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Hopanoid lipids in *Bradyrhizobium* and other plant-associated bacteria and cloning of the *Bradyrhizobium japonicum* squalene-hopene cyclase gene

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Abstract

Hopanoid lipids have been discovered recently in a number of nitrogen-fixing soil bacteria and in *Bradyrhizobium* bacteria which fix nitrogen in association with legume plants. We report here an investigation of the hopanoid content in an additional number of soil bacteria capable of living in close association with plants. Of the strains investigated, hopanoids were discovered in phototrophic, nitrogen-fixing bacteria and in an extended number of *Bradyrhizobium* strains. Strains in which hopanoids so far have not been found belong to the following genera: *Rhizobium, Sinorhizobium, Phyllobacterium, Agrobacterium,* and *Azoarcus.* To address the function of hopanoids in *Bradyrhizobium,* we cloned the gene coding for a key enzyme of hopanoid biosynthesis, the squalene-hopene cyclase, and expressed the gene in *E. coli.* The recombinant enzyme catalyzed in vitro the cyclization of squalene to hopanoid derivatives.

Abbreviations: SHC – squalene-hopene cyclase, *shc* – squalene-hopene cyclase gene.

Introduction

Hopanoids are a class of pentacyclic triterpenoid lipids (Figure 1) widely distributed in the domain *Bacteria* (Ourisson et al., 1987). Among nitrogen-fixing bacteria of the soil, hopanoid lipids were discovered in Gram-negative *Azotobacter* and *Beijerinckia* (Vilcheze et al., 1994) as well as in Gram-positive *Frankia* bacteria (Berry et al., 1993) which fix nitrogen in symbiotic association with plants. Recently, we discovered the presence of hopanoid lipids in Gram-negative symbiotic bacteria of the genus *Bradyrhizobium* but not in *Rhizobium* bacteria (Kannenberg et al., 1995).

Hopanoids are thought to comprise a class of bacterial membrane stabilizers which condense the lipid phase of the cell membrane (Kannenberg et al., 1980; Poralla et al., 1980). However, hopanoids may have additional functions, as a variety of structurally related molecules have been described that have not yet been explained biologically (for a recent review, see Sahm et al., 1993). For example, in bradyrhizobia besides diplopterol a series of hopanoid derivatives with a modified side chain are currently under investigation (Kannenberg et al., 1995; Figure 1). It has been suggested that a particular class of hopanoid lipids may also play a role in the oxygen protection mechanism employed by *Frankia* to protect the nitrogenase complex from oxygen destruction (Berry et al., 1993). This raises the question of whether the unique ability of bradyrhizobia to fix nitrogen ex planta could be partially ascribed to the occurrence of hopanoids in these bacteria. Preparatory to addressing this question and that of the general function of hopanoids in plant-associated bacteria, we investigated (i) the hopanoid content in additional Gram-negative bacteria capable of living in close association with plants, and (ii) the cloning and partial characterization of a gene encoding a key enzyme of hopanoid biosynthesis, the squalene-hopene cyclase.

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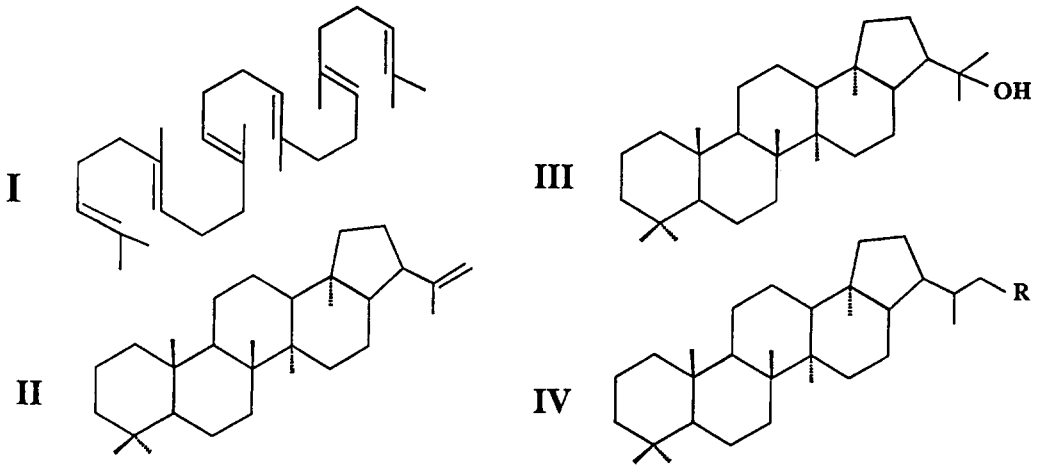

Figure 1. Hopanoid derivatives (and their biosynthetic precursor squalene) identified in *Bradyrhizobium japonicum* USDA 110. I, squalene; II, hopene; III, diplopterol; IV, elongated hopanoids (R = side chain of different polyol variants).

