Squalene-hopene cyclase from *Bradyrhizobium japonicum*: cloning, expression, sequence analysis and comparison to other triterpenoid cyclases

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With the help of a PCR-based screening method, the gene encoding squalene-hopene cyclase (SHC) of *Bradyrhizobium japonicum* USDA 110 was isolated from a cosmid library. The SHC catalyses the cyclization of squalene to hopanoids, a class of triterpenoid lipids recently discovered in nitrogen-fixing, root-nodule-forming *Bradyrhizobium* bacteria. Hybridization experiments showed that the gene is present in bacteria of all *Bradyrhizobium* strains tested and in photosynthetic bacteria forming stem nodules on tropical legumes of the genus *Aeschynomene*. The *Bradyrhizobium shc* gene is 1983 bp in length and encodes a protein of 660 amino acid residues with a calculated molecular mass of 73671 Da. Comparison of the deduced amino acid sequence with the sequences of other SHCs revealed highest similarity (70%) to the SHC from the Gram-negative *Zymomonas mobilis* and lower similarity (48%) to the SHCs from the Gram-positive *Alicyclobacillus acidocaldarius* and *Alicyclobacillus acidoterrestris*. *Bradyrhizobium* SHC also showed similarity (38–43%) to eukaryotic oxidosqualene cyclases. The *B. japonicum shc* gene was expressed in *Escherichia coli*. The recombinant SHC catalysed the cyclization of squalene to the hopanoids hopene and diplopterol in vitro. However, the formation of the gammacerane derivative tetrahymanol, which is produced in addition to hopanoids in *B. japonicum* strains in vivo, could not be detected in vitro. Therefore, the presence of a second squalene cyclase in *B. japonicum* can be assumed. Sequence analysis of 0.5 kb upstream from the *shc* gene identified a partial ORF with significant similarity to the C-terminus of an ORF located immediately upstream from the *shc* gene in *Z. mobilis*. Both ORFs also showed similarity to phytoene desaturases from cyanobacteria and plants. The 3′-end of this ORF from *B. japonicum* overlaps with 13 bp at the 5′-end of *shc*. The close proximity of this ORF to *shc* suggests that *shc* and this ORF may be part of an operon.

Keywords: *Bradyrhizobium japonicum*, triterpenoids, hopanoids, squalene-hopene cyclase

INTRODUCTION

Hopanoids are a class of pentacyclic triterpenoid lipids occurring in a wide range of Gram-positive and Gram-negative bacteria (Sahm et al., 1993). Among aerobic bacteria with the ability to fix atmospheric nitrogen, hopanoids were found in free-living *Azotobacter* and *Beijerinckia* (Vilcheze et al., 1994), and in the symbiotic actinomycete *Frankia* (Berry et al., 1991). Recently, hopanoids were also discovered in nitrogen-fixing *Bradyrhizobium* bacteria (Kannenberg et al., 1995) that form root nodules in symbiosis with various legume plants, and in photosynthetic bacteria forming stem
The key enzyme in hopanoid biosynthesis is the squalene-hopene cyclase (SHC). This enzyme catalyses the cyclization of the linear triterpenoid squalene to hopene and diplopterol (Fig. 1). These reaction products are the presumed precursors of elongated hopanoids formed in prokaryotes from squalene by SHC activity. Elimination of a proton leads to hopene; addition of a hydroxyl group derived from water leads to formation of diplopterol. In some cases such as B. japonicum, R. palustris or the ciliate Tetrahymena, addition of a hydroxyl group can lead to formation of the gammacerane derivative tetrahymanol. However, neither hopanoids nor tetrahymanol have been detected. In the case of the nitrogen-fixing Rhizobium, Bradyrhizobium contains another pentacyclic triterpenoid, the gammacerane derivative tetrahymanol (unpublished results). The only prokaryote in which tetrahymanol has been discovered in addition to hopanoids is the purple non-sulphur bacterium Rhodopseudomonas palustris (Kleemann et al., 1990). Phylogenetic studies based on 16S rRNA sequence analysis revealed that R. palustris is a close relative of Bradyrhizobium (Jarvis et al., 1986). Neither hopanoids nor tetrahymanol have been detected so far in Rhizobium species or in additional members of the Rhizobiaceae (Kannenberg et al., 1995, 1996).

