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BTS1 Encodes a Geranylgeranyl Diphosphate Synthase in *Saccharomyces cerevisiae**

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Protein prenylation utilizes different types of isoprenoids groups, namely farnesyl and geranylgeranyl, to modify proteins. These lipophilic moieties attach to carboxyl-terminal cysteine residues to promote the association of soluble proteins to membranes. Most prenylated proteins are geranylgeranylated. Geranylgeranylation is catalyzed by two different prenyltransferases, the type I and type II geranylgeranyl transferases, both of which utilize geranylgeranyl diphosphate as a lipid donor. In the yeast *Saccharomyces cerevisiae*, the *BET2* gene encodes the β-subunit of the type II geranylgeranyl transferase. Mutations in this gene cause a defect in the geranylgeranylation of small GTP-binding proteins that mediate vesicular traffic. In an attempt to analyze those genes whose products may interact with Bet2, we isolated a suppressor of the *bet2–1* mutant. This suppressor gene, called *BTS1*, encodes the yeast geranylgeranyl diphosphate synthase. *BTS1* is not essential for the vegetative growth of cells; however, disrupting it impedes the geranylgeranylation of many cellular proteins and renders cells cold sensitive for growth. Our findings imply that *BTS1* suppresses the *bet2–1* mutant by increasing the intracellular pool of geranylgeranyl diphosphate.

Protein prenylation is a post-translational lipid modification that involves the covalent attachment of isoprenoid groups onto cysteine residues at or near the carboxyl termini (Casey, 1992; Schafer and Rine, 1992; Sinensky and Lutz, 1992). The attachment of a lipophilic isoprenoid group to proteins is believed to increase their hydrophobicity, allowing otherwise hydrophilic proteins to associate with membranes. Up to 0.5% of total cellular proteins are estimated to be prenylated (Epstein *et al.*, 1991). Known prenylated proteins include small GTP-binding proteins of the Ras superfamily, nuclear lamins, the yeast mating pheromone a-factor, and trimeric G proteins (Casey, 1992; Schafer and Rine, 1992; Sinensky and Lutz, 1992). These proteins are engaged in a variety of cellular processes, which include the control of cell growth, signal transduction, cytokinesis, and intracellular membrane traffic (Balch, 1990; Barbacid, 1987).

Two different isoprenoid groups, farnesyl (15 carbons) and geranylgeranyl (20 carbons), are post-translationally attached to proteins (Epstein *et al.*, 1991). Farnesyl is added to proteins that terminate in a CAA*X* motif (where C is cysteine, A is an aliphatic amino acid, and *X* can be methionine, cysteine, alanine, glutamine, phenylalanine, or serine), while geranylgeranyl is transferred onto proteins that end in CAAL (where L is leucine), CC, or C*X*C motifs (*X* is any amino acid) (Reiss *et al.*, 1990, 1991; Seabra *et al.*, 1991, 1992). Most known prenylated proteins are geranylgeranylated (Epstein *et al.*, 1991).

Farnesyl and geranylgeranyl groups are attached to proteins from all-trans farnesyl diphosphate (FPP)[1] and all-trans geranylgeranyl diphosphate (GGPP), respectively (Casey, 1992). These lipid precursors are intermediates in the isoprenoid biosynthetic pathway (Goldstein and Brown, 1990). This pathway consists of a series of reactions by which mevalonate is converted into a diverse family of lipophilic molecules that contain a repetitive five-carbon structure. The isoprenoids are subsequently incorporated into a large number of end products, which includes: sterols, ubiquinones, dolichols, tRNAs, and prenylated proteins (Goldstein and Brown, 1990).

FPP is the product of the farnesyl diphosphate synthase. This enzyme, which is the most abundant and widely occurring prenyltransferase, catalyzes the formation of FPP by the sequential addition of isopentenyl diphosphate (IPP) to dimethylallyl diphosphate (DMAPP), and geranyl diphosphate (GPP) (Anderson *et al.*, 1989; Bartlett *et al.*, 1985; Sheares *et al.*, 1989). In some organisms, GGPP is synthesized by a GGPP synthase that catalyzes stepwise additions of IPP to DMAPP, GPP, and FPP. This type of GGPP synthase activity has been detected in mammalian tissue. However, eukaryotic geranylgeranyl diphosphate synthases are known that synthesize GGPP by the addition of a single molecule of IPP to FPP (McCaskill and Croteau, 1993; Sagami *et al.*, 1992, 1993, 1994). But, due to its low activity and the problems in separating this enzyme from FPP synthase, its purification has proven to be difficult (Runquist *et al.*, 1992; Sagami *et al.*, 1985, 1993, 1994).

GGPP is the substrate for two different protein prenyltransferases, the type I (GGTase-I) and type II (GGTase-II) geranylgeranyl transferases (Jiang and Ferro-Novick, 1994; Seabra *et al.*, 1991, 1992). GGTase-I catalyzes the transfer of a geranylgeranyl group from GGPP onto proteins that terminate in a CAAL motif, while GGTase-II attaches geranylgeranyl to terminal CC or C*X*C residues. Its protein substrates include members of the Rab family of small GTP-binding proteins (Jiang and Ferro-Novick, 1994; Seabra *et al.*, 1992). In the yeast *Saccharomyces cerevisiae*, the GGTase-II is composed of three subunits, which are encoded by *BET2*, *BET4* (formerly called *MAD2*), and *MRS6*, respectively (Rossi *et al.*, 1991; Jiang and

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[1] The abbreviations used are: FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; HPLC, high pressure liquid chromatography; kb, kilobase(s).

