingolipid-metabolizing enzymes that can also be active on mammalian sphingosine and FTY720 (22). The effects of several of these genes on cell growth and other cellular functions have been identified previously (12, 23–25).

A comparison of mutant strains shows that those lacking the functional sphingosine kinase LCB4 (lcb4Δ mutant) or LCB4 and LCB5 (lcb4Δlcb5Δ) are resistant to sphingosine but not to FTY720, PHS or DMS, an effective inhibitor of sphingosine kinase activity (26) and a natural metabolite of sphingosine

FTY720 and Phytosphingosine

FIG. 1. Several FTY720 multicopy suppressor genes suppress PHS growth inhibition. Like FTY720, PHS caused S. cerevisiae growth arrest. With the exception of the CAJ1 gene, most of the multicopy suppressors of FTY720 growth sensitivity also made the strain resistant to phytosphingosine. The parent JK9-3d was transformed with pRS426 (pCAW5), TAT1 (pCAW6), TAT2 (pCAW7), UBP5 (pCAW8), UBP11 (pCAW9), and CAJ1 (pCAW10), and the transformants were tested on solid media plates containing either FTY720 or PHS.

FIG. 2. Bul1 and bul2 disruption mutants are resistant to both FTY720 and PHS. JK9-3d, a strain missing either Bul1, an Rsp5-binding protein or Bul2, a BUL1-homologue, is resistant to 15 μM FTY720 and 25 μM PHS.
more resistant to PHS than the isogenic observed with FTY720, the prototroph is of the parent strain.

gave the same results. The indicated concentration of each compound represents the minimal concentration sufficient to have the growth inhibition /H9262

a sphingosine concentration of 10 /H9262

MH338-2a-u was resistant to phyto-

mutant strains showed the same resistance/sensitivity to FTY720 and PHS. Nevertheless, some mutants showed some sensitivity difference between FTY720 and sphingosine or between FTY720 and DMS. The sensitivity to dihydrosphingosine was also tested up to a concentration of 100 /H9262

phorylatable enantiomers (-(R)-AAL and -(S)-AAL) of an ana-

The observation that both the phosphorylatable and non-phos-

phorylation plays a significant role in the yeast tox-

These results suggest that either other kinases are responsible

does not render the strain resistant to sphingosine. A mutant for the phosphatase YSR3 is resistant to sphingosine and PHS. These results suggest that either other kinases are responsible for the phosphorylation of FTY720, PHS, and DMS in yeast or that phosphorylation plays a significant role in the yeast toxicity of only sphingosine and not that of FTY720, PHS, or DMS. The observation that both the phosphorylatable and non-phosphorylatable enantiomers (-(R)-AAL and (S)-AAL) of an analogue of FTY720 (8) also inhibit yeast growth (data not shown) suggests that FTY720 growth inhibition is independent of its phosphorylation. Nevertheless, this result does not exclude the possibility that both non-phosphorylated and phosphorylated forms of FTY720 can kill yeast and that other enzymes besides the ones that were knocked out in our study are involved in growth sensitivity.

The reversal of the PHS growth inhibitory effect upon the deletion of YSR3 that we have observed seems to be in contra-

some experimental differences might explain this discrepancy.

Sensitivity of S. cerevisiae gene disruption mutants to FTY720, PHS, sphingosine, and DMS