Hopanoids and tetrahymanol are reinforcing of cellular membranes (Kannenberg et al., 1980; Ourisson et al., 1987), condensing membranes in a similar way to cholesterol in eukaryotic membranes. However, hopanoids and tetrahymanol may have additional functions besides membrane reinforcement that have not yet been detected. In the case of the nitrogen-fixing Frankia, it has been suggested that hopanoids are involved in oxygen protection of the nitrogenase complex by forming a diffusion barrier (Berry et al., 1993). This may also be true in the case of Bradyrhizobium, which, unlike Rhizobium, is able to fix nitrogen non-symbiotically in the free-living state (Wilcockson & Werner, 1976).

The key enzyme in hopanoid biosynthesis is the squalene-hopene cyclase (SHC). This enzyme catalyses the cyclization of the linear triterpenoid squalene to hopene and diplopterol (Fig. 1). These reaction products are the presumed precursors of elongated hopanoids with a great diversity of polar side chains. Mechanistically, this cyclization reaction is not well understood and the cyclization products can differ depending on the organism involved; for example, in the ciliate Tetrahymena an analogous reaction leads predominantly from squalene to the formation of tetrahymanol (Caspi et al., 1968; Abe et al., 1993).

SHCs from several bacteria (Alicyclobacillus acidocaldarius, Zymomonas mobilis and R. palustris) have been purified and characterized recently (Ochs et al., 1992; Tappe, 1993; Kleemann et al., 1994). The genes of the SHCs from A. acidocaldarius, Alicyclobacillus acidoterrestris and Z. mobilis have been isolated and their nucleotide sequences determined (Ochs et al., 1992; EMBL accession no. X89584; Reipen et al., 1995). Prokaryotic SHCs exhibit similarity to oxidosqualene cyclases (OSCs) from plants, animals and fungi, supporting the hypothesis that both types of cyclases have evolved from a common ancestor cyclase (Ourisson et al., 1987).

We chose Bradyrhizobium to start an investigation on the molecular biology of hopanoid biosynthesis for several reasons. First, bradyrhizobia live and thrive in two environments: as soil colonizers and as symbiotic endophytes. This characteristic allows us to address the function of hopanoids in these different habitats and as part of plant–microbe interactions. Second, bradyrhizobia contain a range of hopanoid derivatives and structurally related compounds (e.g. tetrahymanol) whose biological function has not been explained. Since bradyrhizobia are genetically accessible, they are ideal for addressing the functions of these different compounds. Third, the ease of genetically manipulating the amino acid sequence of the SHC protein will allow us to address questions about the cyclization reaction mechanism and important features in the SHC enzyme structure.

In this paper, we describe the cloning, DNA sequencing, expression and sequence analysis of the shc gene from B. japonicum as the first step in an examination of hopanoid biosynthesis and in vivo function.

**METHODS**

**Strains, plasmids and growth conditions.** B. japonicum USDA 110spc4 (Regensburger & Hennecke, 1983), Bradyrhizobium sp. (Lupinus) (Deutsche Sammlung von Mikroorganismen und Zellkulturen 30140) and the photosynthetic Aeschynomene stem-nodulating strain MKA3 (USDA 4088; Wong et al., 1994) were cultivated in YM medium (Vincent, 1970) at 28 °C. Escherichia coli strains (S17-1, XL-1 Blue and DH5α) were grown in LB medium (Gibco) routinely at 37 °C. E. coli strain S17-1 (Simon et al., 1983) was used for construction of the cosmid library, and strains XL-1 Blue and DH5α were used for cloning procedures. Cloning and subcloning were carried out with plasmid vectors pUC18 and pUC19 (Vieira & Messing, 1982), respectively. For construction of the cosmid library, cosmid vector pVK100 (Knauf & Nester, 1982) was used. The concentrations of antibiotics were 100 mg ampicillin L⁻¹, 10 mg tetracycline L⁻¹ and 30 mg kanamycin L⁻¹.

