High hopanoid/total lipids ratio in *Frankia* mycelia is not related to the nitrogen status

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Vesicles are specific *Frankia* structures which are produced under nitrogen-limiting culture conditions. Hopanoids are the most abundant lipids in these vesicles and are believed to protect the nitrogenase against oxygen. The amounts and quality of each hopanoid were estimated in different *Frankia* strains cultivated under nitrogen-depleted and nitrogen-replete conditions in order to detect a possible variation. Studied *Frankia* strains nodulating *Eleagnus* were phylogenetically characterized by analysis of the *nifD-K* intergenic region as closely related to genomic species 4 and 5. Phylogenetically different strains belonging to three infectivity groups were cultivated in the same medium with and without nitrogen source for 10 d before hopanoid content analysis by HPLC. Four hopanoids together accounted for 23–87 % and 15–87 % of the total lipids under nitrogen-replete and nitrogen-depleted culture conditions, respectively. Two of the hopanoids found, bacteriohopanetetrols and their phenylacetic acid esters, have previously been described in *Frankia*. Two new hopanoids, moretan-29-ol and a bacteriohopanetetrol propionate, have also been identified. The moretan-29-ol and bacteriohopanetetrols were found to be the most abundant hopanoids whereas the bacteriohopanetetrol propionate and phenylacetates were present at a concentration close to the limit of detection. The ratio of (bacteriohopanetetrols + moretan-29-ol)/(total lipids) varied in most of the strains between nitrogen-depleted and nitrogen-replete culture conditions. In most of the strains, the hopanoid content was found to be slightly higher under nitrogen-replete conditions than under nitrogen-depleted conditions. These results suggest that remobilization, rather than neosynthesis of hopanoids, is implicated in vesicle formation in *Frankia* under nitrogen-depleted conditions.

Keywords: *Frankia* strains, lipids, hopanoids, nitrogen

INTRODUCTION

Pentacyclic triterpenoids of the hopane series are widespread among bacteria (Rohmer *et al.*, 1984). *Zymomonas mobilis* (Hermans *et al.*, 1991), *Alicyclobacillus acidocaldarius* (Poralla *et al.*, 1984) and *Frankia* (Berry *et al.*, 1991) are known to contain a large percentage of hopanoids among their total lipids. According to their planar amphiphilic structure, hopanoids were proposed to act as sterol surrogates, i.e. as membrane stabilizers in prokaryotes (Rohmer *et al.*, 1979). In *Z. mobilis*, they

were supposed to be implicated in the high degree of ethanol tolerance of this bacterium (Hermans *et al.*, 1991; Schulenberg-Schell *et al.*, 1989). In *Al. acidocaldarius*, an acidothermophilic bacterium, hopanoids were proposed to stabilize the membrane under high temperature and low pH conditions (Poralla *et al.*, 1984). Under such extreme conditions, the adaptive response of the bacteria is to increase the amount of total hopanoid content.

Frankia is a nitrogen-fixing actinomycete which can form root nodules with woody plant hosts in eight angiosperm families. It is also able to fix nitrogen as a free-living organism over a wide range of oxygen concentrations (Silvester *et al.*, 1990). Biological ni-

The GenBank/EMBL/DBJ accession numbers for the sequences in this paper are AJ251388–91 and AJ251393–4.

trogen fixation constitutes a paradox for aerobic organisms as it relies on the oxygen-labile nitrogenase enzyme while, at the same time, the process is an energy-demanding function which implies aerobic respiration. Aerobic nitrogen-fixing micro-organisms have developed strategies to regulate their intracellular oxygen concentration (for a review, see Gallon, 1992). Because *Frankia* can fix nitrogen under aerobic conditions, adaptive mechanisms for oxygen protection must exist.

In *Frankia*, the nitrogenase is localized in specialized structures called vesicles (Meesters *et al.*, 1987). The vesicles differentiate mainly under nitrogen-limiting conditions. Each vesicle is surrounded by an external multilamellate lipid envelope which presumably functions as an oxygen diffusion barrier (Lamont *et al.*, 1988; Harriott *et al.*, 1991). Structural studies have shown that in cultured *Frankia*, the thickness of vesicle envelopes and the number of lipid layers increased in response to an increased oxygen concentration (Parsons *et al.*, 1987). Four hopanoids, two bacteriohopanetetrol isomers, (Ia) and (Ib), and two bacteriohopanetetrol phenylacetyl monoesters, (IIa) and (IIb), represented 84% of the dry weight of purified vesicle envelope preparations (Berry *et al.*, 1993), and 80% of the total lipids of vesicle clusters (Kleemann *et al.*, 1994). Thus the bacteriohopanetetrols (Ia) and (Ib) together with their phenylacetic acid esters (IIa) and (IIb) were proposed to form the major physical barrier to protect nitrogenase against oxygen (Berry *et al.*, 1993). It is not yet known whether these lipids might play an additional role in other stages of the *Frankia* life cycle. In *Frankia* strains HFPArl3 (Berry & Torrey, 1979) and HFPCc13 (Zhang & Torrey, 1985), the two bacteriohopanetetrols represented a large fraction (20–50%) of total lipids under nitrogen-replete conditions (Berry *et al.*, 1991), but a comparison of hopanoids in different genomic species of *Frankia*, in cells grown under nitrogen-replete versus nitrogen-depleted conditions, has not yet been carried out.