| Strains       | 15 μM FTY720 | 25 μM PHS | 15 μM Sphingosine | 15μM DMS | Description                                      |
|---------------|-------------|-----------|------------------|----------|-------------------------------------------------|
| JK9-3da      | –           | +         | –                | <±       | Parent strain                                   |
| JK9-3da bul1Δ| +           | –         | –                | ±        | Deletion of BUL1, RSP5 ligand                   |
| MH272-1d bul2Δ| +           | –         | –                | ±        | Deletion of BUL2, RSP5 ligand                   |
| JK9-3da ubi1Δ| –           | +         | –                | ±        | Deletion of UBI1, ubiquitin structural gene     |
| JK9-3da ubi2Δ| –           | –         | +                | ±        | Deletion of UBI2, ubiquitin structural gene     |
| JK9-3da ubi4Δ| +           | +         | +                | ±        | Deletion of UBI4, ubiquitin structural gene     |
| MH272-1d gnp1| –           | –         | +                | ±        | Deletion of LCB3, dihydrosphingosine-1-P phosphatase |
| JK9-3da lcb4Δ| –           | –         | –                | ±        | Deletion of LCB4, sphingosine kinase            |
| JK9-3da lcb5Δ| –           | –         | –                | ±        | Deletion of LCB5, sphingosine kinase            |
| JK9-3da lcb4A lcb5Δ| – | – | – | ± | Deletion of LCB4 and LCB5, sphingosine kinases   |
| JK9-3ds dpl1Δ| –           | –         | +                | ±        | Deletion of DPL1, dihydrosphingosine-P lyase    |
| JK9-3ds ysr3Δ| –           | +         | –                | ±        | Deletion of YSR3, dihydrosphingosine-P phosphatase |
| JK9-3ds tat1Δ| –           | –         | –                | ±        | Deletion of TAT1, amino acid transporter         |

* Resistant at 10 μM where the parent is already inhibited.

Fig. 3. The prototrophic strain MH338-2a-u was resistant to phytosphingosine (25 μM). As was previously observed with FTY720, the prototroph is more resistant to PHS than the isogenic auxotroph.

(27) (Table IV). It is interesting to note the differential sensitivity of the two kinase mutants with respect to sphingosine. LCB4 is the major sphingosine kinase in yeast, and its deletion reverses growth sensitivity to sphingosine (Table IV). The deletion of the minor kinase LCB5 did not alter sensitivity, and the double disruption behaves like the single LCB4 gene deletion (Table IV). Finally, the absence of the phosphatase LCB3 does not render the strain resistant to sphingosine. A mutant for the phosphatase YSR3 is resistant to sphingosine and PHS. These results suggest that either other kinases are responsible for the phosphorylation of FTY720, PHS, and DMS in yeast or that phosphorylation plays a significant role in the yeast toxicity of only sphingosine and not that of FTY720, PHS, or DMS. The observation that both the phosphorylatable and non-phosphorylatable enantiomers (-(R)-AAL and (S)-AAL) of an analogue of FTY720 (8) also inhibit yeast growth (data not shown) suggests that FTY720 growth inhibition is independent of its phosphorylation. Nevertheless, this result does not exclude the possibility that both non-phosphorylated and phosphorylated forms of FTY720 can kill yeast and that other enzymes besides the ones that were knocked out in our study are involved in growth sensitivity.

The reversal of the PHS growth inhibitory effect upon the deletion of YSR3 that we have observed seems to be in contra-

diction with results reported by Chung et al. (12). However, some experimental differences might explain this discrepancy.
FIG. 5. An experimental clustering of the microarray samples shows the relationship between the individual samples.

