

## The *BST1* Gene of *Saccharomyces cerevisiae* Is the Sphingosine-1-phosphate Lyase\*

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**Sphingolipids elicit a wide variety of eukaryotic cellular responses, most involving regulation of cell growth, differentiation, and apoptosis. Sphingosine 1-phosphate, a sphingolipid catabolite, is mitogenic in fibroblasts and inhibits the chemotactic mobility and invasiveness of human tumor cells. Sphingosine 1-phosphate degradation requires cleavage at the C<sub>2-3</sub> carbon bond by sphingosine phosphate lyase. A yeast genetic approach was used to clone the first sphingosine phosphate lyase gene, *BST1*. *BST1* overexpression conferred resistance to sphingosine in yeast. *BST1* deletion produced sensitivity to exogenous *D*-erythro-sphingosine and phytosphingosine and intracellular accumulation of sphingosine 1-phosphate upon exposure to exogenous sphingosine. This study confirms that sphingoid base metabolism is similar in all eukaryotes and suggests that yeast genetics may be useful in the isolation and identification of other genes involved in sphingolipid signaling and metabolism.**

Sphingolipids and their metabolic derivatives elicit a wide variety of eukaryotic cellular responses (1, 2). Although the stimuli and biological end points differ in each cell type, the role of sphingolipid by-products as second messengers in specific, growth regulatory signal transduction pathways appears to be a universal theme among eukaryotic cells (3). Sphingosine and sphingosine 1-phosphate (S-1-P)<sup>1</sup> are both catabolites of sphingolipid breakdown, which have been shown to modulate DNA synthesis and cellular proliferation in mammalian cells (4). Evidence suggests that S-1-P is largely responsible for these effects. In addition, S-1-P has recently been shown to inhibit the growth, motility, and invasiveness of tumor cells (5, 6). Free sphingosine and S-1-P are maintained at very low levels in mammalian cells (7), consistent with the notion that potent second messengers would be tightly regulated in the absence of a particular stimulus. The mechanism(s) by which the intracellular levels of sphingosine and S-1-P are regulated have not been established. Such control could occur at the

synthetic stage, via regulation of the activities of ceramidases and sphingosine kinase (8). Alternatively, control could occur at the catabolic stage, through regulation of the activity of sphingosine phosphate lyase (9). Sphingolipids exist in yeast where they provide vital yet unknown functions (10). In this study, we cloned the sphingosine phosphate lyase gene through its ability to suppress sphingosine-induced growth suppression when present on a high-copy vector in *Saccharomyces cerevisiae*.

### EXPERIMENTAL PROCEDURES

**Yeast Strains and Transformations**—The following yeast strains were used: SGP3 (*leu2-3,112 trp1 ura3-52 his3 ade8 ras1::HIS3*), JS14 (SGP3 [*pRS202-BST1*]); JS16 (SGP3 *bst1Δ::NEO*); JS29 (SGP3 *bst1Δ::NEO* [*pYES2-BST1*]); Jk93d (*ura3-52 leu2-3,112 his4 trp1 rme1*).

The pRS202 library used in all transformations was constructed by Connelly and Heiter.<sup>2</sup> The vector is a modified version of the pRS306 vector, into which a 2-micron plasmid piece was inserted. Inserts from this library are approximately 6–8 kilobases in length. Wild type yeast were transformed with the high-copy library, selected for uracil prototrophy, and transformants were pooled and replated at a concentration of 10<sup>6</sup> cells/plate onto 1 mM *D*-erythro-sphingosine plates.

**Sphingosine Plate Assay**—Six transformants which grew large colonies on 1 mM *D*-erythro-sphingosine plates were grown in selective medium, and control SGP3 colonies were grown in minimal medium, at 30 °C until saturated. Absorbance at 660 nm was used to correct for small variations in cell concentration between cultures. Serial dilutions were performed, and cells were template-inoculated onto 1 mM *D*-erythro-sphingosine plates, incubated at 30 °C for 48 h.

**Creation of a *bst1Δ* Strain**—The creation of genomic *bst1Δ* alleles was performed by replacing *BST1* with kanMX, which confers resistance to G418. The kanMX marker replaced all sequence between nucleotide 396 and 1770 of the 1770-base pair open reading frame, creating a 1373-base pair deletion. Disruption was confirmed using polymerase chain reaction amplification of genomic DNA from G418-resistant clones, using primers to genomic sequence just 5' and 3' to the region replaced by the disruption.