**Standard DNA manipulation procedures.** Genomic DNA of B. japonicum USDA 110 was isolated according to the procedure of Somasegaran & Hoben (1994). Digestion with restriction enzymes on tropical legumes of the genus Aeschynomene (Kannenberg et al., 1996). Besides hopanoids, Bradyrhizobium contains another pentacyclic triterpenoid, the gammacerane derivative tetrahymanol (unpublished results). The only prokaryote in which tetrahymanol has been discovered in addition to hopanoids is the purple non-sulphur bacterium Rhodopseudomonas palustris (Kleemann et al., 1990). Phylogenetic studies based on 16S rRNA sequence analysis revealed that R. palustris is a close relative of Bradyrhizobium (Jarvis et al., 1986). Neither hopanoids nor tetrahymanol have been detected so far in Rhizobium species or in additional members of the Rhizobiaceae (Kannenberg et al., 1995, 1996).

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endonucleases, ligation and transformation of E. coli were carried out as described in Sambrook et al. (1989). Large-scale isolation of plasmid DNA was performed with a Macherey and Nagel Nucleobond AX-100 kit. Small-scale isolation of plasmid DNA was done using the alkaline lysis method (Birnboim & Doly, 1979).

Southern hybridization was performed with digoxigenin-labelled probes using the random primed method according to the protocol of the manufacturer (Boehringer Mannheim). Digested DNA was separated on agarose gels and blotted with a Vakublot apparatus (Pharmacia) on positively charged nylon membranes (Boehringer Mannheim). Hybridization was done at 65 °C (2 h prehybridization, at least 12 h hybridization) in a hybridization oven (Hybaid). Washing was performed twice with 2× SSC (0.3 M NaCl; 0.03 M tri-sodium citrate, pH 7.4); 0.1% SDS at room temperature, followed by washing twice with 0.1× SSC, 0.1% SDS at 65 °C. Detection was carried out with anti-digoxigenin-antibody conjugates (anti-digoxigenin–alkaline phosphatase Fab fragments) and the chemiluminescence substrate CSPD (Boehringer Mannheim) according to the instructions of the manufacturer.

**Construction of the cosm id library.** Genomic DNA of *B. japonicum* USDA 110 was partially digested with EcoRI and separated by gel electrophoresis. Fragments of 20–25 kb were cloned in the single EcoRI site of cosm id vector pVK100, packaged in vitro in phage λ, and propagated in *E. coli* S17-1 (Parniske et al., 1993).

**PCR screening of the cosm id library.** Clones of the cosm id library from *B. japonicum* USDA 110 harbouring the *shc* gene were identified with the help of a PCR-based screening method utilizing a method described by Griffin et al. (1993). The PCR conditions were as follows: denaturation at 94 °C for 1 min; annealing at 48 °C for 1 min; and elongation at 73 °C for 1 min. A 50 μl reaction contained 1 U vent DNA polymerase (Biolabs), 0.5 μl 10× polymerase buffer (Biolabs), 1 mM each dNTP, 0.5 M MgSO₄, 10 pmol each primer and 1 μg genomic DNA or 1 μg cosmid DNA. The reaction mixture was overlaid with 50 μl mineral oil.

Conserved regions of known SHCs and OSCs (Fig. 2) were used to design a pair of degenerate oligonucleotide primers (P1/P2 in Fig. 2). All oligonucleotides were custom-synthesized by Eurogentec.

P1 (5’-TGAGTTC/TCGG/TTTGGG-3’) and P2 (5’-CGCCCAG/ACCG/ACCG/ATCCGGTGGTC-3’) amplified a 150 bp fragment of the *shc* gene from genomic DNA of *B. japonicum* USDA 110. Homologous primers B1 (5’-CATCTAAGAACCTGTGTC-3’) and B2 (5’-TCGAGG-CCAGCCAGTCGG-3’) were synthesized according to the nucleotide sequence of the 150 bp PCR fragment. PCR with these two primers was expected to result in amplification of a 98 bp PCR fragment (for more details see Results and Discussion and Fig. 2). The conditions in both PCRs were identical with the exception of an annealing temperature of 52 °C in the case of primers B1 and B2. These primers were used to PCR-screen a *B. japonicum* cosm id library for clones harbouring the *shc* gene. The cosm id library was divided into pools of 100 clones that were grown on agar plates. The clones were washed off with 5 ml LB medium, and the cosm id DNA of these clones was collectively isolated. An aliquot of the isolated cosm id DNA was used as PCR template. Only those pools that allowed amplification of the expected 98 bp fragment were submitted to the next screening step. Positive pools were subdivided into pools containing 10 clones, cultivated in 1 ml LB medium, and the isolated cosm id DNA was screened by PCR. The indicative 98 bp fragment could be amplified from two of these pools. The remaining 20 clones were now individually assayed by PCR.