Table V

| Pathway          | Gene name | Function                     | p value FC (FTY720) | p value FC (PHS) |
|------------------|-----------|------------------------------|---------------------|------------------|
| Mating           | AGA1      | α-Agglutinin                 | 2.27E-03            | -2.7             | 2.00E-04         | -5.7             |
|                  | CSN12     | Adaptation to pheromone signalling | 3.46E-03            | -3.2             | 2.80E-03         | -2.8             |
| Amino acid metabolism-related genes | SNO1      | Pyridoxine metabolism       | 4.52E-03            | +20.7            | 1.10E-02         | +36.5            |
|                  | GDH3      | Glutamate biosynthesis      | 1.11E-03            | +2.1             | 2.80E-03         | +2.3             |
| Respiration      | COX5B     | Cytochrome oxidase chain    | 3.08E-03            | +12.0            | 3.80E-03         | +7.1             |
| Fatty acid metabolism | AGP2     | Camitine transporter       | 1.65E-03            | +2.5             | 1.60E-03         | +2.1             |
| Nucleotides metabolism | URA7     | Pyrimidine biosynthesis    | 4.26E-03            | -4.0             | 2.10E-03         | -4.2             |
|                  | IMD3      | Purine biosynthesis        | 5.90E-04            | -2.8             | 4.00E-04         | -2.2             |
| Ribosome biogenesis | RSA3     | Ribosome assembly          | 4.52E-03            | -4.2             | 1.20E-03         | -4.4             |
|                  | BRX1      | Ribosome synthesis         | 4.72E-03            | -3.0             | 1.10E-03         | -3.3             |
|                  | NOP13     | Ribosome synthesis         | 3.20E-03            | -2.5             | 2.40E-03         | -2.4             |
| Energy           | ATP11     | Chaperone                   | 3.61E-03            | -2.6             | 1.00E-03         | -2.6             |
|                  | YTA7      | ATPase                      | 4.03E-03            | +3.0             | 1.80E-03         | +2.8             |
| Unknown function | FUN11     | Unknown                     | 5.59E-04            | -5.2             | 8.00E-04         | -7.5             |
|                  | GAS3      | Unknown                     | 3.89E-03            | -5.3             | 8.00E-04         | -5.1             |
|                  | YMR069W   | Unknown                     | 1.54E-03            | -3.8             | 1.50E-03         | -4.5             |
|                  | NLR079C   | Unknown                     | 3.71E-03            | -3.8             | 4.50E-03         | -3.2             |
|                  | FUT19     | Unknown                     | 4.18E-03            | +3.1             | 2.20E-03         | +2.5             |
|                  | ZTA1      | Unknown                     | 2.30E-03            | -6.3             | 3.50E-03         | -4.7             |
|                  | YBLO48W   | Unknown                     | 3.32E-03            | +5.0             | 5.00E-04         | +5.4             |

These genes are significantly (p < 0.005) differentially regulated at least 2-fold in the JK9–3d strain upon treatment with 15 μM FTY720 and 25 μM PHS. The p value and the fold change (FC) obtained for each gene between untreated samples and samples treated with FTY720 and PHS are reported.

The JK9-3d α strain using the nourseothricin resistance gene. Thus, our strain backgrounds are slightly different. However, we have also observed at 30 μM PHS (unlike at 20 and 25 μM) that growth of both parent and mutant strains remained sensitive. Thus, the window for sensitivity is rather narrow. Our results indicate that the growth advantage of the YSR3 deletion is observable at a lower concentration (up to 25 μM in our conditions), suggesting a differential sensitivity and a growth advantage as compared with the isogenic parent.

Another interesting observation is that bul1Δ mutant strain is resistant to FTY720, PHS, and DMS but is sensitive to sphingosine, whereas a mutant for BUL2 (bul2Δ) is only sensitive to DMS (Table IV). Overall, these results suggest that the metabolisms and targets of PHS and FTY720 are more similar than those of sphingosine and DMS.

With respect to DMS, we have observed that the bul1Δ, ubi4Δ, and gnp1Δ mutants were resistant to N,N-dimethylsphingosine under conditions where the parent was sensitive. Interestingly, bul2Δ is hypersensitive to growth inhibition, whereas bul1Δ is more resistant (Table IV). This pattern is inverse to that observed with sphingosine, suggesting again that we are looking at discrete effects mediated by similar yet distinct compounds of the same family. Because DMS inhibits growth and because the sphingosine kinase double mutant is not resistant, one can conclude that DMS interferes with targets other than these two sphingosine kinases.

A significant difference between these results and what is reported in the literature is that DHS did not inhibit the growth of JK9-3d. DHS was claimed to be as or more efficient than sphingosine under conditions where the parent was sensitive. Another interesting observation is that bul1 Δ mutant strain is resistant to FTY720, PHS, and DMS but is sensitive to sphingosine, whereas a mutant for BUL2 (bul2Δ) is only sensitive to DMS (Table IV). Overall, these results suggest that the metabolisms and targets of PHS and FTY720 are more similar than those of sphingosine and DMS.