**Sphingosine Uptake and Metabolism**—Uptake and metabolism studies were performed using [<sup>3</sup>H]sphingosine (NEN Life Science Products). Specific activity of sphingosine was 22 Ci/mmol, 0.1 mCi/ml. Cells were incubated in a volume of 5 ml for varying times with sphingosine at a final concentration of 1 μM, final specific activity of 0.4 mCi/μmol and cell density of 5 × 10<sup>7</sup> cells/ml. Cells were harvested, washed in sterile water, and subjected to Bligh-Dyer extraction (14). Radioactivity present in the aqueous (upper) phase of the Bligh-Dyer extraction of whole cells was determined using a standard scintillation counter. TLC conditions for initial evaluation of sphingosine recovery were butanol:acetic acid:water (3:1:1). Identification of the unknown lipid as sphingosine 1-phosphate was performed under the following conditions, with the resulting *R<sub>F</sub>* values: butanol:water:acetic acid (3:1:1), 0.47; chloroform:methanol:water (60:35:8), 0.22; chloroform:methanol:water:acetic acid (30:30:2:5), 0.33.

**Sphingosine Phosphate Lyase Assay**—Sphingosine phosphate lyase activity was determined essentially as described (11), using unlabeled *D*-erythro-dihydrosphingosine-1-phosphate (Biomol, Plymouth Meeting, PA) and *D*-erythro-dihydrosphingosine [4,5-<sup>3</sup>H]1-phosphate (American Radiolabeled Chemicals, Inc., St. Louis, MO). Specific activity was 100 mCi/mmol.

### RESULTS AND DISCUSSION

We had previously observed that long chain sphingoid bases inhibit proliferation of *S. cerevisiae* cells in liquid culture. To determine whether this inhibition could be employed in a selection scheme, 1 × 10<sup>6</sup> exponentially growing yeast cells were inoculated onto 1 mM *D*-erythro-sphingosine plates and incu-

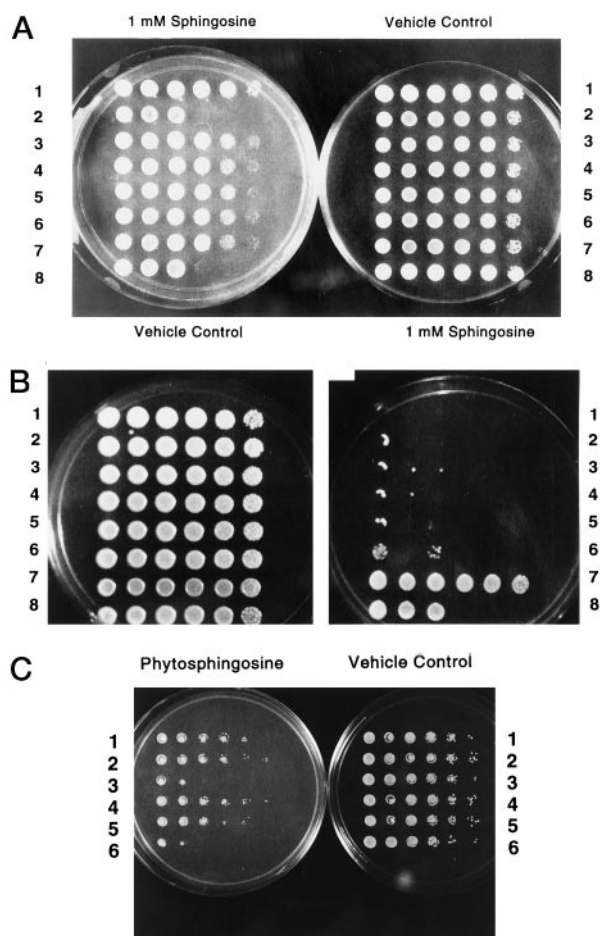
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<sup>1</sup> The abbreviation used is: S-1-P, sphingosine 1-phosphate.

<sup>2</sup> C. Connelly and P. Hieter, unpublished data.



