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Molecular Characterization of Squalene Synthase from the Green Microalga *Botryococcus braunii,* Race B[1]

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The green microalga *Botryococcus braunii* produces large amounts of liquid hydrocarbons and is classified into three races, depending on the type of the hydrocarbon produced. The B race produces two types of triterpenoid hydrocarbons, squalene and botryococcene, both of which are putative condensation products of farnesyl diphosphate. In an attempt to better understand the regulation involved in the production of squalene and botryococcene, we have isolated and characterized a squalene synthase (SS) gene from the B race of *B. braunii.* A 366-bp cDNA fragment was initially obtained from the B race utilizing a reverse transcription/polymerase chain reaction and degenerate primers based on conserved amino acid sequences found in all SS enzymes. Using this putative SS fragment as a probe, a 2632-bp cDNA clone was isolated from a cDNA library. This cDNA contained an open reading frame coding for a protein with 461 amino acids and a predicted molecular mass of 52.5 kDa. Comparison of the *Botryococcus* SS (BSS) with SS from different organisms showed 52% identity with *Nicotiana tabacum,* 51% with *Arabidopsis thaliana,* 48% with *Zea mays,* 40% with rat, 39% with yeast, and 26% with *Zymomonas mobilis.* Expression of full-length and carboxy-terminus truncated BSS cDNA in *Escherichia coli* resulted in significant levels of bacterial SS enzyme activity but no botryococcene synthase activity. RNA blot hybridization analysis of algal cultures during a culture cycle indicated that BSS gene expression is preferential during rapid growth. Given that the DNA blot analysis indicated only a single copy of the SS gene in the algal genome, these results suggest either that there exists coordinate expression of separate synthase genes for squalene and botryococcene biosynthesis or that there might be unique physiological conditions controlling the SS vs botryococcene synthase activity of a single peptide species. © 2000 Academic Press

***Key Words: Botryococcus braunii;* squalene; botryococcene; squalene synthase.**

Botryococcus braunii is a green microalga which grows as a colony of individual cells held together by a colony matrix that contains a large mixture of liquid hydrocarbons (1, 2). This alga is classified into three races (A, B, and L), depending on the type of hydrocarbons synthesized (3, 4) (see Fig. 1). The B race produces triterpenoid hydrocarbons, referred to as botryococcenes, as the major matrix component, while the A race accumulates nonterpenoid alkadienes and alkatrienes derived from fatty acids (3, 5, 6). The L race produces a tetraterpene hydrocarbon called lycopadiene (4). Botryococcenes are promising as a renewable energy source because they accumulate to very high levels in the algae (30–40% of algal dry weight) (7, 8) and they have a high "octane rating" as a fuel source because of their highly branched structures (9). There are various homologues of botryococcenes and their composition in the colony matrix depends on the algal strain, culture conditions, and growth stage (10, 11). Feeding experiments using radiolabeled methionine or carbon dioxide have demonstrated that C30 botryococcene is the precursor of all botryococcenes and is converted into homologues of up to C34 by methylation with *S*-adenosylmethionine (12–14). Despite the extensive study of botryococcene chemistry, the biochemistry and physio-

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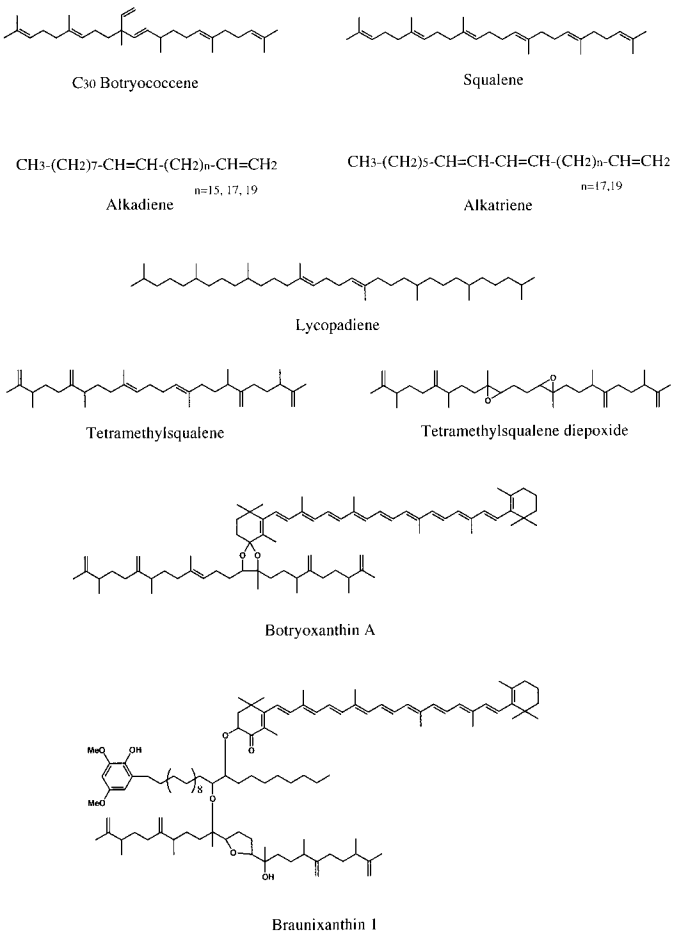

FIG. 1. Various lipids found in the colony matrix of different *B. braunii* races. Race B accumulates botryococcenes and methylated squalenes (3, 20). Race A produces alkadienes, and alkatrienes while race L accumulates lycopadiene (3, 6). Race B also accumulates squalene derivatives, such as tetramethylsqualene epoxides (23), botryoxanthins (21), and braunixanthins (22), which may be incorporated into the resistant biopolymer.

logical regulation of C30 botryococcene biosynthesis has received less attention.

The hydrocarbons and their derivatives produced by *B. braunii* accumulate in the extracellular colony matrix as a combination of organic extractable lipids and condensed, chemically resistant biopolymers (15). These resistant biopolymers of the A race are polymers of polyaldehydes derived from fatty acids (16). The liquid hydrocarbons generated by the A race are also fatty acid-derived alkadienes and alkatrienes. In contrast, while the resistant biopolymers of the B race colony matrix are thought to be derived from fatty acids (17, 18), the free liquid hydrocarbons are isoprenoid derivatives (7, 8). Thus, there is no obvious biosynthetic relationship between the free hydrocarbons and resistant biopolymers of the colony matrix for the B race.

The B race's free hydrocarbons are actually a complex triterpenoid mixture consisting of botryococcenes and squalene derivatives (19, 20). The relative abundance of squalene and methylated squalenes in the hydrocarbon fraction is usually low compared to that of botryococcenes. Recently, however, several compounds derived from tetramethylsqualene, such as botryoxanthins (21), braunixanthins (22), and tetramethylsqualene epoxides (23), have been isolated from the B race (Fig. 1). The chemical structures of these squalene derivatives, especially tetramethylsqualene epoxides, suggest that they could be used in constructing the resistant biopolymers by condensation with polyaldehydes derived from fatty acids (16, 23). Therefore, the B race may produce and accumulate more squalene than the other races, which synthesize small amounts of squalene as a normal requirement for sterol biosynthesis.