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A significant difference between these results and what is reported in the literature is that DHS did not inhibit the growth of JK9-3d. DHS was claimed to be as or more efficiently imported and metabolized by yeast cells than phytosphingosine (12). In addition, even though growth inhibition in the presence of phosphorylated compounds has been extensively reported (23, 24), concentrations as high as 100 μM of the phosphorylated compounds FTY720-P, PHS-P, or S1P did not inhibit JK9-3d strain growth in our hands (data not shown). This difference can be explained by the fact that exogeneous phosphorylated compounds were tested. Previous work tested only indirectly the effect of the accumulation of...
these substances. We can therefore not exclude the possibility that phosphorylated sphingolipids cannot be imported into the cells. It is important to note the fact that several mutants behave identically in their response to FTY720 and PHS while exhibiting differential sensitivity to the close mammalian homologue of PHS (sphingosine) and to DMS. This suggests that FTY720 may be more like PHS in its cellular action. In this context it is interesting to mention that Candelore et al. (28) have shown that PHS-P binds to S1P4 receptor much more effectively than does S1P.

**FTY720 and PHS Treatments Do Not Induce Massive Changes in Gene Expression**—To further elucidate the responses of the FTY720 and PHS treatment in yeast cells, we have compared the transcriptional effect of both compounds on the FTY720- and PHS-sensitive parent strain JK9-3d and on the FTY720- and PHS-resistant bul1/H9004 mutant using Affymetrix microarrays.

The JK9-3d parent strain and the bul1 mutant were treated with FTY720 and PHS for 1 h, and total RNA from untreated and treated samples was extracted for expression profiling by gene chips (see “Experimental Procedures”). The data were analyzed using GeneSpring software (Silicon Genetics) with the primary focus on those open reading frames whose function is reported in the Stanford Genome Data Base and in the literature. Analysis of the data was based on fold change (minimum 2-fold) combined with the statistical significance of the change (Welch t test) after an initial filtering for genes with a robust expression. The level of fold change was confirmed by reverse transcription PCR with selected genes (Table III).

After the application of these selection criteria, 87 open reading frames were found to be differentially regulated (up and down) by FTY720 in the parent strain, and 97 were found to be influenced by PHS (up- and down-regulation) (see supplementary data in the on-line version of this article). These numbers indicate that the two compounds did not induce massive quantitative changes at the transcriptional level under the present experimental conditions and the above analysis criteria. This observation is consistent with our previous proposition that the primary effects of FTY720 is at the post-transcriptional level (15). However, it is important to emphasize that we are looking at a snapshot of expression profiles that are pertinent to the present experimental conditions but do not possibly encompass all potential changes influenced by the varying experimental conditions such as changes in the concentration of these compounds as well as the duration of the treatment.

**FTY720 and PHS Largely Induce Similar Changes in Gene Expression in Both the Parent and Mutant Strains**—A clustering experiment was performed to analyze the relationship between the different samples. Clustering all the individual samples resulted in a correct grouping of all duplicates that were clustered close to each other, confirming the quality of the microarray data (Fig. 5).

In addition, the untreated samples of the parent and the mutant strains clustered very close together and were well separated from the rest of the (treated) samples. Interestingly, the PHS-treated mutant sample is the closest to the untreated samples, confirming that the resistant mutant is not much affected by the treatment. The next closest sample in the hierarchy is the FTY720-treated mutant. Clustered together and separated from all the other samples are the parent strains that were treated by PHS and FTY720; these are the samples that are expected to be most affected by the treatment. These results indicate that both compounds induce similar changes in gene expression in the parent strain and that both compounds have clearly less effect on the growth-resistant mutant strain, although the samples were taken at a time point (1 h after treatment) when only slight differences in growth were expected. The relatively short 1-h incubation time point was chosen to enable the detection of the early effects of the compounds on gene expression and to avoid seeing the general effects of growth differences. In addition, Gasch et al. (29) reported that yeast cells respond rapidly (within 1 h) to a wide variety of environmental changes.