**Sequence determination.** Double-stranded DNA was sequenced with the AutoRead Sequencing kit and an ALF DNA Sequencer (Pharmacia). As sequencing primers, fluorescein-labelled M13 universal and reverse primers or fluorescein-labelled *shc*-specific primers (custom-synthesized by Pharmacia) were used. Sequence analysis was done with the PCGENE program package (IntelliGenetics). Database searches were performed with the BLASTN and BLASTP programs of NCBI (Altschul et al., 1990).

**Enzyme activity assay.** *E. coli* DH5α cells, containing plasmid DNA with or without the *B. japonicum* shc gene, were grown overnight at 28 °C in liquid LB medium, harvested by centrifugation, and washed in a 0.9% NaCl solution. The cells were resuspended in buffer (100 mM Tris/HCl, 250 mM saccharose, 20 mM ascorbic acid, pH 8). The frozen cells (−20 °C) were passed five times through an X-press homogenizer for cell rupture. The homogenate was thawed, lysozyme and DNase were added, and debris and unbroken cells were removed by centrifugation. Appropriate aliquots (between 100 and 400 μl) of the different homogenates were added to individual assay solutions containing the substrate squalene (100 μl of a squalene stock solution of 500 μM squalene; 0.1% Triton X-100; 100 mM sodium citrate, pH 6.5), 100 μ1 1 M sodium citrate (pH 6.5) and water to make up a final volume of 1 ml. The mixture was incubated for 2 h at 28 °C and then extracted with 2 ml hexane/2-propanol (3:2, v/v). The organic phase was collected and evaporated, and the remaining lipids were redissolved in 30 μl hexane/2-propanol for analysis.

The reaction products were analysed by GLC analysis with a Shimadzu GC-9 gas chromatograph on a capillary column (DB-1, methylsilicon; 0-32 mm × 20 m). The oven temperature was set from 250°C to 320°C with 4°C min⁻¹ increase followed by 10 min at 320°C. Detection was made with a flame ionization detector at 330°C. Triterpenoids were identified either by comparison with the retention times of standard compounds or by GLC-Ms fragmentation analysis.

**RESULTS AND DISCUSSION**

**Isolation of the *shc* gene.**

The importance of hopanoid lipids for bradyrhizobia in the soil and as symbiotic endophytes has yet to be elucidated. As a first step in the analysis of hopanoid function and biosynthesis, we attempted to isolate the gene encoding one of the key enzymes in biosynthesis, the SHC.

For isolation of the *shc* gene from *B. japonicum* USDA 110, a two-step PCR-based screening strategy was used. In the first step, genomic DNA was used to amplify a 150 bp DNA fragment with homology to the *shc* gene. The degenerate primers P1 and P2 were designed according to two of the most conserved regions in known SHCs and OSCs (Fig. 2). The deduced amino acid sequence of the amplified DNA fragment from strain USDA 110 had significant identity (57% in the case of *Z. mobilis* SHC) to the corresponding region of other SHCs and OSCs (Fig. 2), indicating that we had amplified a DNA fragment that should be part of a
BamHI fragment was found to be entirely part of the 5 kb EcoRI fragment (Fig. 3).

Hybridization signals with the 150 bp PCR fragment were also found with genomic DNA of other Bradyrhizobium strains (data not shown), e.g. Bradyrhizobium sp. (Lupinus) and also MKAa3 (USDA 4088), a stem-nodulating, photosynthetic isolate from the tropical legume Aeschynomene (Wong et al., 1994). These strains are grouped in one cluster together with R. palustris, which also contains hopanoids and tetrahymanol (Kleemann et al., 1990), on several recently suggested phylogenetic trees for the α-subdivision of proteobacteria (e.g. Young et al., 1991; Martinez-Romero & Caballero-Mellado, 1996). Our findings about the distribution of the shc gene confirm the phylogenetic relatedness of these strains.