**FIG. 1. Effects of 1 mM D-erythro-sphingosine on *BST1* overexpression and *bst1* $\Delta$  yeast strains.** *A*, overexpression of *BST1*. Lanes 1 and 3–7 represent sphingosine-resistant clones. Lanes 2 and 8 represent wild type SGP3. Dilutions across are 0, 1:2, 1:4, 1:40, 1:400, 1:4000. *B*, *bst1* $\Delta$  strains. Lanes 1–6 represent six different *bst1* $\Delta$  strains. Lane 7 represents the *BST1* overexpression strain JS14. Lane 8 represents wild type SGP3. The *bst1* $\Delta$  strains were subjected to the dilutional plate assay as in *A*. Disruption of *BST1* and phenotypic analysis were also performed in the JK93d strain background. Shown are experiments performed in the original SGP3 strain. *C*, phytosphingosine. *BST1* wild type, overexpression, and *bst1* $\Delta$  strains were template inoculated onto 100  $\mu$ M phytosphingosine or control plates, as in *A*. Lanes 1 and 4 represent JS14; lanes 2 and 5 represent wild type, and lanes 3 and 6 represent JS16.

bated for several days. Under these conditions growth was completely inhibited, whereas no growth inhibition occurred with either L-erythro-sphingosine or stearylamine. The stereospecificity of sphingosine-induced growth inhibition indicated interaction with a biological target or receptor.

Our strategy was to transform wild type yeast cells with a yeast genomic library carried on the pRS202 high-copy shuttle vector (12) containing a selectable nutritional marker (*URA3*) and then to subject pooled transformants to D-erythro-sphingosine selection. Utilizing this approach, we expected to identify *S. cerevisiae* genes involved in sphingolipid signal transduction and/or metabolism. Numerous clones which demonstrated between 100- and 1000-fold greater resistance to sphingosine than the parent strain were identified (Fig. 1A). The most highly represented insert, 13-1, was subcloned and sequenced identifying a novel gene on *S. cerevisiae* chromosome IV, which we named *BST1* (bestower of sphingosine tolerance) (GenBank™ accession number U51031; *S. cerevisiae* genome data base accession number YDR294C). The *BST1* nucleotide sequence encodes a predicted protein of 65,523 kilodaltons and

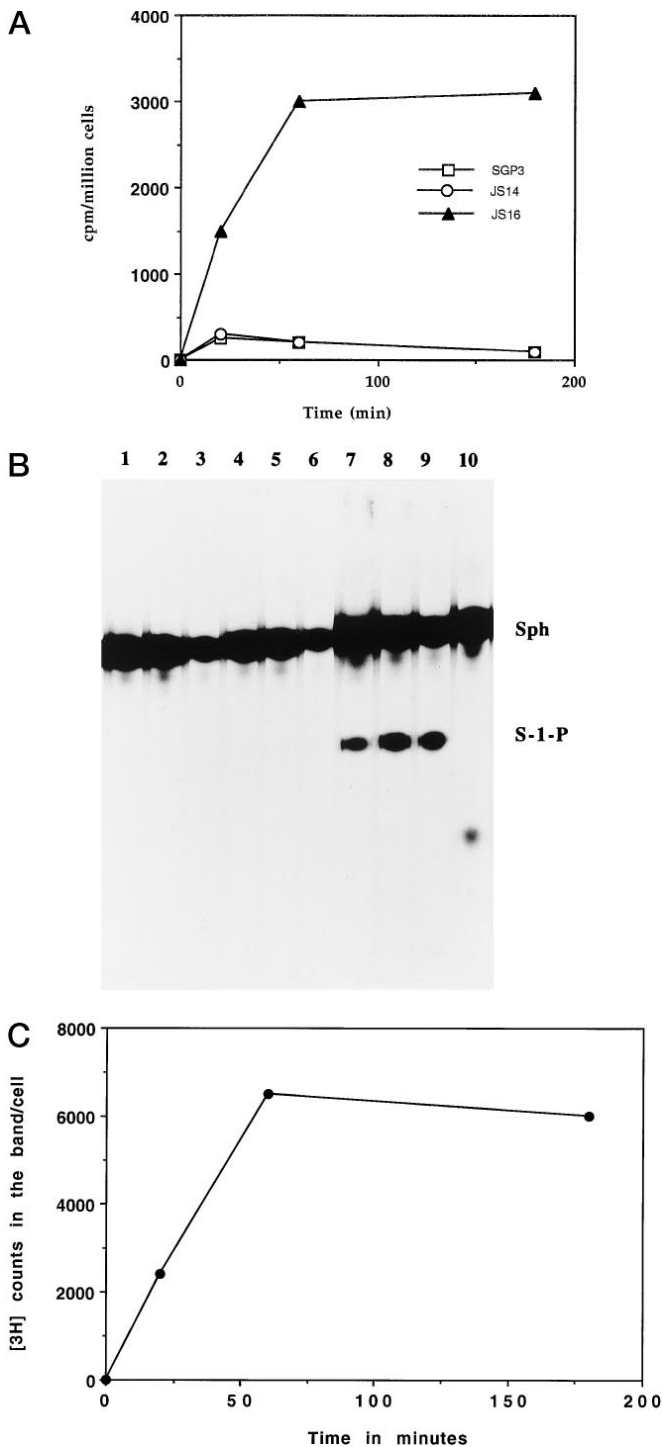
589 amino acids in length. This sequence is 23% identical to *gadA* and *gadB*, two nearly identical *E. coli* genes encoding glutamate decarboxylase, a pyridoxal-5'-phosphate-dependent enzyme that catalyzes synthesis of the neurotransmitter  $\gamma$ -amino butyric acid.