Both botryococcene and squalene are C30 compounds derived from the isoprenoid pathway with a common backbone of two C15 farnesyl residues. While it is well established that squalene is derived from the condensation of two farnesyl diphosphate (FPP)[4] molecules (24), the actual farnesyl precursors for botryococcene are not known. Botryococcenes may be synthesized from FPP (25, 26), but other evidence suggests that 3-hydroxy-2,3-dihydrofarnesal may be the botryococcene precursor (27). A biochemical rationalization for the structural difference between botryococcene and squalene predicts a difference in the condensation of the two farnesyl residues. Squalene has a 1′-1 condensation between two FPP molecules, while C30 botryococcene has an unusual 1′-3 fusion between two farnesyl residues (28). A logical extension from this rationalization is that the enzyme(s) catalyzing these reactions may share structural motifs in common, including those important for substrate binding and perhaps catalysis.

Molecular clones for squalene synthase (SS) have been isolated and characterized from several organisms (29–33). SS is an endoplasmic reticulum (ER)-associated enzyme with a predicted carboxy terminus tethering it to the ER membrane (34). The association with the ER has hampered conventional purification of this enzyme in an active state. However, soluble SS enzyme activity has been isolated and characterized from many organisms, including yeast (30, 31), rat (32), tobacco (29), and *Arabidopsis* (33), by expressing various forms of the cDNA in bacteria. Alignments of

[4] Abbreviations used: FPP, farnesyl diphosphate; SS, squalene synthase; ER, endoplasmic reticulum; PSPP, presqualene diphosphate; BSS, *Botryococcus* SS; gDNA, genomic DNA; CTAB, cetyldimethylethylammonium bromide; ORF, open reading frame; RT/PCR, reverse transcriptase/polymerase chain reaction; IPTG, isopropylthio-β-D-galactoside; DTT, dithiothreitol; NTR, nontranslated region.

SS from these organisms at the amino acid level has revealed highly conserved amino acid sequences (domains I–V), which are thought to be important for the catalytic reactions of the enzyme (34). The formation of the 1′-1 linkage between two FPP residues in squalene results from at least two partial reactions. The first is a condensation of two molecules of FPP to form presqualene diphosphate (PSPP), which contains a cyclopropane ring. The second step involves cleavage of the cyclopropane ring followed by rearrangement and reduction by NADPH (35). Mutational analysis of amino acids in domains III, IV, and V are consistent with the essentiality of these consensus regions for SS enzyme activity (35). Asp^{219} and Asp^{223} of domain IV appear to be responsible for binding of the diphosphate moiety of FPP through magnesium salt bridges, and Tyr^{171} in domain III is essential for the first step of the reaction (35). PSPP accumulates in mutants of Phe^{288} of domain V, indicating that this domain is involved in the second step of the reaction and possibly in the cleavage of the cyclopropane ring to form the 1′-1 linkage (35). In contrast to squalene, little is known about C30 botryococcene biosynthesis. Feeding experiments using stereospecifically deuterated farnesol, for example, suggest the possibility that C30 botryococcene is also produced via a PSPP intermediate (28, 36).

To better understand the reaction mechanisms for squalene and botryococcene biosynthesis, as well as regulation of isoprenoid metabolism in *B. braunii*, cDNAs corresponding to SS and botryococcene synthase have been sought. Here, we report the isolation and characterization of a SS gene from *B. braunii*.

MATERIALS AND METHODS

Culture of the Berkeley strain. *B. braunii* Berkeley (Showa) strain (37) belonging to the B race was cultured in modified Chu 13 medium (38) and grown under illumination of 150 microeinstein $\cdot m^{-2} \cdot s^{-1}$ on a 12L:12D cycle at 20°C and aerated with filter-sterilized air containing 2% CO_2. Subculturing was carried out every 30 days by transferring 300 ml of the 30-day-old culture to 900 ml of newly prepared medium. Aliquots (10 ml) were collected every 3 days by vacuum filtration onto preweighed Whatman GF/C filters (Whatman, Madison, England). The filters with algal cells were freeze-dried and then weighed.

Cloning strategies for Botryococcus squalene synthase (BSS). Genomic DNA (gDNA) was extracted from freeze-dried algal cells by the cetyldimethylethylammonium bromide (CTAB) method (39). APCR was carried out using this gDNA and a degenerate forward 17-base primer (5′-TA(T/C) TG(T/C) CA(T/C) TA(T/C) GT(T/C/A/G) GC-3′) based on the amino acid sequence YCHYVA and a degenerate reverse 18-base primer (5′-(T/C)TG IA(A/G) (A/G)AA IA(A/G) ICC CAT-3′) based on the amino acid sequence MGLFLQ. The forward sequence (YCHYVA) is located within domain III of the SS protein and the reverse sequence (MGLFLQ) is located within domain IV of the SS protein according to the nomenclature proposed by Robinson *et al.* (34). A PCR product of 1.3 kb (gBSS1.3) was amplified from the gDNA using these degenerate primers and isolated from an agarose gel using DE-81 ion exchange chromatography paper (Whatman) (40). The PCR product was ligated into the *Eco*RV site of the plasmid pBluescript II KS+ (Strategene, La Jolla, CA) (pBKS-gBSS1.3), transformed into *Escherichia coli* strain TB1 according to a standard $CaCl_2$ transformation procedure (40), and sequenced using the dideoxy nucleotide chain termination method according to the manufacturer (Amersham Life Science Inc., Cleveland, OH). A similar strategy was employed to obtain a genomic fragment amplified using a forward degenerate primer to domain IV (5′-AA(A/G) ACI AA(C/T) AT(A/T/C) AT(A/T/C) (A/C)GI GA(C/T) T-3′) and a reverse degenerate primer to domain V (5′-AC(C/T) TG(A/T/G/C) GG(A/G/T) AT(A/T/G/C) GC(A/G) CA(A/G) AA-3′).

First-strand cDNA was produced by isolating total RNA from cells in rapid growth (9 days after subculturing), using a SDS/LiCl method (41), followed by isolation of poly(A)$^+$ RNA using an oligo(dT)-cellulose column (Gibco Life Technologies, Gaithersburg, MD). First-strand cDNA was prepared using an oligo(dT_{27}) primer and Superscript II (Gibco Life Technologies). A gene-specific forward 18-base primer (GTT GCT GGG GTG GTG GGT) was synthesized based on the sequence found in pBKS-gBSS1.3, which corresponds to the conserved amino acid sequence (VAGVVG) located within domain III of the SS protein. A 50-μl PCR was done using this gene-specific forward primer (200 ng), an oligo(dT_{27}(G/A/C)) primer (200 ng), and first-strand cDNA as a template. Since this first reaction did not produce a clear reaction product when analyzed by agarose gel electrophoresis, a 1-μl aliquot of the reaction mixture was subjected to a second nested PCR using the same forward primer and 750 ng of degenerate reverse primer (5′-AC(T/C) TG(G/A/T/C) GG(G/A/T) AT(G/A/T/C) GC(G/A) CA(G/A) AA-3′), which corresponds to the amino acid sequence (FCAIPQV) located within domain V of the SS protein. The nested PCR gave a 366-bp fragment (BSS366), which was isolated, cloned, and sequenced as above.

A cDNA library was prepared with the ZAP-cDNA Synthesis Kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). The cDNA library was amplified once in *E. coli* strain XL-1-Blue MRF' and then screened with the BSS366 cDNA. After a tertiary screening, positive clones were used for *in vivo* plasmid excision with ExAssist helper phage and *E. coli* strain SOLR according to the manufacturer (Strategene). The resulting colonies were screened by PCR to check for clones containing the BSS366 primer sequences. Several clones were subsequently sequenced by cycle sequencing using an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA) and the clone containing an apparent full-length ORF was designated pBKS-BSS2632.