**Gene and protein structure**

Sequencing of almost the entire 5 kb EcoRI fragment led to the identification of an ORF that was designated the *shc* gene. The *shc* gene is 1983 bp long and encodes a protein of 660 amino acids with a calculated molecular mass of 73671 Da (Fig. 4). These values agree with those of known SHCs, which have a molecular mass ranging between 71 and 74 kDa and a length of between 631 and 658 amino acids.

A putative ribosome-binding site (GGAGTAA) is located at position −16 to −10 upstream of the ATG start codon of *shc*. Downstream of the *shc* gene a region capable of forming a hairpin (ΔG° = −20 kcal mol⁻¹ (−84 kJ mol⁻¹)) was found representing a putative termination sequence. The *shc* gene has a GC ratio of 64–8%, which is in the range of the GC ratio of *B. japonicum* (61–65%; Jordan, 1982). The sequence of the 150 bp PCR fragment amplified from genomic DNA was found to be identical with the sequence obtained from the isolated cosmid clones with the exception of base changes in the P1/P2 primer regions.

An alignment of the deduced amino acid sequence of the *Bradyrhizobium* SHC with other SHCs and OSCs revealed significant overall homology. The highest amount of homology (70% similarity; 59% identity) was found with the SHC from Z. mobilis, which belongs like *B. japonicum* to the α-subgroup of proteobacteria. The homology to the SHC from the Gram-positive *A. acidocaldarius* and *A. acidoterrestris* is significantly lower (48% similarity; 38% identity). Among the SHCs, 165 amino acid residues are strictly conserved. The eukaryotic OSCs show about 38–43% similarity and 25–27% identity to the *B. japonicum* SHC. Overall homology of SHC to other terpenoid cyclases, e.g. mono-, sesqui- or diterpene cyclases, is negligible, indicating that triterpenoid cyclases form a separate group of enzymes.

Interestingly, database searches revealed an amino acid sequence from an ORF from the cytochrome P-450 gene cluster of *B. japonicum* USDA 110 (Tully & Keister, 1993; EMBL accession no. BJ12678) that has some
Fig. 3. (a) Orientation of three EcoRI fragments of approximately 5, 6 and 12 kb derived from a cosmid (pJB81) isolated from a B. japonicum USDA 110 cosmid library by PCR screening. (b) Restriction map of the 5 kb EcoRI fragment (cloned as pMPE2) harbouring the shc gene. The black box indicates the location of the 150 bp fragment. E, EcoRI; S, SacI; B, BamHI; K, KpnI.

Fig. 4. Nucleotide sequence and deduced amino acid sequence of a partial ORF and of the shc gene from B. japonicum USDA 110. A putative ribosome-binding site upstream of the start codon of the shc gene and a termination signal downstream of shc in the nucleotide sequence are underlined. In the amino acid sequence, the QW motifs are in bold type. The base numbers are shown on the right side of the nucleotide sequence.
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penoid cyclases is unusually high (ranging from 10.4% in Saccharomyces cerevisiae compared to the normal amount of aromatic residues in all known triterpenoids.)

Assay stopped immediately after addition of cell homogenate; forward about some of the molecular properties of these enzymes and their relatedness. Basically, it is expected that these enzymes derive from a common ancestor and that they show common molecular features necessary for the cyclization reaction (Poralla, 1994). B. japonicum SHC contains an aspartate-rich amino acid motif (Fig. 4) with the consensus sequence DV/LDDTA. The QW motif occurs seven to eight times in SHCs (Fig. 4) and among SHCs. Similar aspartate-rich motifs are found in other enzymes of isoprenoid biosynthesis. In these enzymes, the aspartate-rich motifs may be involved in binding and/or stabilization of the diphosphate moiety of the allylic substrates via divalent cations (Mg2+ or Mn2+; Ashby et al., 1990). However, SHC activity does not depend on the presence of divalent cations, and squalene does not possess a diphosphate group.

One possible function of these conserved negatively charged amino acids and also of the conserved aromatic amino acid residues could be stabilization of intermediate carbocations that occur during the cyclization process (Abe et al., 1993). Recently, site-directed mutagenesis of the second and third aspartate residue of the DV/LDDTA motif of the SHC from A. acidocaldarius led to complete inactivation of the SHC, indicating an important role for these residues in enzymatic activity (Feil et al., 1996). In total, 11 aspartate and 5 glutamate residues are strictly conserved throughout the known SHCs.