Ferro-Novick, 1994; Jiang *et al.*, 1993; Li *et al.*, 1993). The *BET2* gene product binds to the product of the *BET4* gene to form the catalytic component of this enzyme. *MRS6* encodes the accessory protein that binds the protein substrate. Mutations in these genes abolish the geranylgeranylation of Ypt1p and Sec4p, two small GTP-binding proteins that mediate intracellular membrane traffic (Jiang and Ferro-Novick, 1994; Li *et al.*, 1993; Rossi *et al.*, 1991). The *bet2–1* gene is a recessive temperature-sensitive mutant allele that fails to grow at 37 °C (Newman and Ferro-Novick, 1987). In this mutant, a failure to geranylgeranylate Ypt1p and Sec4p leads to a defect in the membrane association of these proteins. This deficiency results in a block in intracellular membrane trafficking (Rossi *et al.*, 1991). In an attempt to identify new genes that may interact genetically with *BET2*, *BET4*, or *MRS6*, we isolated a suppressor of the *bet2–1* mutant. This suppressor gene, named *BTS1*, suppresses the growth defect of *bet2–1* when expressed on a low (*CEN*) or high (2 μm) copy vector. Sequence analysis revealed a significant homology between *BTS1* and the geranylgeranyl diphosphate synthase from *Neurospora crassa*, suggesting that *BTS1* encodes the homologue of this gene in *S. cerevisiae*. In accordance with this proposal, the *BTS1* gene product was found to be required for the membrane attachment of Ypt1p and Sec4p, a process that is known to require geranylgeranylation. When *BTS1* was expressed in bacterial cells, it generated an activity that was able to convert FPP to GGPP, thereby conclusively demonstrating that the *BTS1* gene product is the yeast geranylgeranyl diphosphate synthase. This enzyme is a previously unidentified component of the yeast isoprenoid biosynthetic pathway.

MATERIALS AND METHODS

Strains, Media, and Growth Conditions—The following strains were used in this study: ANY119 (*MAT*α, *bet2–1*, *ura3–52*, *his4–619*), NY648 (*MAT a*/α, *leu2–3*, *112*/*leu2–3*, *112*, *ura3–52*/*ura3–52*), NY180 (*MAT*α, *ura3–52*, *leu2–3*, *112*), SFNY26–6A (*MAT*α, *his4–619*), and SFNY368 (*MAT*α, *ura3–52*, *leu2–3*, *112*, *URA3::BTS1*). Yeast strains were grown at 25 or 37 °C in either YP or selective minimal medium that was supplemented with 2% glucose.

Isolation of BTS1—The yeast genomic DNA library used in this study was prepared by ligation of genomic DNA that was prepared from ANY119. This DNA was partially digested with *Sau*3A and inserted into the *Bam*HI site of pRS316 (*CEN*, *URA3*). The library was used to transform the *bet2–1* mutant (ANY119), and the transformants were (1×10^5) selected on minimal medium lacking uracil. After a 3-day incubation at 25 °C, the cells were stamped onto YPD plates and incubated overnight at 37 °C. 11 positive transformants were obtained. Plasmids (pS1-pS11) retrieved from these transformants were amplified in *Escherichia coli* and retested in ANY119.

DNA Sequence and Analysis—The DNA sequence of the *BTS1* gene was determined by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). The reactions were performed using the Sequenase (U. S. Biochemical Corp.) protocol, and the data were analyzed with GCG software. Homology searches were performed with the EMBO/GenBank and Swiss-prot data bases.

Disruption of the BTS1 Gene—To disrupt *BTS1*, a 1.7-kb *Dra*I-*Nru*I fragment containing the *BTS1* gene was excised from pS8 and cloned into the *Pvu*II site of pUC118 to generate pSJ30. The plasmid-borne disruption of *BTS1* was constructed by replacing a 0.65-kb *Sac*I-*Eco*RI fragment in pSJ30 with a 1.2-kb *Sac*I-*Eco*RI fragment containing the *URA3* gene. The resulting plasmid (pSJ31) was digested with *Ssp*I and *Bgl*II and transformed into NY648. The transformants were sporulated, and tetrad analysis was performed. After 3 days at 25 °C, all 48 tetrads examined displayed two large and two small colonies. The large colonies were Ura$^-$, and the small ones were Ura$^+$.

DNA Hybridization Analysis—Yeast genomic DNA prepared from NY180 or SFNY368 was examined by DNA-DNA hybridization as described before (Naumvoski and Friedberg, 1984). Genomic DNA digested with *Bgl*II was fractionated on a 0.8% agarose gel and transferred to a BioTrans membrane (ICN). The blot was probed with a radiolabeled 0.65-kb *Sac*I-*Eco*RI fragment, containing *BST1*, prepared by random-primer labeling and visualized by autoradiography.

Cell Fractionation Studies—Wild type (NY180) and the Δ*BTS1* strain (SFNY368) were grown overnight at 30 °C in YPD medium to early exponential phase. 1 aliquot of cells (150 A_{599} units) was pelleted and washed once with ice-cold 10 mM sodium azide. The remaining cells were shifted to 14 °C, and the incubation was continued for 12 h before the cells were harvested. To generate spheroplasts, cells were resuspended in 0.7 ml of 10 mM ice-cold sodium azide and mixed with an equal volume of 2 × spheroplast medium (2.8 M sorbitol, 100 mM Tris-HCl (pH 7.5), 20 mM sodium azide) containing 100 units of zymolyase. After a 1-h incubation at 25 °C, the spheroplasts were harvested by centrifugation in a clinical centrifuge during a spin at 1400 rpm for 5 min, washed, and lysed in 1.4 ml of ice-cold lysis buffer (0.8 M sorbitol, 10 mM triethanolamine (pH 7.2), 1 mM EDTA) as described before (Rossi *et al.*, 1991). Cell debris was removed during a 3-min spin at 450 × *g*, and the supernatant from this spin was centrifuged at 100,000 × *g* for 1 h to generate a soluble fraction. The pellet was resuspended in a volume of lysis buffer equal to the supernatant. Samples were electrophoresed and subjected to Western blot analysis using anti-Ypt1p or anti-Sec4p antibodies (1:2000 dilution).

Expression of the BTS1 Gene in E. coli—To express *BTS1* in *E. coli*, the *BTS1* open reading frame sequence was generated by polymerase chain reaction using two primers that overlapped the initiation codon or the region 100 base pairs downstream from the stop codon. *Eco*RI and *Cla*I sites were also incorporated into the 5′- and 3′-ends, respectively. The polymerase chain reaction product was digested with *Eco*RI and *Cla*I and cloned into the pUC118 expression vector. The resulting gene fusion encodes a Bts1 protein with six additional NH_2-terminal amino acids from β-galactosidase. This construct was then transformed into JM101 bacterial cells and expressed as described before (Jiang and Ferro-Novick, 1994).

