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 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.
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Perversely, this cyclization reaction is not well understood and the cyclization products can differ depending on the hopene and diplopterol (Fig. 1). These reaction products are derived from water leads to formation of diplopterol. In some cases such as B. japonicum, R. palustris or the ciliate Tetrahymana, addition of a hydroxyl group to the formation of the gammacerane derivative tetrahymanol. Not known if this reaction is catalysed by SHC.

Fig. 1. Enzymic cyclization of squalene. I, Squalene; II, hopene; III, diplopterol; IV, tetrahymanol. Hopene and diplopterol are 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 other cases such as B. japonicum, R. palustris or the ciliate Tetrahymana, addition of a hydroxyl group to the formation of the gammacerane derivative tetrahymanol. Not known if this reaction is catalysed by SHC.

Hopping and tetrahymanol are reinforcing agents of cellular membranes (Kannenberg et al., 1980; Ourisson et al., 1987), condensing membranes in a similar way to cholesterol in eukaryotic membranes. However, hoppoanoids 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 Tetrahymana 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. X89854; 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: 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 elucidated. 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 MAa3 (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
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 0.2 x 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 x 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 cosmid 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 cosmid vector pVK100, packaged in vitro in phage λ, and propagated in E. coli S17-1 (Parniske et al., 1993).

**PCR screening of the cosmid library.** Clones of the cosmid library from B. japonicum USDA 110 harbouring the shc gene were identified with the help of a PCR-based screening method modifying 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), 5 µl 10x polymerase buffer (Biolabs), 1 mM each dNTP, 0.5 mM 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.

**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 from the shc gene from genomic DNA of B. japonicum USDA 110. Homologous primers B1 (5'-CATCTATGGAACTCTTGTT-3') and B2 (5'-TCAGGGG-CCGCGCTC-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 cosmid library for clones harbouring the shc gene. The cosmid 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 cosmid DNA of these clones was collectively isolated. An aliquot of the isolated cosmid 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 cosmid 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 ascorbic acid, pH 8). The frozen cells (~20 g) 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. Aqueous 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 µM 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 x 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.
The OSCs of \textit{Candida albicans} and \textit{Bradyrhizobium} were isolated and characterized. Partial amino acid sequences of the \textit{C. albicans} cycloartenol squalene cyclase were aligned with the amino acid sequence of the \textit{Bradyrhizobium} cycloartenol squalene cyclase. The position of the first and last amino acid residues of the fragment sequences in the proteins are indicated as numbers. Gaps in the alignment are indicated by a dash. Conserved amino acid residues and residues that have been conservatively exchanged are in bold type. The 150 bp PCR fragment was obtained with degenerate PCR primers P1/P2 (their location is indicated by arrow). The homologous primers B1/B2 were used to PCR-screen in a cosmid library of \textit{B. japonicum}. An alignment of the deduced amino acid sequence of the \textit{Bradyrhizobium} cycloartenol squalene cyclase gene 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 \textit{Bradyrhizobium} strains (data not shown), e.g. \textit{Bradyrhizobium} sp. (\textit{Lupinus}) and also MKAA3 (USDA 4088), a stem-nodulating, photosynthetic isolate from the tropical legume \textit{Aeschynomene} (Wong et al., 1994). These strains are grouped in one cluster together with \textit{R. palustris}, which also contains hopanoids and tetrahymanol (Kleemann et al., 1990), on several recently suggested phylogenetic trees for the \textit{x}-subdivision of proteobacteria (e.g. Young et al., 1991; Martinez-Romero & Caballero-Mellado, 1996). Our findings about the distribution of the \textit{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 \textit{shc} gene. The \textit{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 \textit{shc}. Downstream of the \textit{shc} gene a region capable of forming a hairpin \[ \Delta G^0 = -20 \text{ kcal mol}^{-1} \] was found representing a putative termination sequence. The \textit{shc} gene has a GC ratio of 64.8\%, which is in the range of the GC ratio of \textit{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 \textit{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 \textit{Z. mobilis}, which belongs like \textit{B. japonicum} to the \textit{x}-subgroup of proteobacteria. The homology to the SHC from the Gram-positive \textit{A. acidocaldarius} and \textit{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 \textit{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 \textit{B. japonicum} (Tully & Keister, 1993; EMBL accession no. BJ12678) that has some similarities with the \textit{shc} gene.
**Fig. 3.** (a) Orientation of three EcoRI fragments of approximately 5, 6 and 12 kb derived from a cosmid (pB81) 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 ORF. The parts of the EcoRI and BamHI fragments that were sequenced are hatched. 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 and underlined. The DV/LDDTA motif is surrounded by a box. Amino acid residues that are identical in all known SHCs are marked with an asterisk. The deduced amino acid residues of the partial ORF (start of sequence indicated by an arrow) of *B. japonicum* that are identical with amino acid residues from an ORF located upstream of the *shc* gene are in bold type. The base numbers are shown on the right side of the nucleotide sequence.
The chemistry and enzymology of the complex ring-forming reaction fostered by cyclases has attracted a great deal of attention, and ideas have been put 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 shares two striking amino acid motifs with other SHCs. First, a characteristic feature of SHCs and also of OSCs is a repetitive non-tandem motif (QW motif) with the consensus sequence (K/R)(G/A)X_{2-4}(F/Y/W)LX_{4-6}GXW. The QW motif occurs seven to eight times in SHCs (Fig. 4) and seven times in OSCs in alignable positions (Poralla et al., 1994). This motif contains highly conserved residues of aromatic amino acids. It is noteworthy that the total amount of aromatic amino acids in all known triterpenoid cyclases is unusually high (ranging from 10.4% in the case of B. japonicum to 13.5% in the case of Saccharomyces cerevisiae OSC; Corey et al., 1994) compared to the normal amount of aromatic residues found in proteins, which is in the range of 8.4% (Robinson & Robinson, 1991). A high proportion of the aromatic amino acids are strictly conserved throughout the SHCs (30 of 69 aromatic amino acid residues from B. japonicum SHC).

Second, the B. japonicum SHC contains an aspartate-rich amino acid motif (Fig. 4) with the consensus sequence DV/LDDTA which is also highly conserved 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 moieties of the allylic substrates via divalent cations (Mg$^{2+}$ or Mn$^{2+}$; 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 enzymic 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

![Graph](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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