A Venn diagram comparing the gene lists based solely on fold regulation confirms that FTY720 and PHS elicit similar transcriptional responses. ~77% of the genes regulated >2-fold by
FTY720 also respond to PHS in the parent (data not shown). When screening the data with both a t test (p < 0.005) and a fold change >2, the percentage of overlap is reduced (Table V) because of the more stringent selection criteria, but the vast majority of the genes that are changed upon treatment with one compound show the same trend in expression after treatment with the other (Fig. 6).

**PHS and FTY720 Have Similar Effects on a Variety of Genes Involved in Amino Acid and Nucleotide Synthesis**—Among the genes whose expression did change significantly and >2-fold upon treatment with both FTY720 and PHS in the parent strain (Table V), several were linked to amino acid and nucleotide biosynthesis. SNO1, up-regulated >20-fold, is involved in the metabolism of pyridoxine, which is the metabolic precursor of pyridoxal phosphate. Pyridoxal phosphate functions as a cofactor of many enzymes in amino acid biosynthesis and was proposed to exert its effect through GCN4, the transcriptional activator of amino acid biosynthetic genes (30). The strong up-regulation of SNO1 can be a response related to the negative effects of both PHS and FTY720 on amino acid uptake (12, 15), as SNO1 encodes a precursor of a cofactor known to increase enzymes of amino acid biosynthesis. PHS was shown previously to induce GCN4 expression (11), but we did not observe a similar induction with either PHS or FTY720 in these studies (data not shown).

GDH3, a glutamate dehydrogenase, is involved in the biosynthesis of glutamate (31), which serves as a precursor to the synthesis of several amino acids (32). Alternatively, glutamate can also be produced from glutamine, a precursor to amino acid as well as purine and pyrimidine biosynthesis. The expression of enzymes involved in glutamate synthesis can coincide with an increased expression of genes involved in the import of amino acids that serve as non-preferred cellular nitrogen sources (32). It is interesting to note that not only do we see increases in gene expression of GDH3 and SNO1 genes, but we reported previously that mutated glutamine permease GNP1 confers resistance to both FTY720 and PHS (15).

Another interesting group of genes commonly regulated by both compounds is associated with the metabolism of nucleotides. URA7 and IMD3 genes involved in pyrimidine and purine biosynthesis, respectively, were found to be down-regulated in both the FTY720- and PHS-treated parent strain (Table V). It has been reported that there is a significant cross-regulation between nucleotide and amino acid biosynthesis pathways. For example, the genes of the purine biosynthesis pathway, namely ADE1, ADE2, ADE5, ADE7, and ADE9, are regulated by the bioavailability of adenine as well as by the transcription factors BAS1 and BAS2, which are also associated with the histidine biosynthetic pathway (33). GCN4, the pan-specific amino acid transcription factor that regulates, among many other genes, histidine biosynthesis genes, is also required for the maximal induction of ADE4. Conversely, purine starvation leads to post-translational de-repression of GCN4 and the subsequent activation of histidine biosynthesis genes (34). The activity of GCN4 itself is also reported to be post-translational activated (32). Supporting this observation, we have also observed that GCD14, a translational repressor of GCN4, is significantly down-regulated (3,3-fold) by treatment with FTY720 (see the supplementary data in the on-line version of this article) though GCD7, a second negative regulator of GCN4 expression that turned out to be unchanged upon treatment with FTY720 in the parent strain (Table III).