Construction of the BSS expression vectors. The entire ORF region of the BSS cDNA was amplified by PCR using a forward primer (5′-ATATAC<u>AT</u>**ATG**<u>G</u>GGATGCTTCGCTGG-3′) (*Nde*I restriction site underlined and the translation start codon in boldface), a reverse primer (5′-GCCT<u>GATCA</u>**TTA**GGCGCTGAGTGTGGG-3′) harboring a *Bcl*I site (underlined), and a stop codon (boldface), and the pBKS-BSS2632 as a template. The resulting 1403-bp PCR product (BSS1403) was digested with *Nde*I and *Bcl*I, gel purified, and ligated into the *Nde*I and *Bam*HI sites within the pET11c vector (Novagen, Madison, WI). The construct, pET11c-BSS1386, was transformed into *E. coli* strain BL21(DE3) according to the manufacturer's recommendations (Novagen). Carboxy-terminal truncated BSS was also generated by PCR using the forward primer harboring *Nde*I restriction site (mentioned above), a reverse primer (5′-GCCT<u>GATCA</u>**T-CA**CCACCAATGTTGGGTTAC-3′) harboring a *Bcl*I site (underlined), and a TGA stop codon located 26 amino acids upstream of the native TAA stop codon in the pET11c-BSS1386. The 1325-bp PCR product (BSS1325) was digested with *Nde*I and *Bcl*I, subcloned into the *Nde*I and *Bam*HI sites within the pET11c vector (pET11c-BSS1308), and transformed into *E. coli* strain BL21(DE3) as above.

Comparison of SS protein sequences. The sequence of BSS was compared with known sequences of SS proteins from *Nicotiana tabacum* (29), *Arabidopsis thaliana* (33), *Zea mays* (42), rat (*Rattus rattus*) (32), yeast (*Saccharomyces cerevisiae*) (30), and *Zymomonas mobilis* (43). Sequences were aligned using CLUSTAL W (44) and a

phylogenetic tree was generated by Phylogenetic Analysis Using Parsimony (PAUP version 3. 1. 1) (45).

Expression of BSS in E. coli. The BSS cDNAs were expressed in *E. coli* by adding isopropylthio-β-D-galactoside (IPTG) to a final concentration of 1 mM to exponentially growing recombinant cells (OD_{600} = ~0.5) harboring the pET11c-BSS1386 or the pET11c-BSS1308 plasmid. One milliliter aliquots of the cultures were subsequently removed for enzyme assay and collected by centrifugation at 5000*g* for 2 min, resuspended in 100 μl lysis buffer (25 mM potassium phosphate, pH 7.0, 1 mM dithiothreitol (DTT), and 2 mM phenylmethylsulfonyl fluoride) by vortexing, and sonicated two times for 5 s each time. The sonicate was centrifuged at 16,000*g* for 10 min at 4°C and 5 μl of the supernatant was used for the enzyme activity assay as described below. Protein levels were determined by the Bradford method (Bio-Rad, Hercules, CA). The presence of the induced BSS was monitored by SDS–PAGE by harvesting 100-μL aliquots of bacterial culture at the indicated times and centrifuging at 5000*g* for 2 min. The pellet was resuspended in 100 μl of 50 mM Tris–HCl, pH 6.8, 10 mM DTT, 2% SDS, 0.01% bromophenol blue, and 10% glycerol. Twenty-microliter aliquots were separated on a precast 8–16% gradient Tris–HCl polyacrylamide gel (Bio-Rad) and stained with Coomassie blue for protein detection.

Enzyme assays. The SS enzyme assays of a microsomal fraction of *B. braunii* and proteins expressed in *E. coli* were carried out as follows. A crude microsomal fraction of *B. braunii* for the assay was prepared by homogenizing 1 g of cells in 5 ml of 100 mM potassium phosphate buffer (pH 7.0), 250 mM sucrose, 4 mM magnesium chloride, and 5 mM β-mercaptoethanol. The homogenate was filtrated through two layers of MIRACLOTH (CALBIOCHEM, La Jolla, CA) and centrifuged for 10 min at 2000*g*. The supernatant was further centrifuged for 10 min at 10,000*g*. The resulting supernatant was centrifuged for 60 min at 100,000*g* to obtain a crude microsomal pellet. The pellet was dissolved in 50 μl of 20 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$, 2.5 mM β-mercaptoethanol and used for the SS assay. Two microliters of a microsomal fraction or 5 μl of the protein extracts from *E. coli* in a final reaction volume of 50 μl of 0.5 M potassium phosphate buffer (pH 6.0) containing 10 mM NADPH, 25 mM β-mercaptoethanol, 40 mM MgCl2, 20 μM [^{3}H]FPP (86.9 μCi/μmol) were incubated for 30 min at 37°C. The reaction was terminated by placing the reaction tubes on ice and adding 100 μl of *n*-hexane, mixing, and then centrifuging. Aliquots of the *n*-hexane phase were applied to TLC plates (PE SIL G; Whatman) with authentic standards of squalene (Aldrich, Milwaukee, WI) and developed with *n*-hexane. The spot of squalene (R_f = 0.14) was visualized with iodine vapor, scraped from the TLC plate, and placed into a scintillation vial to measure the radioactivity. For the enzyme assay of the expressed proteins, purified C30 botryococcene (46) was also spotted with the samples in addition to squalene and radioactivity associated with the spot corresponding to C30 botryococcene (R_f = 0.2) was determined. Squalene synthase activity is expressed in nmol of squalene produced/h/mg protein. Detection limits for squalene synthase activity under these conditions were 0.5 nmol of squalene/h/mg protein (corresponding approximately to 50 dpm on the TLC plate) and 0.5 nmol of botryococcene/h/mg protein (corresponding approximately to 50 dpm on the TLC plate). Subsequent work has determined that a Tris buffer (50 mM, pH 7.5) is superior to the phosphate buffer used in these experiments for measurement of the *Botryococcus* squalene synthase activity.

Southern blot analysis. Genomic DNA was isolated using the CTAB method (39). Genomic DNA (8 μg) was digested with the indicated restriction enzymes, size fractionated on 0.8% agarose gels, and transferred to a nylon membrane (Zeta probe, Bio-Rad). DNA blots were hybridized with an [α-^{32}P]dCTP-radiolabeled 1386-bp full-length cDNA (BSS1386) using the Prime-It kit (Strategene) at 42°C in 5 ml of hybridization buffer [5× SSPE (1× SSPE = 0.6 M NaCl, 34.6 mM sodium phosphate buffer, 5 mM EDTA), 2× Denhardt's solution, 0.2% SDS, 100 μg/ml salmon sperm DNA, 50% formamide]. Blots were washed twice at 42°C for 5 min with 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1% SDS and twice at 42°C for 10 min with 0.2× SSC, 0.1% SDS. Hybridization was detected using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

RNA blot analysis. Total RNA was extracted from the cells at the indicated days after subculturing by the SDS/LiCl method (41). The RNA samples heat-denatured at 70°C for 15 min were fractionated on a 1.2% agarose denaturing formaldehyde gel (40) and transferred to a nylon membrane (Zeta probe, Bio-Rad). An [α-^{32}P]dCTP-radiolabeled cDNA probe (BSS1386) was prepared by the Prime-It kit (Strategene) and used as a probe. The blot was prehybridized for 1 h at 42°C in 5 ml of hybridization buffer (5× SSPE, 2× Denhardt's solution, 0.2% SDS, 100 μg/ml denatured salmon sperm DNA, 50% formamide) before the radiolabeled probe (BSS1386) was added. The blots were hybridized for 12 h at 42°C and then washed twice at 42°C for 5 min with 2× SSC, 0.1% SDS and twice at 42°C for 10 min with 0.2× SSC, 0.1% SDS. Hybridization was detected as described above. Similar hybridization results were obtained in duplicate experiments using independently isolated RNA samples.