In vitro cyclization of squalene

The cloned shc from B. japonicum was expressed in E. coli. E. coli produces neither squalene nor hopanoids and is therefore a suitable system for assaying SHC activity. Homogenates of E. coli cells harbouring plasmid pMPE2 were assayed for their ability to catalyse the cyclization of squalene in vitro. shc in plasmid pMPE2 is located in correct orientation of the lacZ promoter. Fig. 5 reveals that after an incubation time of 2 h, squalene was converted partially (to about 20%) to two new compounds with retention times identical to authentic hopene and diplopterol. Both new compounds were further identified by GLC-MS analysis as hopene (m/z 410 with a base peak at m/z 191) and diplopterol (m/z 428 and a base peak at m/z 191). The amount of hopene formed was approximately five times that of diplopterol. However, the formation of tetrahymanol from squalene in vitro by the shc gene product could not be detected. Even a change in assay conditions (e.g. longer incubation times up to 16 h, a change to lower or higher incubation temperatures or a change of the detergent used in the assay) did not lead to the formation of tetrahymanol. This finding agrees with results obtained from R. palustris (Kleemann et al., 1994), where the purified SHC converted squalene to hopene and diplopterol but was unable to produce tetrahymanol in vitro. It remains unclear if there is a second squalene cyclase in B. japonicum and R. palustris responsible for tetrahymanol biosynthesis. Partial sequencing of the downstream region of the shc gene on the 5 kb EcoRI

![Fig. 5. In vitro activity of the shc gene of B. japonicum USDA 110 cloned and expressed in E. coli. E. coli cells harbouring the cloned shc gene (DH5×[pMPE2]) were grown overnight at 28 °C, harvested, and homogenized for release of the cyclase (see Methods for details). Cyclase activity was tested by adding squalene (SQ) and identifying the reaction products of the cyclase (hopene (H) and diplopterol (D)) by GLC analysis. (a) Assay stopped immediately after addition of cell homogenate; (b) assay stopped after 2 h incubation time at 28 °C. The arrow indicates the position where tetrahymanol should appear as determined with authentic tetrahymanol.](image-url)
fragment (Fig. 3) did not lead to identification of sequence homologies to genes encoding triterpenoid cyclases, indicating that a second squalene cyclase gene is not located immediately downstream of shc. On the basis of the reaction mechanism and biochemical data from the purified cyclase from the ciliate Tetrahymena thermophila (Saar et al., 1991; Abe et al., 1993), one can assume that a squalene-tetrahymanol cyclase should resemble the SHCs in its primary structure. Another possible explanation for the lack of tetrahymanol biosynthesis could be the need of the cyclase for a special membrane environment in the form of certain lipids or proteins to enable the squalene cyclase from B. japonicum and R. palustris to convert squalene to tetrahymanol. In that case, it is possible that tetrahymanol could only be synthesized under physiological conditions.

Analysis of the upstream region of shc

The sequence analysis of 0.5 kb upstream of the shc gene from B. japonicum on plasmid pMPE2 revealed significant homology between this DNA region of B. japonicum and the upstream region of the shc gene from Z. mobilis. A part of a potential ORF (ORF1'; 163 amino acids; Fig. 4) lacking the 5'-end was discovered that has 64% similarity and 51% identity to the C-terminal 140 amino acids (of 415) of an ORF located immediately upstream of shc from Z. mobilis (Reipen et al., 1995). Both ORFs have similarity to a stretch of amino acids found in phytoene desaturases, enzymes of the carotenoid biosynthesis pathway, of cyanobacteria and plants (Sandmann, 1994). Bradyrhizobium and Zymomonas are usually non-pigmented, indicating that this ORF in these two organisms might not be involved in biosynthesis of carotenoids. In the case of B. japonicum, this ORF overlaps with shc for 13 bp. The close proximity of ORF1' to shc may indicate that these two ORFs are members of an operon. Future genetic analysis of this identified gene region, harbouring the shc gene and the neighbouring ORFs, will allow the function of these gene regions in the biosynthesis of hopanoids in Bradyrhizobium and the role of hopanoids in general in this and other bacteria to be addressed.

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