Another function group affected by both FTY720 and PHS in the parent strain is the fatty acid metabolism. The AGP2 gene, up-regulated upon FTY720 and PHS treatment, codes for a carnitine transporter. Carnitine is an amino acid derivative that is essential for the oxidation of fatty acids (35). Fatty acids and sphingoid bases are the main components of sphingolipids. Several other genes commonly down-regulated in both FTY720- and PHS-treated parent strains are genes involved in ribosome biogenesis (Table V). These include the ribosome assembly gene RSA3 and the ribosome synthesis genes BRI1 and NOP13. Down-regulation of these genes, along with genes involved in RNA metabolism and the inhibition of protein synthesis are observed in response to multiple cellular stresses and may be a consequence of general environ-

| Pathway                        | Gene name | Function                        | p value  |
|--------------------------------|-----------|---------------------------------|----------|
| Cytoskeleton organization      | BEM2      | Actin cytoskeleton organisation  | 4.75E-03 |
|                                | GIC2      | Axial budding                    | 2.99E-03 |
| Vesicular transport            | VPS33     | Golgi to endosome transport      | 3.09E-03 |
|                                | ERY25     | ER to Golgi transport            | 3.86E-03 |
| Lipids and fatty acids metabolism | ITR1    | myo-Inositol transporter         | 1.38E-03 |
|                                | MCR1      | Ergosterol biosynthesis          | 3.46E-03 |
|                                | TAF4      | RNA polymerase II transcription  | 3.21E-03 |
| Transcription                  | DAT1      | Negative regulator of transcription from POL II promoter | 1.53E-03 |
|                                | ITC1      | Negative regulator of transcription from POL II promoter | 2.63E-03 |
| DNA replication                | MCM2      | DNA replication                  | 7.89E-04 |
|                                | CDC21     | DNA replication                  | 1.34E-03 |
|                                | NUP100    | Nuclear pore complex subunit     | 4.28E-03 |
| Transport                      | STP2      | Branched-chain amino acid uptake | 4.08E-03 |
|                                | ATX2      | Ion transporter                  | 2.63E-03 |
|                                | SUL1      | Sulfate permease                 | 1.33E-03 |
|                                | TE(CUC/D) | tRNA-Glu                        | 3.99E-03 |
|                                | SHM2      | Serine and glycin biosynthesis   | 3.79E-03 |
|                                | GTT1      | Glutathione transferase          | 1.82E-03 |
|                                | K02207    | Centromere                      | 3.89E-03 |
| Unclassified                   | YDL124W   | Unknown                          | 4.07E-03 |
|                                | YNL217W   | Unknown                          | 3.42E-03 |
|                                | YLR199C   | Unknown                          | 3.07E-03 |
|                                | YPS4      | Unknown                          | 1.41E-03 |
|                                | YOL019W   | Unknown                          | 2.75E-03 |
|                                | YNL158W   | Unknown                          | 4.75E-03 |
FTY720 and Phytosphingosine

TABLE VII

| Pathway                  | Gene name | Function                     | p value | FC (FTY720) | p value | FC (PHS) |
|--------------------------|-----------|------------------------------|---------|-------------|---------|----------|
| Cell wall biosynthesis   | HOC1      | Mannosyl transferase         | 4.84E-03| -2.4        | 1.6E-03| -3.3     |
| rRNA modification        | AMS1      | α-mannosidase                | 3.9E-03 | +5.1        | 3.6E-03| +6.0     |
| Protein folding          | GAR1      | Nucleolar protein            | 3.56E-03| -3.7        | 5.5E-04| -3.1     |
| Protein folding          | CCT2      | Chaperonine                  | 1.50E-03| -2.3        | 1.4E-03| -2.1     |
| Unknown function         | YLR179C   | Unknown                      | 9.59E-04| -3.3        | 9.4E-04| -2.91    |
| Unknown function         | CEN9      | Centromere                   | 4.11E-03| -2.2        | 4.9E-04| -3.3     |

The second group in which FTY720 and PHS differentiate from each other in the parent strain is the one that contains vesicular transport genes (VPS33 and ERV25) (Table VI). It is well known that ceramides and sphingolipids are necessary for the vesicular transport of glycosylphosphatidylinositol-anchored proteins from the endoplasmic reticulum to the Golgi as reviewed (38). Most yeast cell wall proteins are believed to be glycosylphosphatidylinositol-anchored from their synthesis place in the endoplasmic reticulum to the plasma membrane. Although FTY720 has never been reported to be related to vesicular transport, myriocin, the precursor of FTY720, has been shown to interfere with the ceramides biosynthesis and transport of glycosylphosphatidylinositol-anchored proteins (1). It would be interesting to further dissect the subtle nature of these pathways and analyze these differences.