RESULTS

Isolation of BSS cDNAs. SS-like cDNAs were isolated from *B. braunii* using a combination of PCR and RT/PCR strategies (Fig. 2). Preliminary PCRs were carried out using genomic DNA from *B. braunii* Berkeley and degenerate primers based on conserved regions of all SS proteins. The forward degenerate primer was based on a consensus sequence in domain III (YCHYVA) and the reverse primer was based on a consensus sequence in domain IV (MGLFLQ) (see Fig. 3). The resulting amplification product was 1.3 kb (gBSS1.3), portions of which predicted peptides with a high degree of identity to domains III and IV of other squalene synthase proteins. A RT/PCR strategy was then employed to isolate clones lacking introns. Poly(A)$^+$ RNA isolated from the 9-day-old algal cells was used as the initial template. Based on the sequence information of gBSS1.3, a gene-specific forward primer was designed based on the conserved sequence VAGVVG in domain III and used in combination with an oligo(dT) primer for the PCR portion of the reaction. No clear amplification product was observed in the primary reaction. Consequently, an aliquot of this RT/PCR was subjected to a second PCR amplification using the same forward primer and a nested degenerate reverse primer based on a conserved sequence within domain V of SS (FCAIPQ). This resulted in a 366-bp cDNA fragment (BSS366). The BSS366 clone contained sequences predicting conserved amino acids found in domains III, IV, and V of typical SS proteins. This RT/PCR cDNA was then used to screen a *B. braunii* cDNA library constructed with poly(A)$^+$ RNA isolated from 9-day-old cells. Four positive clones were selected and one containing an insert of 2632 bp, BSS2632, was sequenced (Fig. 2). This clone contained an ORF of 1386 bases, preceded by 171 bases of 5′ nontranslated region (NTR) and followed by 1009 bases of 3′ NTR and 66 bases of a poly(A)$^+$ tail. The

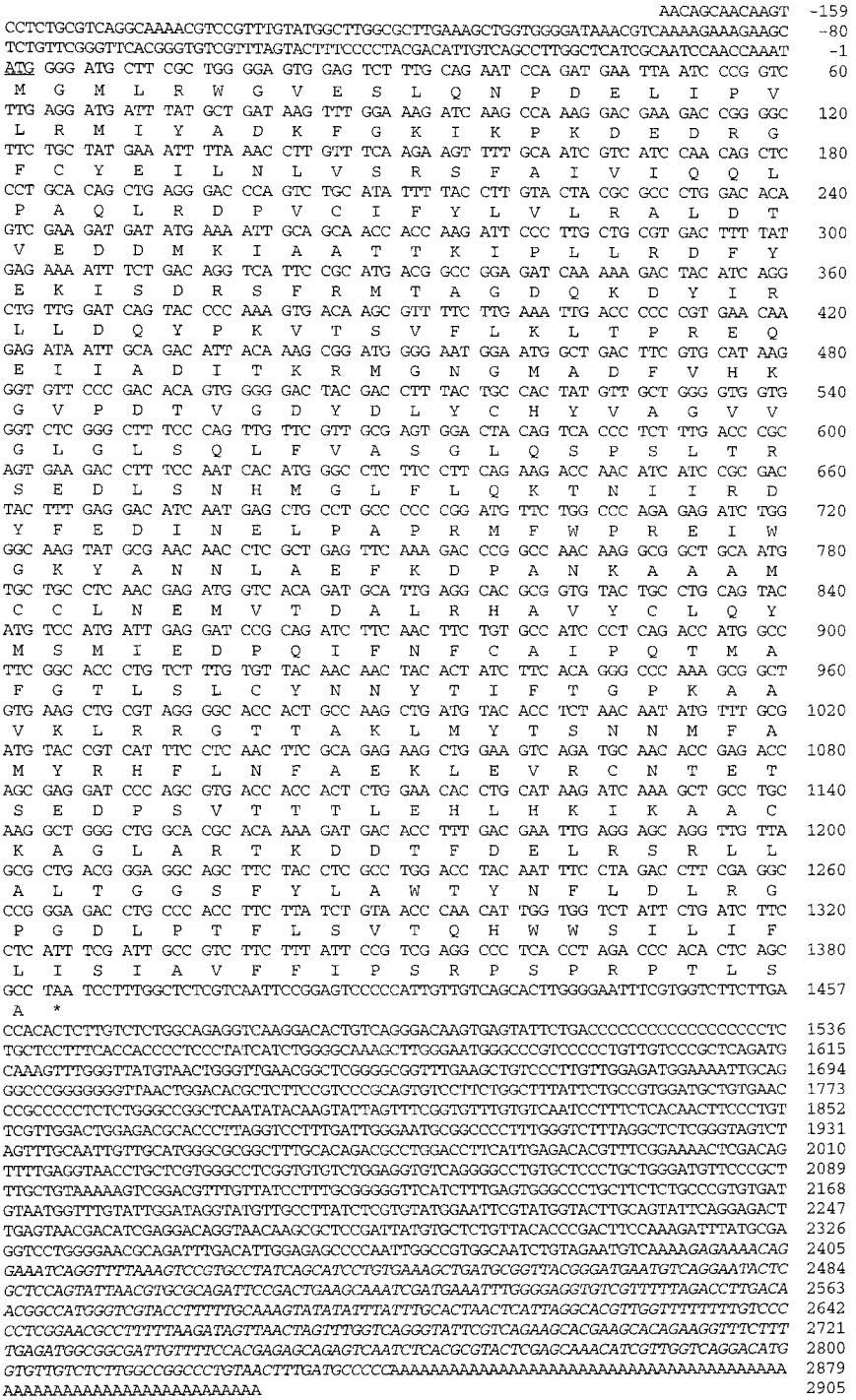

FIG. 2. Nucleotide and predicted amino acid sequence in single-letter code for the BSS cDNA. Nucleotides are numbered starting at the A in the ATG start codon (underlined). An open reading frame coding for 461 amino acids is shown below the nucleotide sequence. An additional 464 nucleotides found within the 3′ NTR of only one cDNA clone (BSS3031) is shown in italic.

ORF corresponded to a predicted polypeptide of 461 amino acid residues. Subsequent work identified another clone, BSS3031, which is identical to BSS2632 except for an additional 444 bases at the 3′ NTR.

Comparison of BSS with other SS sequences. The predicted molecular weight of BSS is 52.5 kDa, which is somewhat larger than the 46.9–48.1 kDa reported for mammalian and higher plant SS (29, 32, 33). In

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B. braunii        MGMLRWGVESLQNPDELIPVLRM-IYADKFGKIKPKDEDRG----FCYEILNLVSRSFAI   55
N. tabacum        MGSLRA---ILKNPDDLYPLVKLKLAARHAEKQIPPSPHWG----FCYSMLHKVSRSFAL   53
A. thaliana       MGSLGT---MLRYPDDIYPLLKMKRAIEKAEKQIPPEPHWG----FCYSMLHKVSRSFSL   53
Z. mays           MG-------ALSRPEEVLALVKLRVAAGQIKRQIPPEEHWA----FAYSMLQKVSRSFAL   49
R. rattus         MEFVK----CLGHPEEFYNLLRFRMGGRRNFIPKMDRNSLSNSLKTCYKYLDQTSRSFAA   56
S. cerevisiae     MGKLLQ---LALHPVEMKAALKLKFCRTPLFSIYDQSTSPY--LLHCFELLNLTSRSFAA   55
Z .mobilis        -------------------MWKGRAQARNRSVSIKTKNKLN--AAALVSGKGHQDENFPV   49
Consensus         MG...........P.E...L.K............P.........FCYS.L..VSRSFA.