To explore the function of *BST1*, a deletion strain was created through homologous recombination using a *NEO*-selectable marker (13). Whereas our original selection was performed in the SGP3 yeast strain due to its high transformation efficiency, we were concerned that the deletion of the *RAS1* gene in this strain might complicate phenotypic analysis of *BST1* deletion. Therefore, deletion of *BST1* and all subsequent biological studies were performed in both SGP3 and another strain, JK93d. Heterozygous diploids were sporulated and spores segregated 2:2 for G418 resistance. Both G418-resistant and-sensitive progeny were viable, indicating that *BST1* is not an essential gene.

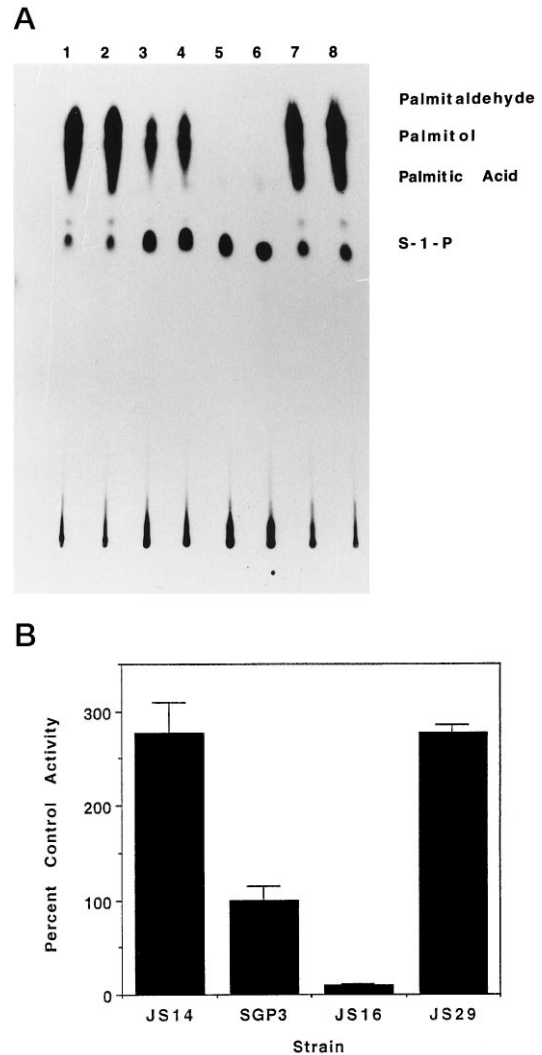
Despite weak nucleotide sequence homology between *BST1* and *gadA/gadB*, analysis of glutamate decarboxylase activity in cytosolic extracts from wild type, *BST1* overexpression and *bst1* $\Delta$  strains refuted the notion that *BST1* encodes the *S. cerevisiae* homologue of glutamate decarboxylase (data not shown). However, deletion of *BST1* was associated with another interesting phenotype, severe sensitivity to D-erythro-sphingosine (Fig. 1B). Concentrations as low as 10  $\mu$ M sphingosine completely inhibited growth of *bst1* $\Delta$  strains but had no effect on the viability of wild type cells. In comparison with the control strain, the *bst1* $\Delta$  strain also demonstrated greater sensitivity to 100  $\mu$ M phytosphingosine, the long chain base endogenous to *S. cerevisiae* (Fig. 1C). We did not appreciate a noticeable difference between the growth of wild type and *BST1* overexpression strains on phytosphingosine, which is only minimally toxic to wild type cells at this concentration.