Another difference between FTY720 and PHS seems to be their effect on lipids and fatty acids metabolism (Table VI). In *S. cerevisiae*, ceramides that are derived from DHS and PHS are modified by the addition of myoinositol phosphate (ITR1 is a myoinositol transporter) to ultimately form sphingolipids (39). In eukaryotic membranes, the existence of microdomains called lipid rafts, which are enriched for sphingolipids and ergosterol (MCR1, ergosterol biosynthesis), has been reported. Lipid rafts were shown to be involved in a variety of cellular processes including endocytosis and transcytosis. Another study using yeast mutant strains that are blocked at different steps in the secretory pathway suggested that raft formation and protein association are initiated in the endoplasmic reticulum for some proteins (40) and in the Golgi for other proteins (41). Our data indicate that FTY720 and PHS have differential effects in these pathways of lipids and fatty acid metabolism. We have also identified genes that would differentiate the two compounds from each other (Table VI). Only 26 genes turned out to be significantly different (by Welch t-test) between FTY720 and PHS. The list was generated by comparing only the sets of FTY720-treated bul1 mutant and PHS-treated bul1 mutant samples without considering the control (untreated bul1 disrupted strain). No restriction on the fold change was applied.

### TABLE VIII

List of genes that were statistically different between the FTY720-treated bul1 mutant strain and the PHS-treated bul1 mutant strain

| Pathway     | Gene name | Function                   | p value |
|-------------|-----------|----------------------------|---------|
| Amino acid metabolism | GDH3     | Glutamate biosynthesis     | 2.61E-03|
|             | LEU1      | Leu biosynthesis           | 4.14E-03|
|             | PRO2      | Proline biosynthesis       | 7.81E-04|
| Stress and detoxification | SGE1     | Drug transport             | 2.10E-03|
|             | SRA1      | Pseudohyphal growth        | 3.09E-03|
| Transcription | PAP1      | mRNA polyadenylation       | 2.35E-03|
|             | RTC2      | Translation regulator activity | 1.86E-03|
| Translation  | SU13      | Translation initiation     | 4.33E-03|
| Ion transport | STV1      | Hydrogen-transporting ATPase | 8.74E-04|
| Unclassified | MEP3      | Ammonium transport         | 1.57E-03|
|             | NKR004C   | snRNA                      | 2.74E-03|
|             | NDR082C   | snRNA                      | 2.04E-03|
|             | NOL015W   | snRNA                      | 1.44E-03|
|             | GHR08     | Centromere                 | 3.31E-03|
|             | GBL04     | Centromere                 | 3.75E-03|
|             | GPH10     | Cell wall organization     | 1.72E-04|
|             | YHL021C   | Unknown                    | 2.56E-03|
|             | YGR161C   | Unknown                    | 3.12E-03|
|             | YMR119W   | Unknown                    | 4.25E-03|
transcriptional effect on LCB1, a subunit of serine palmitoyltransferase (Table III).

In the Mutant Strain, FTY720 and PHS Have Less Effects than in the Parent Strain—Using the same stringent analysis criteria, fewer genes were changed upon treatment with the compound in the mutant strains. 61 genes were changed significantly and 2-fold by FTY720-treatment, although 40 genes were altered by PHS-treatment (see supplementary data in the on-line version of this article). The fact that the expression of far fewer genes is affected by both compounds in the mutant strain as compared with the isogenic parent strain is consistent with the resistance of the mutant. The two lists had six genes in common (Table VII), although most other genes of one list showed the same tendency with the other treatment (Fig. 5). The six genes are HOCl, a mannosyl transferase gene, CCT2, a chaperone gene, GAR1, a leucinerich protein gene, AMS1, an a-mannosidase gene, CEN9, a centromere gene, and YLR179C, a gene with an unknown function.