                        _______I________ _________II_________
B. braunii        VIQQLPAQLRDPVCIFYLVLRALDTVEDDMKIAATTKIPLLRDFYEKISDRSFRMTAGD-  114
N. tabacum        VIQQLPVELRDAVCIFYLVLRALDTVEDDTSIPTDVKVPILISFHQHVYDRERHFSCG--  111
A. thaliana       VIQQLNTELRNAVCVFYLVLRALDTVEDDTSIPTDEKVPILIAFHRHIYDTDWHYSCG--  111
Z. mays           VIQQLGPELRNAVCIFYLVLRALDTVEDDTSIPTEVKVPILQEFYRHIYNRDWHYSCG--  107
R. rattus         VIQALDGDIRHAVCVFYLILRAMDTVEDDMAISVEKKIPLLRNFHTFLYEPEWRFTES--  114
S. cerevisiae     VIRELHPELRNCVTLFYLILRALDTIEDDMSIEHDLKIDLLRHFHEKLLLTKWSFDGNAP  115
Z. mobilis        ASFLINPEYRPIIMAFYQFARQADDVADNVIASKKDRLAILEDMRSSLTG------ES--  101
Consensus         VIQQL..ELR.AVC.FYLVLRALDTVEDD.SI....K.PIL..FH...Y...W....G..


B. braunii        QKDYIRLLDQYPKVTSVFLKLTPREQEIIADITKRMGNGMADFVHKGVP-----DTVGDY  169
N. tabacum        TKEYKVLMDQFHHVSTAFLELRKHYQQAIEDITMRMGAGMAKFICKEV------ETTDDY  165
A. thaliana       TKEYKILMDQFHHVSAAFLELEKGYQEAIEEITRRMGAGMAKFICQEV------ETVDDY  165
Z. maya           TNHYKMLMDKFRHVSTAFLELGEGYQKAIEEVTRRMGAGMAKFICKEV------ETVDDY  161
R. rattus         KEKHRVVLEDFPTISLEFRNLAEKYQTVIADICHRMGCGMAEFLNKDV------TSKQDW  168
S. cerevisiae     DVKDRAVLTDFESILIEFHKLKPEYQEVIKEITEKMGNGMADYILDENYNLGLQTVHDY  175
Z. mobilis        QSEPNAVVLRQTLIN-------HGLDHTIVHGLDLLEAFRRDVSVNRY------ENWDAL  148
Consensus         ...Y..L.D.F..VS..FL.L...YQ..I..IT.RMGAGMA.FI..EV......ETVDDY

                  _______III_________                 ________IV________
B. braunii        DLYCHYVAGVVGLGLSQLFVASGLQSPSLTRSEDLSNHMGLFLQKTNIIRDYFEDINELP  229
N. tabacum        DEYCHYVAGLVGLGLSKLFHASGKEDLAS---DSLSNSMGLFLQKTNIIRDYLEDINEVP  222
A. thaliana       DEYCHYVAGLVGLGLSKLFLAAGSEVLTPDW-EAISNSMGLFLQKTNIIRDYLEDINEIP  224
Z. mays           DEYCHYVAGLVGYGLSRLFYAAGTEDLAL---DSLSNSMGLFLQKTNIIRDYLEDINEIP  218
R. rattus         DKYCHYVAGLVGIGLSRLFSASEFEDPIVGEDTECANSMGLFLQKTNIIRDYLEDQQEG-  227
S. cerevisiae     DVYCHYVAGLVGDGLTRLIVIAKFANESLYSNEQLYESMGLFLQKTNIIRDYNEDLVDG-  234
Z. mobilis        MDYCRYSASPVG----RFVLDVHKESRNL---WPMNDALCTALQVINHLQDCGKDYRMMN  201
Consensus         D.YCHYVAGLVG.GLS.LF.A.G.E.........LSNSMGLFLQKTNIIRDYLEDINE.P


B. braunii        APRMFWPREIWGKYANNLAEFKDPANKAAAMCCLNEMVTDALRHAVYCLQYMSMIEDPQI  289
N. tabacum        KCRMFWPREIWSKYVNKLEELKYEDNSAKAVQCLNDMVTNALSHVEDCLTYMSALRDPSI  282
A. thaliana       KSRMFWPREIWGKYADKLEDLKYEENTNKSVQCLNEMVTNALMHIEDCLKYMVSLRDPSI  284
Z. mays           KSRMFWPREIWSKYADKLEDFKYEENSKKAVQCLNNMVTDALIHAEECLQYMSALKDPAI  278
R. rattus         --RQFWPQEVWGKYVKKLEDFVKPENVDVAVKCLNELITNALQHIPDVITYLSRLRNQSV  285
S. cerevisiae     --RSFWPKEIWSQYAPQLKDFMKPENEQLGLDCINHLVLNALSHVIDVLTYLASIHEQST  292
Z. mobilis        --RIYIPSDIMEAVGATAGDLGCFHASLPLRQAIETAALKTKSLLKRSSSFSVAIHDKRL  259
Consensus         ..RMFWPREIW.KYA.KLEDFK..EN...AVQCLN.MVTNAL.H..DCL.YMS.L.DPSI

                  _______V_______
B. braunii        FNFCAIPQTMAFGTLSLCYNNYTIFTGPKAAVKLRRGTTAKLMYTSNNMFAMYRHFLNFA  349
N. tabacum        FRFCAIPQVMAIGTLAMCYDNIEVFRG---VVKMRRGLTAKVIDQTRTIADVYGAFFDFS  339
A. thaliana       FRFCAIPQIMAIGTLALCYNNEQVFRG---VVKLRRGLTAKVIDRTKTMADVYGAFYDFS  341
Z. maiz           FRFCAIPQIMAIGTCALCYNNVHVFRG---VVKMRRGLTARIIDETNSMTDVYTAFYEFS  335
R. rattus         FNFCAIPQVMAIATLAACYNNHQVFKG---VVKIRKGQAVTLMMDATNMPAVKAIIYQYI  342
S. cerevisiae     FQFCAIPQVMAIATLALVFNNRFVLHG---NVKIRKGTTCYLILKSRTLRGCVEIFDYYL  349
Z. mobilis        GVEVAVIQRLAES-LTECLTKHDPLSE---RVHHNKAETLGLAFVAAAGRLFS-------  308
Consensus         F.FCAIPQ.MAIGTLALCYNN..VF.G...VVK.RRG.TA..I........VY..F..F.


B. braunii        EKLEVRCNTETSEDPSVTTTLEHLHKIKAACK-AGLARTKDDTFDELRSRLLALTGGSFY  408
N. tabacum        CMLKSKVN---NNDPNATKTLKRLEAILKTCRDSGTLNKRKSYIIRS-E-----------  384
A. thaliana       CMLKTKVD---KNDPNASKTLNRLEAVQKLCRDAGVLQNRKSYVNDKGQ-----------  387
Z. mays           SLIESKID---DSDPNAALTQRRVDSIKQTCKSSGLVKQRGYHLEKS-------------  379
R. rattus         EEIYHRVP---NSDPSASKAKQLISNIRTQSLPNCQLISRSHYSPIYLS-----------  388
S. cerevisiae     RDIKSKLA---VQDPNFLKLNIQISKIEQFMEEMYQDKLPPNVKPNETPIFLKVKERS--  404
Z. mobilis        ------------------------------------------------------------
Consensus         .....K.......DPNA.KT......I...C...G....R...................