In this report, we have quantified the ratio of hopanoids/total lipids, including the identification of new hopanoid structures in *Frankia* cells. This was realized in several *Elaeagnus*-compatible *Frankia* strains characterized by the *nifD*–*K* region, and in two reference strains from the two other main infectivity groups, cultivated with and without nitrogen.

METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study belong to the three main infectivity groups and are members of at least three different genomic species as defined by Fernandez *et al.* (1989). *Frankia* sp. HFPArl3 (Berry & Torrey, 1979) and HFPCc13 (Zhang & Torrey, 1985) strains isolated from *Alnus rubra* Bong and *Casuarina cunninghamiana*, respectively, were used as reference strains. *Elaeagnus*-infective strains were isolated directly from nodules after plant trapping from a French soil (Nalin *et al.*, 1997) on a BAP liquid medium without nitrogen (Murry *et al.*, 1984). To ensure optimal vesicle production, each *Frankia* strain was cultivated three times. The first two subcultures were done in

200 ml BAP medium either with or without nitrogen. In the latter case, the only nitrogen source, NH_4Cl , was suppressed. The second subculture was homogenized by syringe-disrupting the *Frankia* cells through a 0.7×30 mm sterile needle. From each culture type, 1 ml homogenized *Frankia* cells was used to inoculate 10 ml of the corresponding BAP medium, with propionate as carbon source for all strains except HFPCc13, for which pyruvate was used. The last culture was maintained for 10 d. Vesicles are formed in response to the limited availability of nitrogen after 1 week static culture (Fontaine *et al.*, 1984; Murry *et al.*, 1984). The presence of vesicles was verified by light microscopy.

Sequencing of PCR-amplified fragments. *Elaeagnus*-infective strains were characterized based on the PCR-RFLP *Hae*III profile of the *nifD*–*K* region (Nalin *et al.*, 1997). One representative strain of each *nifD*–*Hae*III profile was used for the *nifD*–*K* intergenic region identification. PCR amplifications were performed in a total volume of 50 μl , in 0.2 ml Eppendorf tubes, using a thermocycler (9600; Perkin-Elmer).

Amplification of the *nifD*–*K* region directly from cells in liquid cultures was performed with primers FGP-D807 (5'-CACTGCTACCGGTCGATGAA-3') and FGP-K700' (5'-CGAGGTAGGTCTCGAAACCGG-3') as described by Jamann *et al.* (1993). Five microlitres of liquid culture was added to the reaction buffer [10 mM Tris/HCl (pH 8.3), 1.5 mM MgCl_2 , 50 mM KCl, 0.01% (w/v) gelatin], 200 μM each dNTP, 1 μM each primer and 2.5 units *Taq* polymerase (Gibco-BRL). The following program was used: initial denaturation for 2 min at 94 °C, and 35 cycles of denaturation (45 s at 95 °C), annealing (45 s at 55 °C) and extension (45 s at 72 °C), the final extension being longer (2 min at 72 °C). Before sequencing, the amplification reaction mix was purified with Centricon-30 concentrators (Amicon-Grace). The amplicons were sequenced using the Amplicycle Sequencing Kit (Perkin-Elmer) by the direct sequencing method of Winship (1989). The two amplification primers were used as well as FGP-D169 [5'-ATGGACATC-GC(CG)ATCAA-3'], FGP-K333' (5'-CCGGGCGAAGTGG-CT-3') and FGP-K1 (5'-GTGACGACGACTCCC-3'), FGP-K64' (5'-CCTCGTCCTTGAACA-3') described by Navarro *et al.* (1997). The sequences were determined on both strands (GenBank accession nos AJ251388–91 and AJ251393–4).