To determine whether differences in sphingosine uptake or metabolism were responsible for these sensitivity differences, *BST1* wild type, overexpression and *bst1* $\Delta$  strains were exposed to  $^3$ H-labeled sphingosine and subjected to Bligh-Dyer extractions (14). There were no major differences in sphingosine recovery among the three strains. However, the aqueous phase from the *bst1* $\Delta$  strain contained a 10-fold increase in radioactivity over that of control and *BST1* overexpression strains (Fig. 2A). Thin layer chromatography (TLC) analysis of the lipid fractions revealed a sphingosine band, which appeared equivalent in each strain. Interestingly, we observed the presence of another radioactive compound, distinct from sphingosine, in the extracts from the *bst1* $\Delta$  strain, but not in the wild type or *BST1* overexpression strains (Fig. 2B). This compound accumulated rapidly, reaching a plateau by 60 min (Fig. 2C). The presence of significant radioactivity in the aqueous phase and an unknown compound in the organic phase of *bst1* $\Delta$  strain extracts led us to consider whether the unknown lipid might be S-1-P, well known for its unusual solubility properties. We first confirmed that at neutral pH and ambient temperature, S-1-P partitions 65% in aqueous and 35% in organic phases of a (chloroform:methanol:water 1:1:0.9) mixture. We then evaluated the unknown lipid and an S-1-P standard under three separate TLC conditions and found the two compounds to comigrate in each case, confirming the lipid to be S-1-P.

Hyperaccumulation of S-1-P and hypersensitivity to D-erythro-sphingosine could be explained by a failure to metabolize S-1-P. Degradation of S-1-P requires cleavage at the C<sub>2-3</sub> carbon bond by a pyridoxal-5'-phosphate-dependent enzyme, sphingosine phosphate lyase. The enzyme is stereospecific for the D-erythro sphingoid base, but demonstrates less specificity toward the type of sphingoid base. It was, therefore, possible



**FIG. 2. Sphingosine uptake and metabolism in *BST1* overexpression, wild type and deletion strains.** *S. cerevisiae* strains containing the wild type *BST1* allele (SGP3), *BST1* overexpression plasmid (JS14), or *bst1Δ* allele (JS16) were treated with D-erythro-[<sup>3</sup>H]C3-sphingosine for 30, 60, or 180 min, washed, and either assessed for uptake of radioactivity or subjected to lipid extraction. **A**, aqueous phase radioactivity; **B**, TLC determination of sphingosine recovery and metabolism and identification of a novel lipid in the deletion strain; lanes 1–3 represent SGP3 extracts taken at 30, 60, or 180 min, respectively; lanes 4–6 represent JS14 extracts; lanes 7–9 represent JS16 extracts; and lane 10 represents a sphingosine standard; *Sph* = sphingosine and refers to the upper spot identified in each lane; *S-1-P* = sphingosine 1-phosphate and refers to the spot identified only in lanes 7–9. (An additional spot identified in lane 10 well below *S-1-P* and sphingosine is an artifact). **C**, time course for accumulation of sphingosine 1-phosphate in *bst1Δ* strain.



**FIG. 3. *BST1* encodes the *S. cerevisiae* homologue of sphingosine phosphate lyase.** Enzyme activity is demonstrated by representative TLC (as shown in **A**), where lanes 1 and 2 represent JS14, lanes 3 and 4 represent SGP3, lanes 5 and 6 represent JS16, lanes 7 and 8 represent JS29, and *S-1-P* = sphingosine 1-phosphate. **B**, recovery of radioactive products by scraping TLC plates. Student's *t* test was used to determine significance of differences in lyase activity in *BST1* overexpression versus wild type extracts ( $p < .0002$ ), *bst1Δ* versus wild type extracts ( $p < 0.0008$ ), and *BST1* overexpression versus *bst1Δ* extracts ( $p < 1.9 \times 10^{-5}$ ).

that we had isolated sphingosine phosphate lyase through a screen employing a nonendogenous but stereospecifically acceptable sphingolipid. Lyase activity in *BST1* wild type, overexpression, and deletion strains correlated with *BST1* expression (Fig. 3, **A** and **B**), confirming *BST1* to be the yeast homologue of sphingosine phosphate lyase.

Cloning of the first sphingosine phosphate lyase gene from *S. cerevisiae* indicates that phosphorylated sphingoid base signaling is conserved throughout evolution, and thus isolation of other genes responsible for sphingolipid metabolism, transport and signaling should be possible in yeast. Our findings indicate that sphingosine phosphate lyase catalyzes a rate-limiting step in sphingolipid catabolism, and regulation of intracellular *S-1-P* levels should be possible by regulating lyase activity. Further analysis of the *bst1Δ* strain may provide insights into the role(s) of sphingosine 1-phosphate in eukaryotic cell signaling, and knowledge of the yeast sphingosine phosphate lyase sequence should provide a means for the isolation of the sphingosine phosphate lyase genes from higher eukaryotes.

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