                                                  _____________VI_____________
B. braunii        LAWTYNFLDLRGPGDLPTFLSVTQHWWSILIFLISIAVFFIPSRPSPRPTLSA--  461
N. tabacum        ------------PNYSP----------VLIVVIFIILAIILAQLSGNRS-------  411
A. thaliana       ------------PNS-----------VFIIMVVILLAIVFAYLRAN---------  410
Z. maya           ------------PYR-P---------MLIMIVLLLVAILFGVMF-----------  401
R. rattus         ---------------------------FIMLLAALSWQYLSTLSQVTEDYVQREH  416
S. cerevisiae     -----RYDDELVPTQQE------EEYKFNMVLSIILSVLLGFYYIYTLHRA----  444
Z. mobilis        ------------------------------------------------------
Consensus         ............P.............I....I....................
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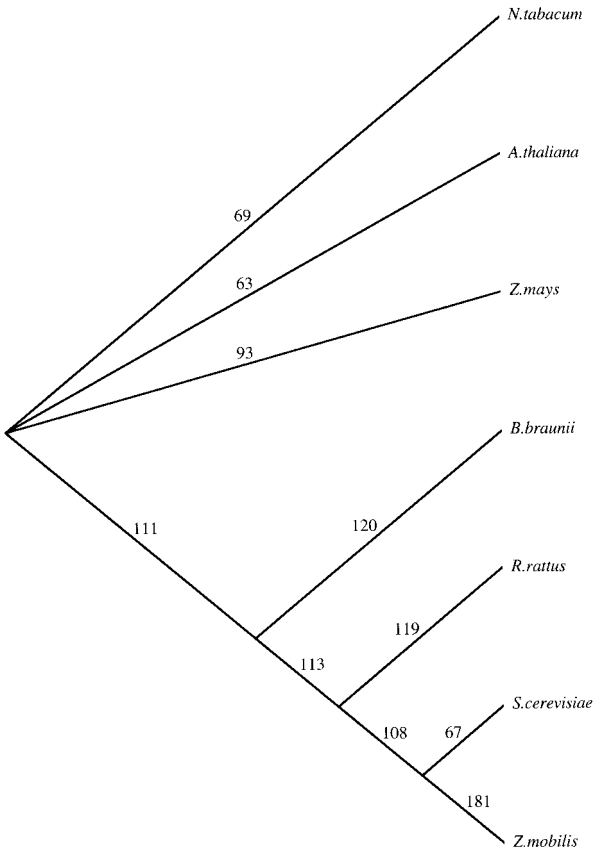

FIG. 4. A phylogenetic tree of SS protein from plant (29, 33, 42), animal (32), fungal (30), and bacterial (43) sources obtained by the parsimony procedure (45).

terms of amino acid sequence comparisons, BSS is 52% identical to the SS cDNA from *N. tabacum* (29), 51% to that of *A. thaliana* (33), 48% to *Z. mays* (42), 40% to *R. rattus* (32), 39% to *S. cerevisiae* (30), and 26% to *Z. mobilis* (43) (Fig. 3). A phylogenetic analysis of BSS positioned it as being more closely related to animal, yeast, and bacterial SS than to higher plant SS on the basis of conservation (Fig. 4). The six peptide sequences identified by Robinson *et al.* (34) are also present in the BSS amino acid sequence (Fig. 3). Three of the six domains (III, IV, and V) are highly conserved, while domains I and II are moderately conserved within the BSS sequence. Domain VI exhibits low sequence identity among all SS proteins; however, the amino acid sequence of domain VI is very hydrophobic in all SS enzymes, including BSS. This is consistent with a membrane-anchoring function for domain VI (34).

Expression of BSS in E. coli. The entire coding region of the BSS cDNA (BSS1386) and a truncated BSS cDNA (BSS1308), consisting of a deletion corresponding to the last 26 amino acids of the carboxy terminus, were inserted into the expression vector pET-11c (pET11c-BSS1386, pET11c-BSS1308), transformed into the *E. coli* strain BL21(DE3), and expression was induced by the addition of 1 mM IPTG. Expression of both the full-length and the truncated BSS cDNAs in *E. coli* gave readily measurable SS activity in the 10,000*g* extracts of the *E. coli* cells (Fig. 5B). This corresponded to the appearance of protein of predicted molecular mass in both cases (52.5 kDa for full length and 49.7 kDa for truncated) as determined by SDS–PAGE of whole-cell extracts (Fig. 5A). The truncated BSS form consistently gave higher expression levels than the full-length BSS. No botryococcene synthase activity with FPP as the substrate was detectable in extracts from *E. coli* expressing either the full-length or the truncated BSS cDNA under a variety of salt and cofactor conditions (data not shown).

Southern blot analysis. Genomic DNA of *B. braunii* digested with *Hin*dIII or *Pst*I, was probed with the 1386-bp BSS cDNA, and the hybridization pattern was compared to that predicted from sequence analysis of the cDNA and a portion of the corresponding genomic DNA (Fig. 6). The sequence and intron–exon organization of the BSS genomic DNA were obtained from cloned PCR amplification products of genomic DNA using degenerate primers designed to conserved domains III, IV, and V. The full-length cDNA contains a single restriction site for *Hin*dIII in the 3′ NTR and one restriction site for *Pst*I within the open reading frame. The position of these restriction sites are conserved in the corresponding genomic DNA. Additional sites for these restriction enzymes are found within the intronic DNA segments examined. One restriction site for *Hin*dIII is found within the intron between domains III and IV of the genomic DNA. Two *Pst*I sites are observed, one between domains III and IV and a second between domains IV and V. Identification of these restriction sites predict minimal sizes of restriction fragments that should arise upon digestion of genomic DNA with the respective enzymes (Fig. 6A). For example, if the BSS gene is a single-copy gene and genomic DNA digested with *Hin*dIII is hybridized with

FIG. 3. Comparison of the BSS amino acid sequence to that of SS amino acid sequences from *Nicotiana tabacum* (29), *Arabidopsis thaliana* (33), *Zea mays* (42), *Rattus rattus* (32), *Saccharomyces cerevisiae* (30), and *Zymomonas mobilis* (43). Numbers indicate the amino acid residues in the sequences. Gaps in the alignment are designated by dashes. Amino acid residues which are common to more than three polypeptides are considered a consensus. Overlines numbered with roman numerals indicate highly conserved regions according to the nomenclature of Robinson *et al.* (34).