The cloning of this gene will allow us to investigate the function of this class of lipids via genetic studies.

Materials and methods

Bacteria were cultured in complex liquid medium (as recommended for the different strains by the Ghent Culture Collection catalogue) to estimate the hopanoid content. Lipids were extracted from bacterial cells as described by Bligh and Dyer (1959), acetylated, and analyzed by GLC (Rohmer et al., 1984). Hopanoids and squalene were identified as previously described (Kannenberg et al., 1995). The experiments were done at least twice. For cloning the squalene-hopene cyclase gene, a PCR-based screen was used to screen a cosmid clone library of strain *B. japonicum* USDA 110 for clones harboring the cyclase gene (for more details, see the legend to Figure 2). The primers for the PCR screen were based on a conserved amino acid motif of characterized cyclase genes (Poralla, 1994). The cosmid library was constructed as described in Parniske et al. (1993). For cloning and subcloning of PCR fragments, DNA was digested with restriction enzymes, ligated into the vectors pUC18 or pUC19, and transformed into *E. coli* using standard procedures.

Results and discussion

Occurrence of hopanoid lipids in plant-associated bacteria

No clear-cut patterns of hopanoid distribution have so far emerged from the bacterial strains investigated (Sahm et al., 1993). The discovery of hopanoids in nitrogen-fixing soil bacteria such as *Azotobacter, Beijerinckia,* and *Bradyrhizobium* (Kannenberg et al., 1995; Vilcheze et al., 1994) raises the question of their function in these bacteria. For example, they could play a role in protecting nitrogenase from oxygen inactivation. Hopanoids might be involved in allowing the bacteria to adapt to changes in the physiological conditions of the soil or plant micro-environments. Taxonomic relatedness may also determine their distribution. Table 1 lists the distribution of hopanoids for a number of recently investigated Gram-negative plant-associated soil bacteria compiled on the basis of the type of plant interaction, physiological considerations, and taxonomic relatedness. The picture emerging from the data is as follows:

(i) Hopanoids seem to occur in some plant-associated bacteria, including the symbiotic *Bradyrhizobium* and phototrophic, nitrogen-fixing bacteria (here called "*Photorhizobium*"). However, they have not been found in other plant-associated bacteria such as in the symbiotic *Rhizobium,* in the growth-promoting endophytes *Phyllobacterium* and *Azoarcus* (although *Azoarcus* contains squalene, the biosynthetic precursor of hopanoids and other triterpenoids), and