DNA sequence analysis. The phylogenetic analyses were based on the sequences of an approximately 420 bp DNA fragment of the *nifD*–*K* intergenic region according to Nalin *et al.* (1999). Sequences were aligned and compared with the alignment of 14 closely related *Frankia* strains described by Navarro *et al.* (1997), using the multiple-alignment CLUSTAL X algorithm (Thompson *et al.*, 1997), with manual refinements in the non-coding regions. Distances were calculated by pairwise comparison according to Kimura's two-parameter model (Kimura, 1980), and phylogenetic analyses were made using the neighbour-joining distance method of Saitou & Nei (1987). The topology of the tree was tested by performing 1000 bootstraps (Felsenstein, 1985) and parsimony analysis (Swofford, 1993).

Lipid extraction and analysis. Total cultures were sonicated for 3 min at 60% of the maximal power and 50% active cycles (Sonifier 250; Branson UltraSonics) for each strain and each treatment and for three replicates. Lipids from the sonicated samples were extracted according to Bligh & Dyer (1959) ($\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$, 2:2:1.8, by vol., 38 ml). Total lipids extracted from each sample were concentrated under nitrogen to a volume of 50 μl immediately prior to the HPLC injection.

Separation and detection of total lipids by HPLC was performed according to Moreau *et al.* (1990) on a silica gel Si-

60 Lichrosorb column of 10 cm with an internal diameter of 5×10^{-4} cm (Chrompack) at a flow rate of 0.5 ml min^{-1} . Quantifications were done with an evaporative light-scattering detector (ELSD IIA model; Varex) by integration of peak areas in the chromatogram (Kleemann *et al.*, 1994).

HPLC peaks containing hopanoids were collected. Compounds were acetylated and identified by direct inlet impact MS according to the characteristic fragmentation ions of the triterpenoid pentacyclic skeleton, as described earlier (Berry *et al.*, 1991). A large-scale culture of *Frankia* afforded enough material for the spectroscopic identification of the hopanoids. The structures of the novel hopanoids moretanol (IV) and bacteriohopanetetrol propionate (IV) and the stereochemistry of bacteriohopanetetrol (Ia) and (Ib) side chains were determined after isolation of the compounds by spectroscopic methods (^1H - and ^{13}C -NMR, MS), by derivatization and finally by comparison of the data with those obtained from synthetic reference compounds (Rosa Putra, 1998). The two bacteriohopanetetrols (Ia) and (Ib) only differed by the configuration at C-34 as shown by the comparison of the ^1H -NMR spectra of their tetra-acetates with those of reference compounds (Bisseret & Rohmer, 1989; Rosa Putra, 1998).

RESULTS

For each new isolate of *Elaeagnus*-infective *Frankia* strains, sequencing of the *nifD*-*K* intergenic spacer was carried out in order to compare them phylogenetically with the reference strains (Table 1). The length of the PCR products was of the expected size of approximately 1600 nucleotides. The length and the sequence composition of the *nifD*-*K* region are highly conserved among the isolated *Frankia* strains. For all sequences,

the *nifD* stop codon is TGA and the *nifK* initiation codon is GTG. A GGAGG sequence seven nucleotides upstream of the *nifK* initiation codon provides a ribosome-binding site (RBS) (Shine & Dalgarno, 1974; results not shown).

Based on the sequences of a 420 bp fragment from the *nifD*-*K* intergenic region, phylogenetic relationships were investigated (Fig. 1). *Elaeagnus*-infective strains were subdivided into five groups corresponding to the four genomic species and an undescribed group. This separation was validated by parsimony analysis and bootstrap resamplings (with a threshold fitted at 99% of bootstraps). They are clustered together and distinct from *Alnus*- and *Casuarina*-infective strains. Most of the *Elaeagnus*-infective strains isolated were clustered with reference strain Ea1-12 corresponding to genomic species 4, which comprised Ea12, Ea13, Ea14 and Ea17. The Ea1 strain was clustered with EaN₁-pec (genomic species 5), whereas strain Ea16 did not cluster with any of the other *Elaeagnus*-infective genomic species but was still in the coherent group of the *Elaeagnus*-infective strains.