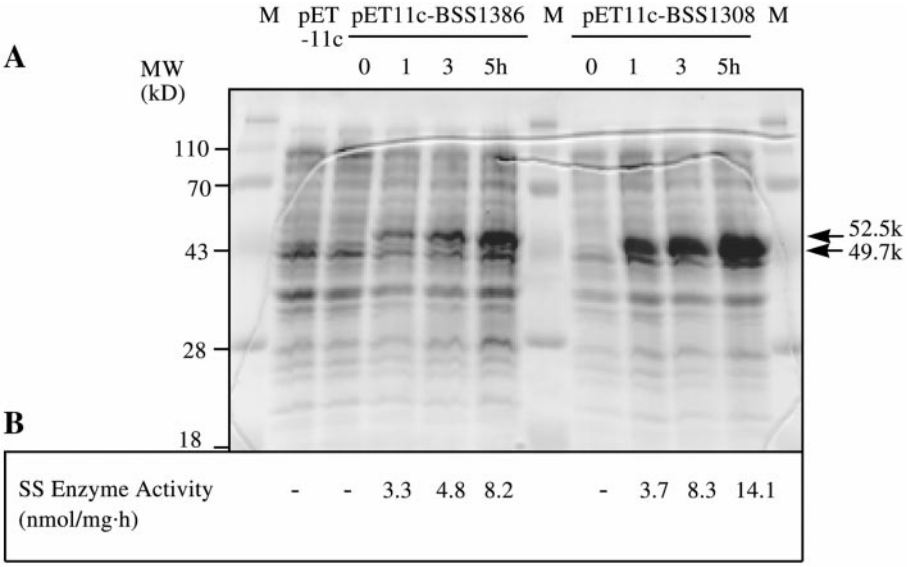

FIG. 5. Time course expression of full-length (BSS1386) and truncated (BSS1308) genes in *E. coli*. The pET11c-BSS1308 cDNA represents a 26-amino acid, carboxy-terminus deletion of the full-length BSS cDNA clone (BSS1386). Bacterial cells harboring these constructs were grown at 37°C up to 5 h after the addition of 1 mM IPTG. Total protein extracts of the *E. coli* cells were used for protein profiling by SDS–PAGE/Coomassie blue staining. The arrows indicate the positions predicted for the full-length and the truncated BSS protein (A). A 10,000*g* supernatant fraction was used to determine SS enzyme activities at the indicated times (B). M, prestained molecular weight markers.

radiolabled cDNA, then two hybridizing bands are expected; one of 2500 bp and a second that is 1337 bp or larger. Similarly, four hybridizing bands are predicted for a hybridization of *Pst*I-digested genomic DNA with labeled cDNA. Even though the hybridization signal for the 384-bp fragment arising from the *Pst*I digestion is weak, the actual hydridzation patterns shown in Fig. 6B closely approximate the expected patterns.

Changes in the level of BSS mRNA and enzyme activity during a culture cycle. *B. braunii* cultures were propagated in a manner similar to those of higher plant cell cultures, and their progression through a culture cycle was monitored as simple dry weight or fresh weight gain. Aliquots of stationary cell cultures inoculated into fresh media underwent a brief lag period of 1 day before entering into a rapid growth phase. The rapid growth phase lasted approximately 18 days and resulted in a net 2.5- to 3-fold gain in dry weight (Fig. 7A). The cultures then underwent a transition into a stationary phase 21 to 24 days after subculturing.

Squalene synthase enzyme activity and mRNA levels were measured in the cells at various points along the growth cycle. The synthase activity was initially low in the cells but rose rapidly to a maximum 7 to 9 days after subculturing (Fig. 7A). The enzyme activity decreased in the middle stages of rapid growth and reached a minimum before the cells entered into their stationary phase. Aliquots of these same cultures were also used to measure the corresponding levels of the squalene synthase mRNA (Fig. 7B). RNA was isolated from cells harvested at the indicated days in a culture cycle and then probed for the squalene synthase mRNA with the BSS cDNA (BSS1386) clone. Parallel to changes in the enzyme activity, the steady state levels of the squalene synthase mRNA were also transiently induced with an apparent maximum occurring 6 to 8 days after subculturing.

DISCUSSION

The amino acid sequence of the *B. braunii* SS enzyme deduced from the cDNA clone described in this report is very similar to that of many of the SS enzymes described from other organisms (29, 30, 32, 33, 42), especially with regard to domains I to VI as proposed by Robinson *et al.* (34). Of these six domains, domains III, IV, and V of the rat SS have been shown by Gu *et al.* (35) to be required for a functional enzyme. Mutations within domain IV disrupt FPP binding, while mutations within domains III and V disrupt the first and second partial reactions of the proposed catalytic mechanism for the enzyme. It is reasonable, therefore, to expect that these three domains would be highly conserved among all SS proteins. Although alignment of the BSS enzyme with that of other species varies from 52 to 26% sequence identity, domains III, IV, and V are as highly conserved as for any other SS enzyme reported to date. The demonstration of SS enzyme activity in extracts of *E. coli* that express the BSS cDNAs

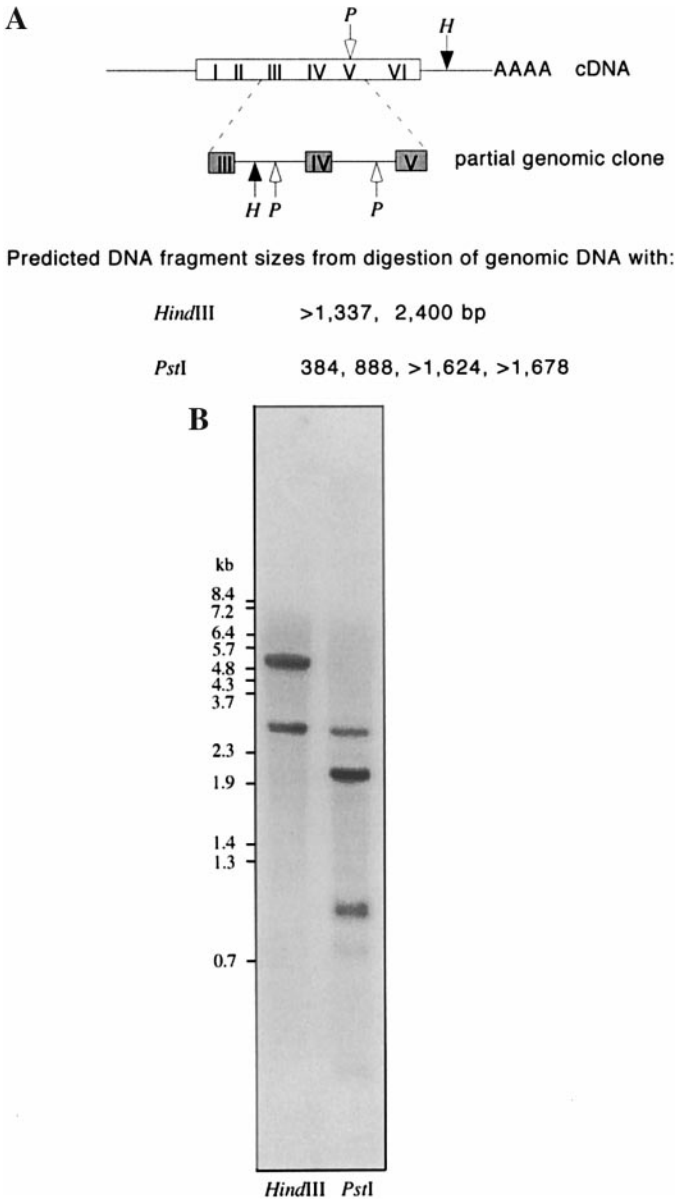

FIG. 6. DNA blot analysis of the BSS genes in the *Botryococcus* genome. (A) Restriction sites identified by sequencing of the full-length BSS cDNA and partial genomic DNA clones were used to predict the minimal size of BSS gene fragments resulting from digestion of *Botryococcus* genomic DNA. (B) Genomic DNA (8 μg) was digested with the indicated restriction enzymes, size-separated on a 0.8% agarose gel, transferred to nylon membrane, and hybridized with a radiolabeled full-length BSS cDNA (BSS1386).

further corroborates out contention that this cDNA represents a *Botryococcus* SS gene.