Synthase Assay and Product Analysis—The standard assay mixture contained 20 mM BHDA buffer (pH 7.0), 10 mM β-mercaptoethanol, 1 mM $MgCl_2$, 0.1% (w/v) bovine serum albumin, 200 μM DMAPP or FPP, 20 μM [1-^{14}C]IPP (10 μCi/μmol purchased from Amersham), and 70–80 μg of protein in a total volume of 200 μl. DMAPP, FPP, and GGPP were synthesized by the method of Davisson *et al.* (1986). After 10 min at 37 °C, 200 μl of CH_3OH-HCl (4:1) was added, and the incubation was continued for 30 min. The reaction mixture was extracted with 1 ml of ligroin, and 0.5 ml of the ligroin layer was mixed with 10 ml of Cytoscint-ES (ICN) for the measurement of radioactivity in a Packard Tri-Carb 4530 liquid scintillation spectrometer. Products were analyzed using HPLC. For the product analysis, bovine serum albumin was omitted from the standard assay mixture, but 10 mM sodium fluoride was present to suppress phosphatase activity. After a 1-h incubation at 37 °C, the reaction was terminated by the addition of EDTA (12.5 mM, final concentration). Unlabeled GGPP (25 μg) was added, and 150 μl of the mixture was injected onto a Shodex Asahipak ODP-50 column (4.6 mm (inner diameter) × 250 mm). 2-min fractions were collected, and the radioactivity in each fraction was determined by liquid scintillation counter after the addition of 15 ml of Cytoscint-ES.

Preparation of Yeast Extracts and Protein Prenylation Assay—Yeast cells were grown in YPD medium at 25 °C to late log phase. The cells were harvested, lysed with glass beads, and centrifuged at 100,000 × *g* for 45 min. The soluble fraction was collected and assayed for GGTase-II activity. Prenylation assays were performed in a 50-μl reaction that contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, 25 μg of extract, 0.4 μM of recombinant Ypt1p, and varying concentrations of [^{3}H]GGPP (American Research Lab, 17,500 dpm/pmol). The reaction mixture was incubated at 30 °C for 30 min before it was terminated with 1 M HCl in ethanol (1 ml) and filtered on a Whatman GF/A filter as described before (Jiang *et al.*, 1993).

RESULTS

Isolation of Suppressors of the bet2–1 Mutant—*bet2–1* is a temperature-sensitive mutant that grows at 25 °C (permissive temperature) but dies at 37 °C. To isolate genes whose products may interact with the Bet2 protein (Bet2p), we screened a yeast genomic library that was prepared from the *bet2–1* mutant for plasmids that conferred growth at 37 °C. After screening (1×10^5 transformants, 11 positive colonies were obtained and retested. The growth of mutant cells containing six of these plasmids (group A) was indistinguishable from that of wild type at 37 °C (data not shown). The other five (group B), however, did not suppress as well. Restriction analysis indicated that the plasmids in group A contained the *BET2* structural gene. Since the genomic library was prepared from *bet2–1*

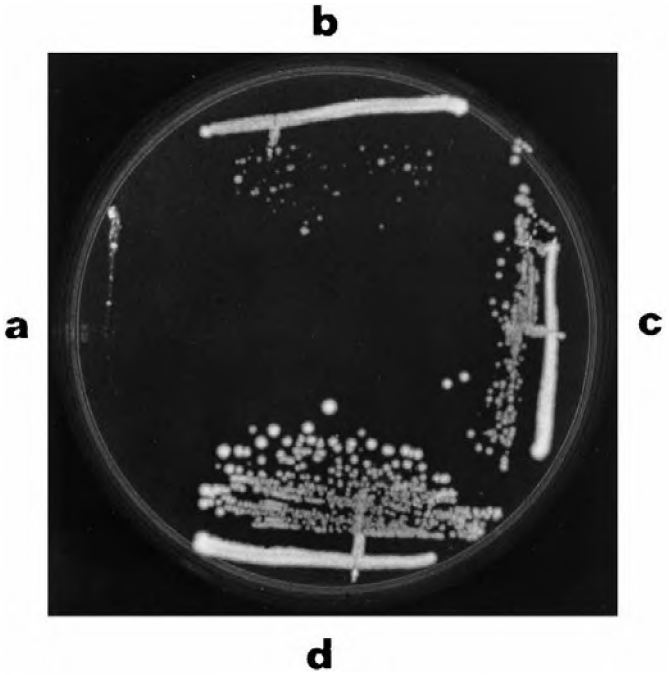

FIG. 1. **Suppression of the *bet2–1* mutant is gene dosage dependent.** *bet2–1* cells (ANY119) were transformed with either pS8 (*CEN*, *URA3*) or pSJ28 (2 μm, *URA3*). The transformants were streaked onto YPD plates and incubated at 37 °C for 3 days. *a*, ANY119; *b*, ANY119 (pS8); *c*, ANY119 (pSJ28); *d*, SFNY26–6A (wild type).

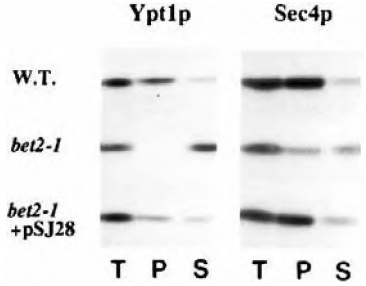

FIG. 2. **Overexpression of the suppressor gene increases the membrane-bound pool of Ypt1p and Sec4p in *bet2–1* mutant cells.** Wild type (*W.T.*) (SFNY26–6A) and *bet2–1* mutant cells (ANY119) that contained pSJ28 were grown to early exponential phase in minimal medium that was supplemented with the appropriate amino acids and 2% gluocse. Cells were harvested, converted to spheroplasts, lysed, and the membrane (*P*) and soluble (*S*) fractions were recovered by centrifuging the lysates (*T*) at 100,000 × *g*. Samples were then subjected to Western blot analysis using anti-Ypt1p and anti-Sec4p antibodies.

mutant cells, the restoration of growth observed at 37 °C is not true complementation. Plasmids in group B contained an overlapping 2.0-kb region of DNA. Therefore, the gene that suppresses the *bet2–1* mutant is located within this 2.0-kb fragment.