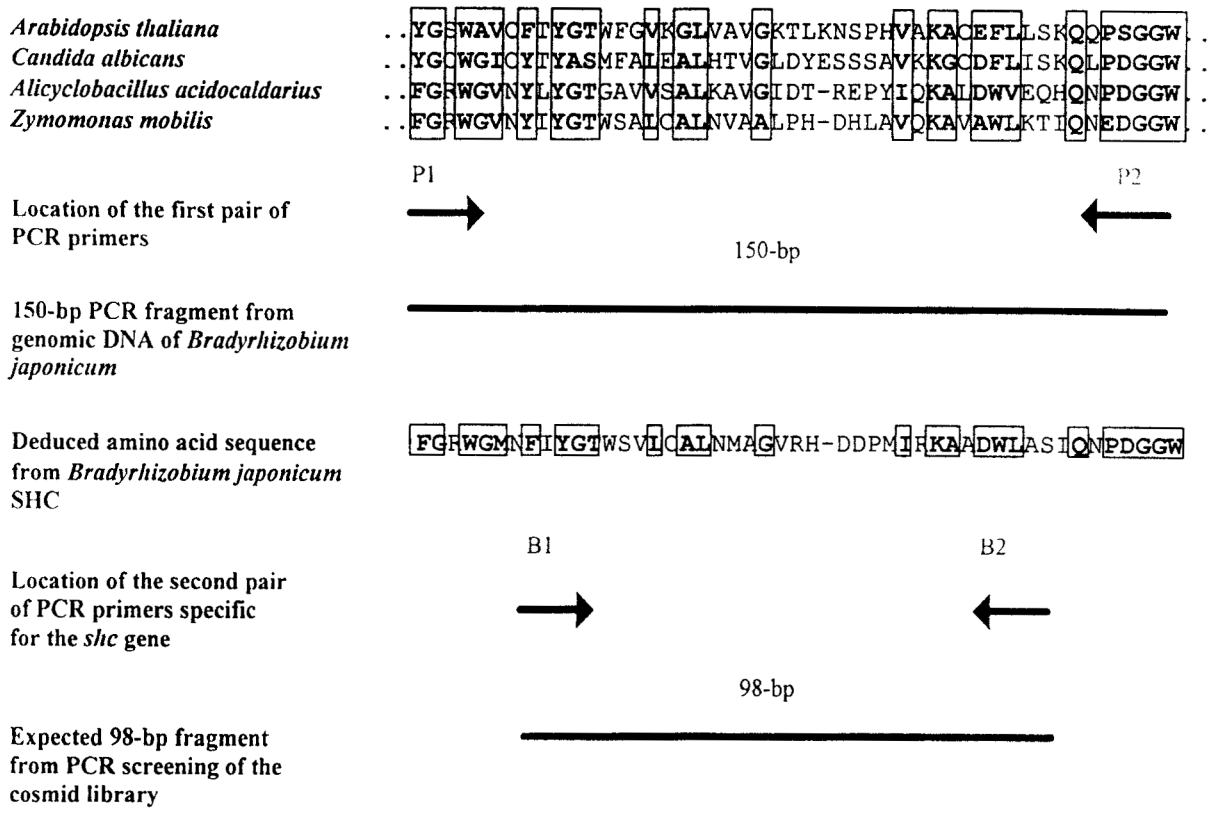

Figure 2. Alignment of a conserved region in the amino acid sequence from several cyclases and the derived PCR primers to screen for homologous DNA in a cosmid gene bank of *Bradyrhizobium japonicum* USDA 110. The two top fragments are from the oxidosqualene cyclases from *Arabidopsis thaliana* and *Candida albicans* and the two at bottom from the squalene-hopene cyclase from *Alicyclobacillus acidocaldarius* and *Zymomonas mobilis* (for a review, see Poralla et al., 1994). Similar parts of the amino acid sequences are highlighted. The screening strategy: P1/P2 were a pair of heterologous, synthetic primers with homology to the coding sequences of the conserved motifs of the various cyclases. With these primers, a 150-bp fragment was PCR-amplified from genomic DNA of *B. japonicum* USDA 110 with a deduced amino acid sequence which showed homology to variant regions of the amino acid sequences of the different cyclases. Under the assumption that this PCR fragment originated from the *Bradyrhizobium* cyclase, we constructed a pair of homologous PCR primers, B1/B2, which we used to screen gene bank clones under stringent conditions for the presence of a 98-bp PCR fragment. The presence of this 98-bp PCR fragment was taken as indicative of the presence of DNA coding for the cyclase gene of *Bradyrhizobium.* In order to avoid a tedious, time-consuming screen of individual gene bank clones, sublibraries (originally containing approximately 100 clones) were constructed from our gene bank and tested in a PCR screen for the presence of the 98-bp fragment. We investigated further only those sublibraries that were positive for the 98-bp PCR fragment by further subdividing them into even smaller clone pools (containing approximately 10 clones). As a final step, a number of individual clones of 98-bp-positive clone pools were analyzed individually by PCR amplification.

in the plant pathogen *Agrobacterium.* Although this sporadic occurrence makes it less likely that they play a generalized role in microbe-plant interactions, there are indications that the expression of triterpenoids is not yet fully understood. In bacteria in which the influence of growth conditions on lipid composition has been investigated, hopanoid content is regulated in response to the physiological conditions encountered by the bacteria (Sahm et al., 1993). An additional complication is that within the same bacteria other structurally related triterpenoid lipids can be present as well as hopanoid lipids. We recently discovered that bradyrhizobia also contain tetrahymanol (a hopanoid-related triterpenoid with a 6-carbon rather than a 5-carbon ring) and additional tetrahymanol derivatives. Overall, no firm conclusions can yet be drawn with regard to a possible role of hopanoids and related lipids in bacteria-plant associations.