SS enzyme activity is rapidly and transiently induced during a culture cycle of *B. braunii* and is correlated with changes in the level of its mRNA. This pattern may or may not be consistent with some of the other physiological changes that are occurring in the algal cultures. Shortly after transfer into fresh culture media, the B race algal cells are thought to undergo a rapid proliferation which entails the generation of new daughter cells and the biosynthesis of cellular membranes containing sterols. The cultures are also thought to initiate synthesis and accumulation of extracellular matrix hydrocarbons and biopolymers which can account for 30 and 10% of the total biological mass, respectively (8, 17). Because the B race of *B. braunii* has been used in the current work, the accumulating extracellular constituents include organic extractable botryococcenes and squalene derivatives, which may be incorporated into chemically resistant biopolymers. Botryococcenes, like squalene, are thought to be derived from the condensation of two FPP moieties in a modified SS-like reaction (25, 26). Therefore, if biomass accumulation can be equated to cell proliferation and accumulation of the extracellular matrix components and if SS enzyme activity was limiting for both of these, then one would expect this enzyme activity to be maximal during that phase of the culture cycle when the rate of biomass accumulation was greatest. Biomass accumulation was greatest on days 6 through 18 while SS activity was maximal on days 3 to 9 of the culture cycle.

One possible explanation for this apparent discrepancy would be that the culture cycle consists of two growth phases. The first phase would be associated with cell proliferation, the accumulation of squalene necessary for sterol and membrane biogenesis, and a limited amount of squalene for extracellular biopolymer assembly. The early induction of SS activity could be sufficient to account for this need. The second phase of biomass accumulation might be correlated with a maturation of cells and a dedication of their biosynthetic capacities to the accumulation of the extracellular components. In the case of the *B. braunii* B race, this would entail the accumulation of large amounts of the triterpenoid botryococcenes. Although there has been speculation that the botryococcenes might be derived from a SS-like reaction (25, 26), perhaps the botryococcenes are derived by a separate and distinct botryococcene synthase enzyme. Our failure to find any botryococcene synthase activity associated with the bacterial-generated SS enzyme activity is consistent with this possibility.

Current biochemical rationalizations for squalene and botryococcene biosynthesis suggest mechanistic features in common between the two reactions. Both require FPP for catalysis and both are thought to proceed through a cyclopropyl intermediate. What has been proposed to distinguish the two reactions from one another is the way in which the cyclopropyl ring is opened to generate either a linear hydrocarbon (squalene) or the branched form (botryococcene). Given the proposed mechanistic features in common, it is not unreasonable to think that botryococcene and squalene

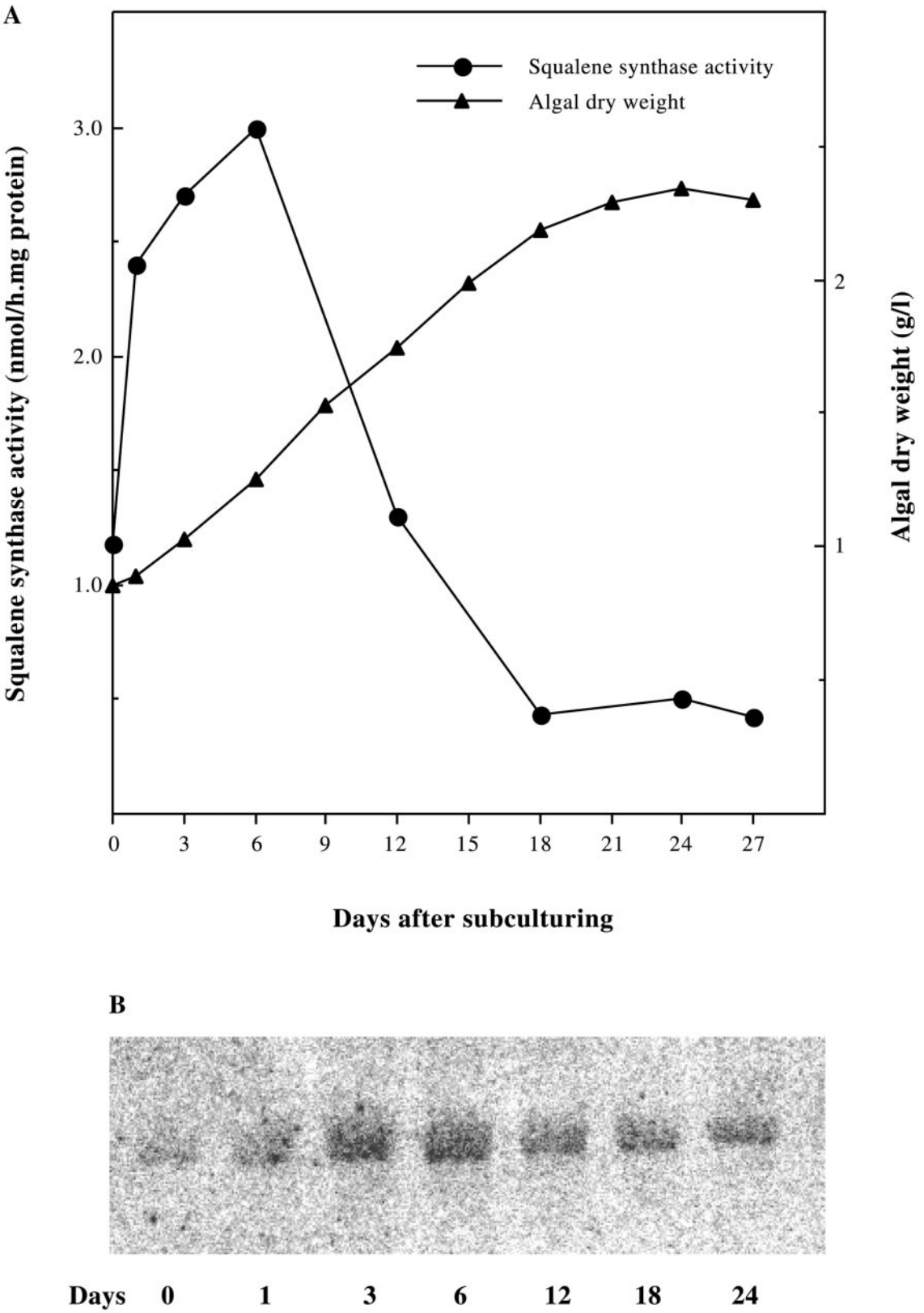

FIG. 7. Growth curve of *B. braunii* Berkeley cell cultures over a 27-day culture period and changes in microsomal SS enzyme activity (A) and the steady-state level of BSS mRNA (B) over this period. Total RNA was isolated from cells at the times indicated; 2-μg aliquots of the RNA were size-separated on a 1.2% formaldehyde-agarose gel, transferred to a nylon membrane, and probed with a full-length BSS cDNA (BSS1386). Microsomal fractions were prepared from the same algal samples and used to determine the SS enzyme activity.

biosynthesis might arise from a single enzyme, modified in some way to give one product or the other, or from two SS-like enzymes. As mentioned above, we have not been able to find conditions under which the algal SS enzyme exhibits any botryococcene synthase activity. The observation of only a single molecular species of SS in the algal genome also does not support the notion of multiple SS-like enzymes with specialized activities. Instead, our results suggest the possibility of a unique botryococcene synthase enzyme and gene.

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