The smallest group B plasmid (pS8) that we isolated contained a 2.8-kb insert (Fig. 1*b*). To analyze the ability of this insert to suppress *bet2–1*, we cloned this fragment into a high copy *URA3* vector (pRS426) to generate pSJ28. When pSJ28 was transformed into *bet2–1* mutant cells, suppression was significantly enhanced (Fig. 1, compare *b* and *c* to the mutant alone in *a*). In fact, growth of the mutant was restored to that of wild type (Fig. 1, compare *c* and *d*), suggesting that suppression was gene dosage dependent.

Plasmid pSJ28 Increases the Membrane-bound Pool of Ypt1p and Sec4p in bet2–1 Mutant Cells—Previous studies have shown that the membrane association of Ypt1p and Sec4p is defective in *bet2* mutant cells (Rossi *et al.*, 1991). This defect is a consequence of the failure to geranylgeranylate these proteins (Jiang *et al.*, 1993; Rossi *et al.*, 1991). Thus, the lethal phenotype of the *bet2–1* mutant is likely to be a consequence of the inability of these proteins to attach to membranes. Since plasmid pSJ28 suppresses the growth defect of the *bet2–1* mutant at 37 °C, it may also cure the membrane attachment defect observed in these cells. To address this possibility, we transformed pSJ28 into *bet2–1*. When the distribution of Ypt1p and Sec4p was examined in these transformants and compared to the mutant and wild type, pSJ28 was found to enhance the membrane association of these small GTP-binding proteins (Fig. 2, compare the amount in the lysate (*T*) to the supernatant (*S*) and pellet (*P*) fractions). The presence of pSJ28 did not lead to an increase in the residual GGTase-II activity that can be measured in *bet2* mutant cells (Jiang *et al.*, 1993). Thus, the restoration of the membrane association of Ypt1p and Sec4p is not a consequence of increasing GGTase-II activity.

Cloning and Sequencing the Suppressor Gene—To localize the suppressor within the 2.8-kb genomic fragment described above, subclones of pSJ8 were constructed and inserted into pRS316 (*URA3*, *CEN*). Suppression studies revealed that the *Sac*I site contained within this fragment is critical for its activity. The smallest region of DNA capable of suppressing *bet2–1* was found to be a 1.6-kb *Ssp*I-*Nru*II fragment. This region of DNA was sequenced in both directions using the strategy shown in Fig. 3. An open reading frame of 1005 base pairs that spans the *Sac*I site was identified. We called the gene that encodes this open reading frame *BTS1* (*B*et *T*wo *S*uppressor). *BTS1* is predicted to encode a protein of 335 amino acids with a calculated molecular mass of 38,627 daltons (Fig. 4). Overall, the amino acid composition of the Bts1p is hydrophilic, and no significant hydrophobic stretches were observed.

Bts1p Is Homologous to Known Prenyltransferases—Comparison of the predicted Bts1p amino acid sequence with the Swiss-Prot protein sequence data base revealed a significant similarity between Bts1p and the *N. crassa albino-3* gene product (Carattoli *et al.*, 1991). These proteins are 40% identical at the amino acid level with the most conserved region localized to the middle of these proteins (Fig. 5). The *albino-3* gene encodes a geranylgeranyl diphosphate synthase in the carotenoid biosynthetic pathway of *N. crassa* (Nelson *et al.*, 1989). Bts1p also contains five conserved regions found in other FPP and GGPP synthases (Chen and Poulter, 1994). These comparisons suggest that *BTS1* encodes GGPP synthase, an unidentified prenyltransferase of *S. cerevisiae*.

Disruption of the BTS1 Gene—To investigate if *BTS1* is required for the vegetative growth of yeast cells, we disrupted one copy of this gene in diploid cells and then performed tetrad analysis. This disruption was constructed by exchanging a 0.65-kb segment of the coding region with the *URA3* gene. The disrupted gene was then transformed into NY648 to replace one of the chromosomal copies. The Ura^+ transformants were selected, sporulated, and analyzed. In all of the 48 tetrads examined, four viable spores were obtained. However, two of the colonies in each of the tetrads displayed a growth defect at 25 °C. The small colonies were Ura^+, indicating that they contained the disrupted *BTS1* gene. This was confirmed by Southern blot analysis using *BTS1* as a probe (see "Materials and Methods"). Thus, *BTS1* is not essential for the vegetative growth of yeast cells. But in its absence, growth is impaired. The growth of the disrupted strain (SFNY368 or Δ*BTS1*) was examined further at different temperatures. As shown in Fig. 6, Δ*BTS1* cells (Fig. 6, *a* and *d*) grew as well as wild type at 30 °C (Fig. 6, *b* and *c*). However, at lower temperatures (25 and 14 °C) a growth defect emerged. Only small colonies appeared