The strains were grown under nitrogen-replete and -depleted conditions and the hopanoids were quantified by HPLC analysis. The two main groups (Fig. 2) in all strains were the C₃₅ bacteriohopanetetrols (Ia) and (Ib) and the C₃₀ moretan-29-ol (IV), whereas the bacteriohopanetetrol phenylacetates (IIa) and (IIb) and propionate (III) were close to the threshold of detection and could not be quantified. Under both culture conditions and for all strains from the three infectivity groups,

Table 1. *Frankia* strains isolated and used in this study

Strain*	GenBank accession no.	Host plant	Reference
Ea11 (U) S	AJ251388	<i>Elaeagnus angustifolia</i>	Nalin <i>et al.</i> (1999)
Ea12 (U) S	AJ251389	<i>Elaeagnus angustifolia</i>	Nalin <i>et al.</i> (1999)
Ea13 (U) S	AJ251390	<i>Elaeagnus angustifolia</i>	Nalin <i>et al.</i> (1999)
Ea14 (U) S	AJ251391	<i>Elaeagnus angustifolia</i>	Nalin <i>et al.</i> (1999)
Ea16 (U) S	AJ251393	<i>Elaeagnus angustifolia</i>	Nalin <i>et al.</i> (1999)
Ea17 (U) S	AJ251394	<i>Elaeagnus angustifolia</i>	Nalin <i>et al.</i> (1999)
Ea1-12 (4)	U63697	<i>Elaeagnus angustifolia</i>	Navarro <i>et al.</i> (1997)
EaN ₁ -pec (5)	U63698	<i>Elaeagnus angustifolia</i>	Navarro <i>et al.</i> (1997)
EUN1f (6)	L37664	<i>Elaeagnus umbellata</i>	Navarro <i>et al.</i> (1997)
HRN18a (7)	U63696	<i>Hypophaë rhamnoides</i>	Navarro <i>et al.</i> (1997)
SCN10a (U)	U63695	<i>Shepherdia canadensis</i>	Navarro <i>et al.</i> (1997)
KP54 (U)	U63694	<i>Gymnostoma poisonianum</i>	Navarro <i>et al.</i> (1997)
CN61 (U)	U63693	<i>Gymnostoma nodiflorum</i>	Navarro <i>et al.</i> (1997)
TC24 (U)	U63692	<i>Gymnostoma chamaecyparis</i>	Navarro <i>et al.</i> (1997)
MG59 (U)	U63691	<i>Gymnostoma glaucescens</i>	Navarro <i>et al.</i> (1997)
RPL61 (U)	U63334	<i>Gymnostoma leucodon</i>	Navarro <i>et al.</i> (1997)
D11 (atypical) (U)	U63700	<i>Casuarina equisetifolia</i>	Navarro <i>et al.</i> (1997)
Ccl3 (9) S	U63699	<i>Casuarina cunninghamiana</i>	Hirsh <i>et al.</i> (1995)
Ar13 (1) S	L35557	<i>Alnus rubra</i>	Nalin <i>et al.</i> (1995)
FaC1	U53363	<i>Alnus viridis</i>	Oh <i>et al.</i> (1997)

* Genomic species are in parentheses. U, Undetermined species; S, strains used in hopanoid analysis.

Fig. 1. Phylogenetic neighbour-joining tree based on *nifD-K* intergenic spacer sequences. Bar, 0.023 substitutions per site. The isolates are in bold, the genomic species described by Fernandez *et al.* (1989) are in parentheses and the *Gymnostoma*-infective strains are in italic. P indicates that the pairwise analysis is confirmed by parsimony analysis. The numbers are the percentages of bootstrap replicates in which the clusters were found.

Fig. 2. Hopanoid structures of *Frankia* strains. Ia and Ib, bacteriohopanetetrols; IIa and IIb, bacteriohopanetetraphenylacetates; III, propionate; IV, moretan-29-ol.

hopanoids represented a large proportion of the total lipids (Table 2). A large variability was found in the hopanoid proportion among *Frankia* strains, ranging from 23 to 87% and from 15 to 88% of the total detected lipids, under nitrogen-replete and nitrogen-depleted culture conditions, respectively. The variation in hopanoid content (hopanoids/total lipids ratio) between the two culture conditions represented between 10 and 20% of the total detected lipids. Under both culture conditions, the highest hopanoid content was found in the *Elaeagnus*-infective strains.

The two bacteriohopanetetrols (Ia) and (Ib) were the most abundant hopanoids in all the strains and for both culture conditions, and moretan-29-ol was the next most abundant triterpenoid (Table 2). However, the percentage of both hopanoids varied among strains and culture conditions in most cases, except in the EaI2 strain, where the bacteriohopanetetrol represented more than 80% of the lipids detected under both conditions. The highest percentage of moretan-29-ol was found in the EaI3 strain, representing 25% of the total lipids under nitrogen-replete conditions and decreasing to 3%

Table 2. Proportion of the main hopanoids as a percentage of the total lipids in *Frankia* strains grown under nitrogen-replete (+N) or nitrogen-depleted (–N) culture conditions for 10 d (mean $\pm$ SE, $n = 3-5$)