(ii) Hopanoids may confer some adaptational advantage to bacteria living under tropical physiological conditions, as they seem to occur mainly in strains of tropical origin (e.g., all strains of *B. japonicum, B. elkanii* and *Bradyrhizobium* sp. tested and in *"Photorhizobium"*, putting aside the as-yet-unresolved sit-

Table 1. Occurrence of triterpenoid lipids in a range of plant-associated bacteria

Strains[a]	Triterpenoid lipids identified[b,c]			
	Squalene	Hopene	Hopane-derivatives	Source[d]
Bradyrhizobium japonicum USDA 110	+	+	+	USDA
Sinorhizobium fredii HH103 (USDA 207)	-	-	-	USDA
Rhizobium sp NGR 234 (USDA 4146)	-	-	-	USDA
Rhizobium leguminosarum B556	-	-	-	N J Brewin
Rhizobium loti NZP 2235	-	-	-	N J Brewin
Azorhizobium caulinodans LMG 6465	-	-	-[e]	LMG
"Photorhizobium" strain BTAi1[f] (USDA 4362)	+	+	+	USDA
Agrobacterium tumefaciens C58 NAL (biovar 1)	-	-	-	M I Boulton
Agrobacterium rhizogenes A4 (biovar 2)	-	-	-	M I Boulton
Phyllobacterium myrcinacearum LMG 2(t2)	-	-	-	LMG
Azoarcus communis LMG 9095	+	-	-	LMG

[a]Taxonomically, the bacteria belong in the α-subdivision of *Proteobacteria* with the exception of *Azoarcus* which is in the β-subdivision (Reinhold-Hurek and Hurek, 1993; Young et al., 1991). The *Bradyrhizobium, Sinorhizobium, Rhizobium, Azorhizobium,* and *"Photorhizobium"* strains form nitrogen-fixing nodules with a range of mainly legume host plants. *Phyllobacterium* forms leaf nodules with some plant species of the genera Rubiaceae and Myrsinaceae; the nodules have a growth-promoting effect on these plants which is poorly understood. *Azoarcus* is a growth-promoting endophyte of Kallar grass whose functional role is not well understood, and *Agrobacterium* is a tumor-forming plant pathogen found in a wide variety of plant species.

[b]For more detailed information on the occurrence of hopanoid lipids in *Rhizobium* and *Bradyrhizobium* strains, see Kannenberg et al. (1995).

[c]Bacteria were cultured and hopanoids extracted and analyzed as described in the Material and methods section. +, compound detected; -, compound not detected.

[d]USDA, United States Department of Agriculture, National *Rhizobium* Culture Collection; LMG, Ghent Culture Collection; Drs. Boulton and Brewin, both at the John Innes Institute, Norwich, UK.

[e]In initial experiments *Azorhizobium* was positive for hopanoids but negative in several subsequent experiments. This inconsistency has not been resolved and is currently under investigation.

[f]We used the phototrophic strain BTAi1 (USDA 4362) and called it *"Photorhizobium"* in these studies (see also Young et al., 1991).

uation in *Azorhizobium*; see Table 1). However, this general assumption is not supported by the finding that hopanoids are absent in the tropical strain *Rhizobium* sp. NGR 234 (Table 1), so it will be interesting to investigate other tropical rhizobia to see if the geographic distribution may play a role in hopanoid occurrence.

(iii) Hopanoid-containing strains of the α-subdivision of *Proteobacteria* seem to fall into clusters of related strains, as defined by the recently suggested phylogenetic tree for this subdivision (Martinez-Romero, 1994), such as the cluster of *Bradyrhizobium, Rhodopseudomonas,* and the phototrophic nitrogen-fixing *"Photorhizobium"* (Kannenberg et al., 1995; Kleemann et al., 1994; Table 1). In a number of other strains including *Rhizobium, Sinorhizobium,* and *Agrobacterium* biovars 1 and 2, hopanoids could not be detected (Kannenberg et al., 1995; Table 1). Since ancestors of the bacteria now grouped into the α-subdivision may have either gained or lost the ability to synthesize hopanoids, hopanoids may either confer an advantage or a disadvantage on bacteria. Much remains to be explained concerning the occurrence of hopanoids.

Cloning and preliminary characterization of DNA coding for the squalene-hopene cyclase gene of Bradyrhizobium japonicum *USDA 110*

The squalene-hopene cyclase (SHC), a key enzyme for biosynthesis, converts squalene to pentacyclic hopene and diplopterol in hopanoid-containing bacteria (for review, see Sahm et al., 1993). Several bacterial squalene-hopene cyclases (and homologous eukaryotic oxidosqualene cyclases) have been characterized (Abe et al., 1993; Reipen et al., 1995) and have been found to display a high degree of homology. Addition-

ally, a conserved and repetitive motif has been identified within the amino acid sequence of all cyclases (Poralla et al., 1994). To see if these features occur in *Bradyrhizobium* and to lay the groundwork for the genetic analysis of hopanoid function in these bacteria, we isolated the gene (*shc*) coding for the squalene-hopene cyclase in these bacteria. We devised a powerful and fast PCR-based screening technique based on the conserved motifs (see the legend to Figure 2) which allowed the identification of two overlapping cosmid clones (BJ64 and BJ81) from a gene bank of *B. japonicum* USDA 110; these clones were further analyzed for the presence of the *shc* gene (see below).