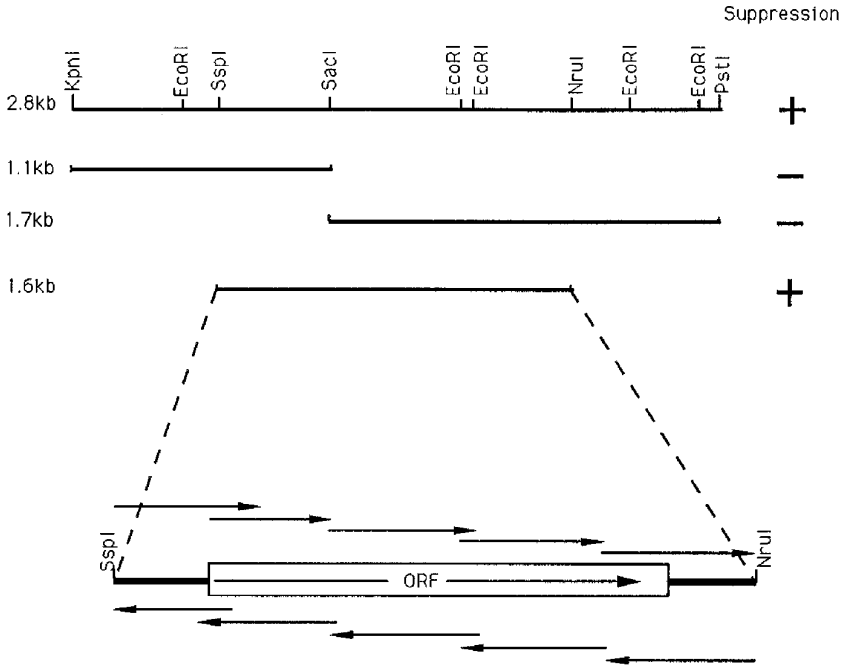

FIG. 3. **Suppression analysis and sequence strategy.** A 1.6-kb *Ssp*I-*Nru*I fragment that fully suppresses the *bet2–1* mutant was sequenced in both directions using the strategy shown above. *ORF*, open reading frame.

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AATATTACATATAGATATAGGACAAGCCCGCATTTTCATACTGAAAGGTAAACTTCTATT
ATTATAGTGGTATCCAACGTTCACCGCTTCCAGCATAGCAGAAATTACGTGTTTTTGCAT
ATGTTATGCTGATCATTGTATGCTTACTACCATTTTTCTTTGCTTCGCCTTGCCTTCTTT
GACGTTTTTTTTGAAGCAAAAAAAAAGTCAAGACAGATGTGCTTACAAAACCATGTAAGGC
TCATTTTCAAAGAAGCTACTAATAGAAAGAGAACAAAGAGTTTACGAGTCTGGAAAATCA
ATGGAGGCCAAGATAGATGAGCTGATCAATAATGATCCTGTTTGGTCCAGCCAAAATGAA
M  E  A  K  I  D  E  L  I  N  N  D  P  V  W  S  S  Q  N  E
AGCTTGATTTCAAAACCTTATAATCACATCCTTTTGAAACCTGGCAAGAACTTTAGACTA
S  L  I  S  K  P  Y  N  H  I  L  L  K  P  G  K  N  F  R  L
AATTTAATAGTTCAAATTAACAGAGTTATGAATTTGCCCAAAGACCAGCTGGCCATAGTT
N  L  I  V  Q  I  N  R  V  M  N  L  P  K  D  Q  L  A  I  V
TCGCAAATTGTTGAGCTCTTGCATAATTCCAGCCTTTTAATCGACGATATAGAAGATAAT
S  Q  I  V  E  L  L  H  N  S  S  L  L  I  D  D  I  E  D  N
GCTCCCTTGAGAAGGGGACAGACCACTTCTCACTTAATCTTCGGTGTACCCTCCACTATA
A  P  L  R  R  G  Q  T  T  S  H  L  I  F  G  V  P  S  T  I
AACACCGCAAATTATATGTATTTCAGAGCCATGCAACTTGTATCGCAGCTAACCACAAAA
N  T  A  N  Y  M  Y  F  R  A  M  Q  L  V  S  Q  L  T  T  K
GAGCCTTTGTATCATAATTTGATTACGATTTTCAACGAAGAATTGATCAATCTACATAGG
E  P  L  Y  H  N  L  I  T  I  F  N  E  E  L  I  N  L  H  R
GGACAAGGCTTGGATATATACTGGAGAGACTTTCTGCCTGAAATCATACCTACTCAGGAG
G  Q  G  L  D  I  Y  W  R  D  F  L  P  E  I  I  P  T  Q  E
ATGTATTTGAATATGGTTATGAATAAAACAGGCGGCCTTTTCAGATTAACGTTGAGACTC
M  Y  L  N  M  V  M  N  K  T  G  G  L  F  R  L  T  L  R  L
ATGGAAGCGCTGTCTCCTTCCTCACACCACGGCCATTCGTTGGTTCCTTTCATAAATCTT
M  E  A  L  S  P  S  S  H  H  G  H  S  L  V  P  F  I  N  L
CTGGGTATTATTTATCAGATTAGAGATGATTACTTGAATTTGAAAGATTTCCAAATGTCC
L  G  I  I  Y  Q  I  R  D  D  Y  L  N  L  K  D  F  Q  M  S
AGCGAAAAAGGCTTTGCTGAGGACATTACAGAGGGGAAGTTATCTTTTCCCATCGTCCAC
S  E  K  G  F  A  E  D  I  T  E  G  K  L  S  F  P  I  V  H
GCCCTTAACTTCACTAAAACGAAAGGTCAAACTGAGCAACACAATGAAATTCTAAGAATT
A  L  N  F  T  K  T  K  G  Q  T  E  Q  H  N  E  I  L  R  I
CTCCTGTTGAGGACAAGTGATAAAGATATAAAACTAAAGCTGATTCAAATACTGGAATTC
L  L  L  R  T  S  D  K  D  I  K  L  K  L  I  Q  I  L  E  F
GACACCAATTCATTGGCCTACACCAAAAATTTTATTAATCAATTAGTGAATATGATAAAA
D  T  N  S  L  A  Y  T  K  N  F  I  N  Q  L  V  N  M  I  K
AATGATAATGAAAATAAGTATTTACCTGATTTGGCTTCGCATTCCGACACCGCCACCAAT
N  D  N  E  N  K  Y  L  P  D  L  A  S  H  S  D  T  A  T  N
TTACATGACGAATTGTTATATATAATAGACCACTTATCCGAATTGTGAAATAAATTGATC
L  H  D  E  L  L  Y  I  I  D  H  L  S  E  L  *
AATCAAATTAGTGGAGGAAGATAGTCAGAAATAAAGCCTTCTCTCCTCCTCTTTCGCATC
TATACATACGATTTCATATATACGTTTCATTGCATCATCTTTTGATATATCTCAAAAA
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FIG. 4. **The nucleotide sequence and predicted amino acid sequence of *BTS1*.** The nucleotide sequence of the 1.6-kb *Ssp*I-*Nru*I fragment is shown *above*. The five conserved regions in all known FPP and GGPP synthases are indicated.

after 3 days at 25 °C (Fig. 6, *a* and *d*), while at 14 °C, the cells did not survive (Fig. 6, *a* and *d*). This result clearly demonstrated that SFNY368 is cold sensitive for growth.