The common regulation of these genes in the mutant strain can be proposed to be part of the drug resistance mechanism. Indeed, a modification of the composition of the plasma membrane (HOCl and AMS1) has been proposed to affect the permeability to toxic compounds (42).

In the Mutant Strain, FTY720 and PHS Have Different Effects on Amino Acid Biosynthesis—We have identified a set of 22 genes that differentiate the response of the bul1 deletion mutant treated with FTY720 and treated with PHS. The genes were classified into six groups, i.e. amino acid metabolism, stress/detoxification, transcription, translation, ion transport, and unclassified (Table VIII). It is interesting to note that, although the expression of a subset of genes involved in amino acid biosynthesis is altered similarly upon treatment with FTY720 and PHS in the parental strain, there are other genes also involved in amino acid biosynthesis that are regulated differently in the resistant mutant strain. This observation indicates that, although amino acid metabolism is a consequence of both treatments, these genes alone are not responsible for the toxicity. These genes, GDH3, LEU1, PRO2, and RTG2, are listed in Table VIII. RTG2 regulates the transcription of glutamate-repressible genes and was proposed to act upstream of the nitrogen catabolism regulation pathway (43). Cowart et al. (37) have previously identified related GDH1 and LEU2 genes as being transcriptionally dependent on sphingolipid synthesis.

The mutant strain shows a differential response to FTY720 and PHS with detoxification and stress genes as indicated by SGE1 and SRA1 gene expression (Table VIII). The SGE1 gene is coding for a multiple drug resistance protein and is proposed to function as a drug export permease (44), whereas SRA1 is involved in triggering pseudohyphal growth upon environmental stresses.

FTY720 and PHS Do Not Affect TAT1 and TAT2 Transcriptional Levels—As we showed that FTY720 and PHS influence the protein levels of amino acid transporters, we have evaluated whether the transcription level of TAT1 and TAT2 genes was changed upon treatment with FTY720 and PHS. The transcription level of both of these genes was not significantly affected by either FTY720 or PHS treatment (data not shown), thereby supporting the post-translational regulation model involving the ubiquitin pathway proposed for the mechanism of action of FTY720.

Concluding Remarks—Our genetic and biochemical studies on the response of S. cerevisiae to FTY720 and phytosphingosine have enabled us to further explore the mechanisms of action of these compounds and to identify some similarities as well as differences.

By genetic studies, we showed that strains that are insensitive to FTY720- and PHS-mediated growth inhibition largely share the same targets. We have shown that both compounds influence TAT1 similarly, although the effect on TAT2 is different. We showed that TAT1 and TAT2 are not affected transcriptionally by FTY720 or PHS. These findings support our previous model for the mechanism of action of FTY720.

By gene expression profiling, FTY720 and PHS are shown to similarly induce a subset of identical genes. Among them are genes for amino acid and nucleotides biosynthesis, lysosome biogenesis, mating, respiration, and ATPase synthesis.

Our studies also shed light on the relevance of sphingolipid pathway enzymes to the sensitivity pattern of various growth-inhibiting compounds of the sphingolipid family. The deletion of the kinase LC84 influences the sensitivity to sphingosine and does not affect the sensitivity to FTY720 and PHS. Overall, our studies indicate that FTY720 is mimicking the natural yeast molecule PHS in many biological effects in this model system. Because of its unique properties, FTY720, in addition to being an immunomodulating compound, is also proving to be a valuable tool for understanding the biological pathways that are normally regulated by the natural lipid molecules in eukaryotic cells.

Acknowledgments—We thank Michael N. Hall for kindly providing the bul1 mutant strain. In addition, we thank Yurie Hata and genome factory colleagues for excellent support. We also acknowledge William L. Rust and Friedrich Ruaf for useful comments on the manuscript.

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*J. Biol. Chem.* 2004, 279:36720-36731.  
doi: 10.1074/jbc.M406179200 originally published online June 9, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406179200

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