Restriction analysis of the two overlapping clones BJ64 and BJ81 showed that they had three *Eco*RI fragments (5, 6, and 12 kb) in common (Figure 3). As explained in the legend to Figure 2, we identified a 150-bp fragment probe that is highly homologous to known cyclases. This probe hybridized with the 5-kb fragment of both cosmids in Southern hybridization experiments, suggesting that this fragment contains the gene of interest. Therefore, the 5-kb fragment was subcloned and its nucleotide sequence determined. The fragment contained an open reading frame of 1977 bp. The deduced amino acid sequence had 59% identical amino acid residues with the SHC from *Zymomonas mobilis* (Reipen et al., 1995) and 34% with that of *Alicyclobacillus acidocaldarius* (Ochs et al., 1992). It also showed the conserved and repetitive amino acid motif common in other characterized cyclases. The nucleotide sequences of the DNA region homologous to the 150-bp fragment probe in the *shc* gene and that of the 150-bp PCR fragment were identical. After cloning the DNA containing the open reading frame into *E. coli*, cell homogenates of these clones synthesized hopene and diplopterol in vitro from supplemented squalene. Our findings suggest that we have cloned the *shc* gene from *Bradyrhizobium japonicum*.

In summary, we have discovered hopanoids (and other triterpenoids) in symbiotic *Bradyrhizobium* bacteria and in the photosynthetic, nitrogen-fixing *"Photorhizobium"*. We could not detect hopanoids in a range of other plant-associated bacteria. However, no definitive picture about the occurrence of hopanoids has so far emerged. Besides the fact that a number of bacteria still have not been analyzed for hopanoid and triterpenoid content, some bacteria may also have escaped analysis due to growth conditions in which hopanoid biosynthesis was strongly reduced or repressed. As a preliminary step to address hopanoid function, we cloned the gene encoding a key enzyme in hopanoid biosynthesis, the squalene-hopene cyclase, from *B. japonicum* USDA 110. The gene showed homology to known cyclase genes from other bacteria and a conserved, repetitive motif within the amino acid sequence present in other cyclases. These traits suggest that the enzyme structure of the squalene-hopene cyclase is relatively conserved among hopanoid-containing bacteria. The cloned gene will allow us to address on a genetic level the function(s) of hopanoids in *Bradyrhizobium*.

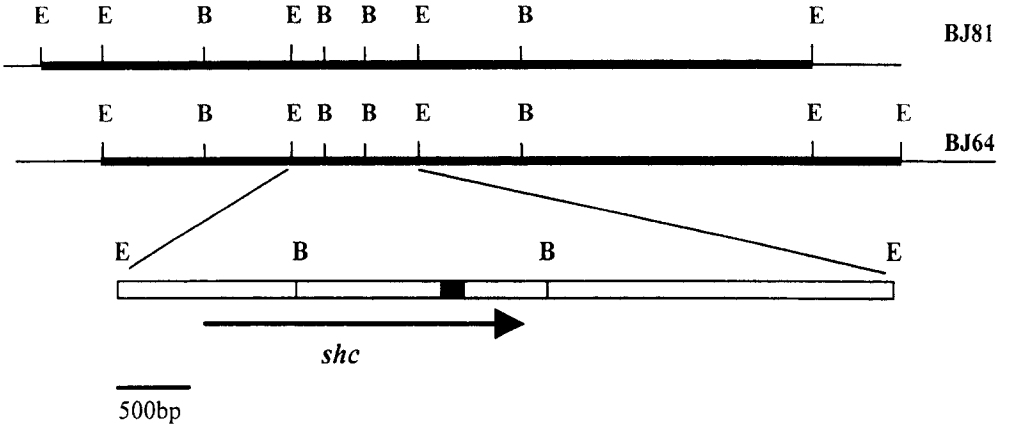

Figure 3. Restriction map of the cloned DNA in cosmids BJ64 and BJ81 containing an open reading frame (ORF) with high homology to known *shc* genes. The coding region of the *shc* gene of *B. japonicum* USDA 110 is part of a 5-kb *Eco*RI fragment of plasmid BJ64 and BJ81, respectively. The location and orientation of the ORF is indicated by the arrow. A series of restriction sites is indicated (E = *Eco*RI; B = *Bam*HI) as well as the approximate position of the 150-bp PCR fragment in the *shc* gene (black box).

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