The BTS1 Gene Product Is Required for the Membrane Attachment of Ypt1p and Sec4p—Ypt1p and Sec4p are two small GTP-binding proteins that regulate intracellular membrane traffic (Ferro-Novick and Novick, 1993). Like many small GTP-binding proteins, they are synthesized in the cytosol but be-

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Bts1      3 AKIDELINNDPVWSSQNESLISKPYNHILLKPGKNFRLNLIVQINRVMNL 52
Al-3    109 ATEDFFSPSRRTWSEEKEKVLTGPYDYLNGHPGKDIRSQMVKAFDAWLDV 158
Bts1     53 PKDQLAIVSQIVELLHNSSLLIDDIEDNAPLRRGQTTSHLIFGVPSTINT 102
Al-3    159 PSESLEVITKVISMLHTASLLVDDVEDNSVLRRGFPVAHSIFGIPQTINT 208
Bts1    103 ANYMYFRAMQLVSQLTTKEPLYHNLITIFNEELINLHRGQGLDIYWRDFL 152
Al-3    209 SNYVYFYALQELQKLKNPKA.....VSIFSEELLNLHRGQGMDLFWRDTL 253
Bts1    153 PEIIPTQEMYLNMVMNKTGGLFRLTLRLMEALSPSSHHGHSLVPFINLLG 202
Al-3    254 T..CPTEDDYLEMVSNKTGGLFRLGIKLMQAESRSPVDC...VPLVNIIG 298
Bts1    203 IIYQIRDDYLNLKDFQMSSEKGFAEDITEGKLSFPIVHALNFTKTKGQTE 252
Al-3    299 LIFQIADDYHNLWNREYTANKGMCEDLTEGKFSFPVIHSIRSNPSNMQ.. 346
Bts1    253 QHNEILRILLLRTSDKDIKLKLIQILEFDTNSLAYTKNFINQLVNMIKND 302
Al-3    347 ....LLNILKQKTGDEEVKRYAVAYME.STGSFEYTRKVIKVLVDRARQM 391
Bts1    303 NENKYLPDLASHSDTATNLHDELLYIIDHLSE 334
Al-3    392 TE.....DIDDGRGKSGGIHKILDRIMLHQEE 418
```

FIG. 5. **A comparison of the *BTS1* and *albino-3* (Al-3) gene products.** The amino acid sequence of Bts1p is compared to Al-3 using the Bestfit program (GCG software package). Identity is indicated by a *line*, and conserved changes are marked by *two dots* (two corresponding bases in a codon) or *one dot* (one corresponding base in a codon) between the sequences. The gaps are designated by *dots* within a sequence. Bts1p and Al-3 share 40% identity at the amino acid level.

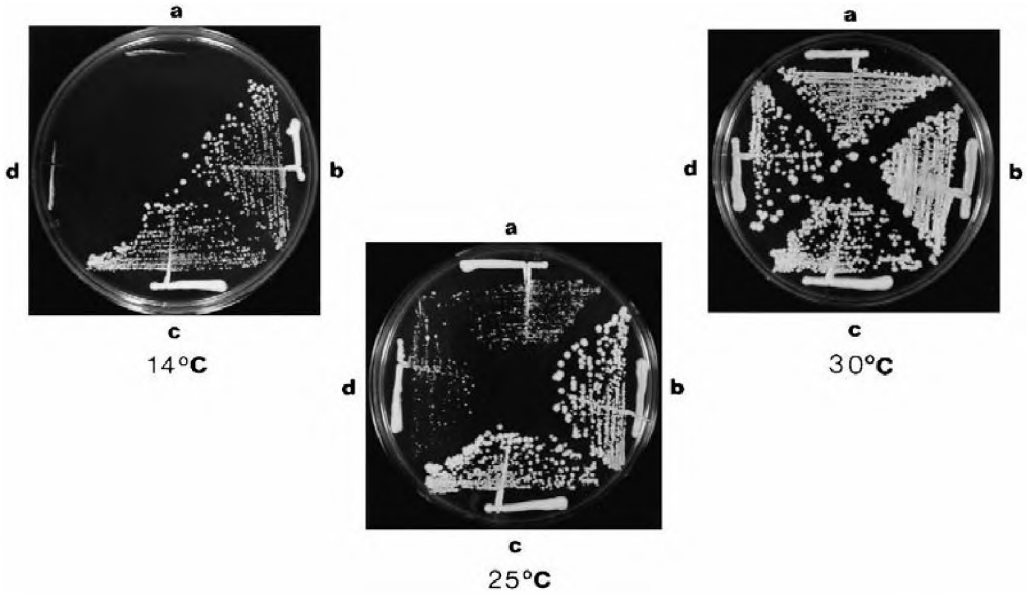

FIG. 6. **SFNY368 (Δ*BTS1*) is cold sensitive for growth.** Diploid cells with one copy of *BTS1* disrupted were sporulated and subjected to tetrad analysis. In each tetrad, two wild type spores (*b* and *c*) and two spores containing the disrupted *BTS1* gene (*a* and *d*) were germinated, purified, and grown at various temperatures (14, 25, and 30 °C). The 25 and 30 °C plates were incubated for 3 days, while the 14 °C plate was incubated for 7 days.

come membrane-bound to perform their function (Rossi *et al.*, 1991). The ability of Ypt1p and Sec4p to bind to membranes is conferred by the addition of the 20-carbon, geranylgeranyl moiety (Jiang *et al.*, 1993). The geranylgeranylation of these proteins is catalyzed by a protein prenyltransferase that utilizes GGPP as a lipid donor. If *BTS1* encodes GGPP synthase, disruption of this gene should result in the depletion of GGPP. Consequently, the geranylgeranylation of Ypt1p and Sec4p will be abolished. To test this hypothesis, we examined the membrane association of these proteins in the Δ*BTS1* strain. SFNY368 was grown at 30 °C for 12 h until the A_{600} was 1.0 prior to shifting the cells to 14 °C for another 12 h. Aliquots of cells were removed at each time point, converted to spheroplasts, lysed, and centrifuged at 450 × *g* to remove unbroken cells and nuclei. Subsequently, these lysates were centrifuged at 100,000 × *g* for 1 h to obtain supernatant and pellet fractions, and the distribution of Ypt1p and Sec4p was examined in each of these fractions by Western blot analysis. In wild type cells (Fig. 7, compare the amount in the lysate (*T*) to the supernatant (*S*) and pellet (*P*)), most of Ypt1p and Sec4p was membrane-bound at both time points, and the change in temperature did not affect their membrane association (Fig. 7, compare 14 and 30 °C). However, in SFNY368, most of the Ypt1p and Sec4p was soluble at both temperatures (Fig. 7, compare the amount in the lysate (*T*) to the supernatant (*S*) and pellet (*P*)), although this defect was more pronounced at 14 °C. Thus, the membrane association of these small GTP-binding proteins is defective in Δ*BTS1* cells.

Prenyltransferase Activity of Crude Extracts—To test the hypothesis that *BTS1* encodes a geranylgeranyl diphosphate synthase, we cloned the gene into a pUC118 vector to express it in *E. coli*. Crude extracts of *E. coli* containing pUC118 (control) or pUC118/*BTS1* were assayed for prenyltransferase activity in the presence of [1-^{14}C]IPP, using DMAPP or FPP as the allylic substrate, and the reaction mixture was analyzed by HPLC. The prenyltransferase activity observed was dependent

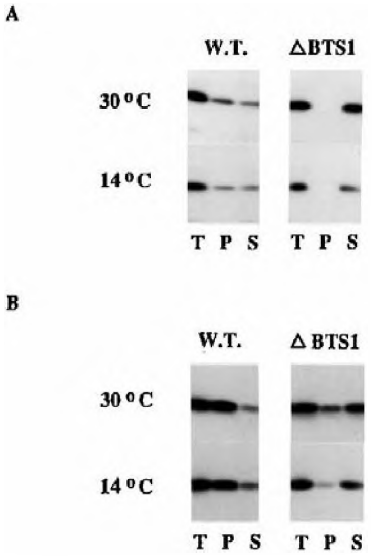

FIG. 7. **The membrane attachment of Ypt1p and Sec4p is defective in Δ*BTS1* cells.** Cells were grown at 30 °C to exponential phase. 1 aliquot of cells was removed, pelleted, converted to spheroplasts, and lysed. The remaining cells were shifted to 14 °C and grown for 12 h before they were harvested and lysed. Lysates (*T*) were centrifuged at 100,000 × *g* to obtain pellet (*P*) and supernatant (*S*) fractions. Samples were electrophoresed on a 12.5% SDS-polyacrylamide gel and subjected to Western blot analysis using anti-Ypt1p (*A*) or anti-Sec4p (*B*) antibodies. *W.T.*, wild type.

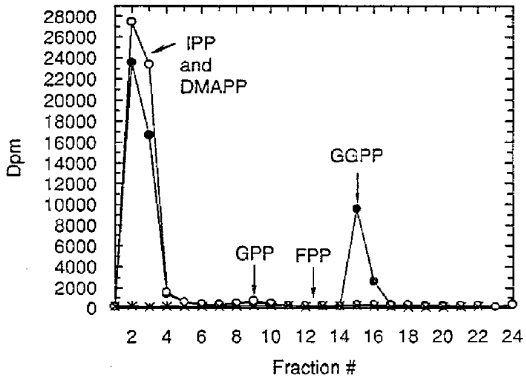

FIG. 8. **Reverse-phase HPLC elution profile of radiolabeled prenyltransferase reaction mixture.** The reaction mixtures from the incubation of crude extracts of *E. coli* containing pUC118 (*open circle*) or pUC118/*BTS1* (*solid circle*) with [1-^{14}C]IPP and FPP were injected onto an Asahipak ODP-50 column, and 2-min fractions were collected. The symbol (*x*) indicates the background that resulted from a run in which only unlabeled GGPP was injected.

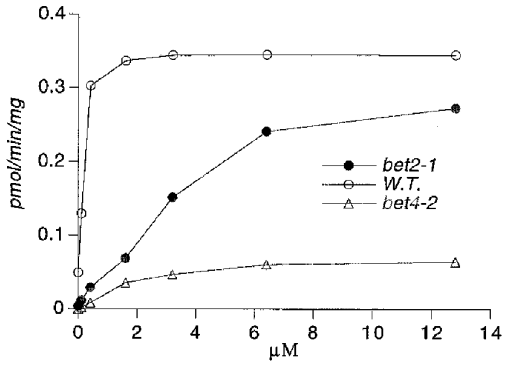

FIG. 9. **Saturation curves for [^{3}H]GGPP using wild type (*W.T.*) (*open circle*), *bet2–1* (*solid circle*), and *bet4–2* (*open triangle*) mutant extracts.** Assays were performed at 30 °C for 30 min with 25 μg of yeast extract and the indicated concentration of [^{3}H]GGPP. Each value is an average of duplicate determinations.

upon the presence of FPP, since no counts were obtained when the pUC118/*BTS1* extract was assayed in the absence of FPP (not shown). The radioactive product of this incubation coeluted with unlabeled synthetic GGPP, indicating that it is GGPP (Fig. 8). No conversion of FPP to GGPP was seen with the pUC118 control. Both extracts also showed low levels of activity in the conversion of DMAPP to an acid-labile product. However, because the extent of conversion was the same for both samples, this activity could not be due to Bts1p (not shown). These findings confirm that *BTS1* encodes a geranylgeranyl diphosphate synthase.

bet2–1 Mutant Extracts Have a Lower Affinity for GGPP—We next investigated the mechanism by which the overexpression of *BTS1* suppresses the lethality of the *bet2–1* mutant. One possibility is that *BTS1* suppresses by increasing the intracellular pool of GGPP, thereby compensating for a mutant GGTase-II that has a lower affinity for GGPP. To test this hypothesis, we measured the GGTase-II activity of wild type and *bet2–1* mutant extracts in the presence of varying concentrations of GGPP. As a control, we also assessed the activity of *bet4–2* mutant extracts. *BET4* encodes the α-subunit of the GGTase-II (Li *et al.*, 1993; Jiang *et al.*, 1993), and extracts prepared from this mutant are devoid of GGTase-II activity. Unlike *bet2–1*, the overexpression of *BTS1* does not suppress the temperature-sensitive growth defect of the *bet4–2* mutant (not shown). As shown in Fig. 9, the GGTase-II activity of the wild type extract was saturated at ~0.8 μM of GGPP. At this concentration, the activity of the *bet2–1* mutant extract was approximately 5–10% of wild type. This activity was significantly enhanced when the GGPP concentration was increased beyond 2 uM, and saturation was achieved at 6 μM. In contrast, the GGTase-II activity of the *bet4–2* mutant extract could not be compensated for by increasing the concentration of GGPP. The calculated K_m values of GGTase-II in the *bet2–1* mutant and wild type were ~3.6 and 0.4 μM, respectively. Therefore, it appears that GGTase-II in the *bet2–1* mutant has a reduced affinity for GGPP, which results in a decrease in prenylation activity. By increasing the amount of GGPP that is added to the assay, prenylation activity is efficiently restored. This result provides a clear explanation for the suppression of *bet2–1* by *BTS1*.

DISCUSSION

Previously, we have shown that the yeast GGTase-II is composed of three subunits (*BET2*, *BET4*, and *MRS6*). Bet2p, the β-subunit of this enzyme complex, forms a complex with Bet4p, the α-subunit (Jiang *et al.*, 1993). Mrs6p is an escort protein that presents protein substrate to the Bet2p-Bet4p complex (Jiang *et al.*, 1994). During geranylgeranylation, the Bet2p-Bet4p complex binds to and transfers GGPP to Ypt1p, Sec4p, and other small GTP-binding proteins. In an attempt to identify new genes whose products may interact with Bet2p, we isolated a suppressor of the *bet2–1* mutant. Our data demonstrates that this suppressor gene, called *BTS1*, encodes a geranylgeranyl diphosphate synthase, an unidentified prenyltransferase of the yeast isoprenoid biosynthetic pathway. The *BTS1* gene product functions on this pathway to convert FPP to GGPP.

The function of *BTS1* was revealed by analyzing the sequence of this gene. The predicted amino acid sequence of Bts1p was found to be significantly homologous to the *albino-3* gene product, the *N. crassa* GGPP synthase (Carattoli *et al.*, 1991). Upon a closer examination, we also found that Bts1p contains five highly conserved motifs that are present in all known FPP and GGPP synthases (Chen and Poulter, 1994), including the aspartate-rich sequences proposed to be involved

in binding and catalysis (Ashby and Edwards, 1990; Joly and Edwards, 1993; Song and Poulter, 1994). This finding suggested that *BTS1* encodes the yeast GGPP synthase. To confirm this hypothesis, we expressed the *BTS1* gene in bacteria. Bacterial lysates that express Bts1p were found to contain an activity that synthesizes GGPP from IPP and FPP.

The suppression of the *bet2–1* mutant by *BTS1* could be explained in several ways. The *BTS1* gene product may itself have GGTase-II activity, or it could directly interact with GGTase-II to stimulate its activity. In either situation, the overexpression of *BTS1* would be expected to increase GGTase-II activity. However, this was not observed. Alternatively, suppression may simply be a consequence of increasing the intracellular pool of GGPP. Since *in vitro* prenylation studies have demonstrated that mutant GGTase-II has a low affinity (increased K_m) for GGPP, which is compensated for by higher concentrations of GGPP, this alternate possibility is most likely. According to this model, additional copies of *BTS1* should result in higher intracellular concentrations of GGPP and enhanced suppression of *bet2–1*, thus explaining why the suppression of *bet2–1* by *BTS1* is gene dosage dependent.

Because each of the subunits of the GGTase-II are essential, we anticipated that *BTS1* would also be required for the vegetative growth of yeast cells. To our surprise, the Δ*BTS1* strain was only cold sensitive for growth. Furthermore, the growth of this strain was not impaired at 30 °C or higher temperatures. When the membrane association of Ypt1p and Sec4p was examined in Δ*BTS1* cells grown at 30 °C, a small fraction of each of these proteins was membrane bound. Thus, *BTS1*-depleted cells are able to prenylate proteins at a level that is sufficient to sustain cell growth at higher temperatures. When these cells were shifted to 14 °C, less membrane-bound Ypt1p and Sec4p was detected, implying that growth ceases as a consequence of the failure to prenylate these essential proteins.

Since *BTS1* is not essential for the growth of yeast cells, the synthase gene may be duplicated. Preliminary DNA hybridization experiments, however, argue against this possibility. Another explanation for the dispensability of *BTS1* is that GGTase-II might utilize FPP as an alternate substrate. However, since GGTase-II cannot transfer FPP to Ypt1p, this possibility seems unlikely (Jiang *et al.*, 1993). Furthermore, extracts prepared from Δ*BTS1* cells do not support the transfer of [^{3}H]FPP onto Ypt1p. Thus, it is more likely that another prenyltransferase, such as hexaprenyl diphosphate synthase, might produce small amounts of GGPP as an intermediate product during the elongation of FPP to longer polyisoprenoid chains. In the Δ*BTS1* strain, GGPP may be formed in this way, enabling yeast cells to survive at certain temperatures in the absence of the geranylgeranyl synthase.

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