Strain	Moretan-29-ol (%)		Bacteriohopanetetrols (%)		Total of the two main hopanoids (%)	
	+N	–N	+N	–N	+N	–N
EaI1	7 $\pm$ 2.0	10 $\pm$ 2.0	62 $\pm$ 3.0	72 $\pm$ 2	69 $\pm$ 3.0	82 $\pm$ 3
EaI2	5 $\pm$ 1.0	2 $\pm$ 0.3	82 $\pm$ 2.0	85 $\pm$ 1	87 $\pm$ 2.0	87 $\pm$ 1
EaI3	25 $\pm$ 5.0	3 $\pm$ 0.3	43 $\pm$ 6.0	49 $\pm$ 1	68 $\pm$ 10.0	52 $\pm$ 1
EaI4	4 $\pm$ 1.0	4 $\pm$ 1.0	83 $\pm$ 1.0	65 $\pm$ 2	87 $\pm$ 1.0	68 $\pm$ 3
EaI5	4 $\pm$ 1.0	4 $\pm$ 2.0	56 $\pm$ 1.0	39 $\pm$ 4	60 $\pm$ 2.0	43 $\pm$ 6
EaI6	14 $\pm$ 0.5	8 $\pm$ 2.0	65 $\pm$ 0.3	53 $\pm$ 1	79 $\pm$ 0.1	61 $\pm$ 0.3
ArI3	5 $\pm$ 1.0	3 $\pm$ 0.3	17 $\pm$ 1.0	40 $\pm$ 1	23 $\pm$ 3.0	42 $\pm$ 1
CcI3	12 $\pm$ 1.0	2 $\pm$ 0.1	21 $\pm$ 0.2	13 $\pm$ 1	33 $\pm$ 1.0	15 $\pm$ 1

under nitrogen-depleted conditions. In contrast, this compound represented only 4% of the total detected lipids in the EaI4 and EaI5 strains under both conditions (Table 2).

DISCUSSION

Adaptation of bacteria to fluctuating environmental conditions represents one of the emerging domains of microbiology. In particular, nitrogen-fixing bacteria must deal with a critical situation with respect to membrane permeability, and hopanoids that would be involved in protecting nitrogenase from oxygen diffusion (Berry *et al.*, 1993) have been postulated to play an important role in that adaptation in their saprophytic and symbiotic lives.

The *nifD*–*K* region, which has been widely used for the characterization of new *Frankia* isolates (Navarro *et al.*, 1997), permits the discrimination between two closely related *Alnus*-infective strains (Nalin *et al.*, 1995). The *nifD*–*K* intergenic spacer sequences have been used to characterize newly detected *Gymnostoma*-infective *Frankia* strains, which were found to be clustered with the *Elaeagnus*-infectivity group confirming the plant nodulation bioassay (Navarro *et al.*, 1997). This region allows for discrimination at the infraspecific level and thus is very useful for strain characterization in the *Frankia* genus.

In *Elaeagnus*-infective strains, we confirm and extend earlier observations on *Frankia* hopanoids (Berry *et al.*, 1991, 1993): bacteriohopanetetrols (Ia) and (Ib) and their phenylacetates (IIa) and (IIb) belong to the main triterpenoids found in these bacteria. In addition, two novel hopanoids were identified: a bacteriohopanetetrol propionate (III) as a minor compound and, surprisingly, moretan-29-ol (IV), which was one of the major hopanoids (Rosa Putra, 1998). Detailed structure determinations will be reported elsewhere. This is the first

report of a (17 β -H, 21 α -H) hopanoid from a prokaryote. The presence of such a C₃₀ triterpene is a striking feature, and its significance is not clear. Over 30 hopanoid structures have been described from bacteria (Ourisson & Rohmer, 1992; Rohmer, 1993). They primarily differ by their side chain structures. Hopanoids have already been found in nitrogen-fixing bacteria, such as *Azotobacter vinelandii*, *Beijerinckia indica* and *Beijerinckia mobilis* (Vilchèze *et al.*, 1994), and in the *Bradyrhizobium* species (Kannenberg *et al.*, 1996), but not in all of them. No common structural features were found, however, for the hopanoid side chains in nitrogen-fixing bacteria. The significance of such structural diversity is not obvious. Hopanoids were assumed to act as membrane stabilizers, in a fashion similar to that of sterols in eukaryotic membranes (Ourisson & Rohmer, 1992). Such a structural role is compatible with molecular diversity, provided the triterpenoids fulfil all structural features required for membrane stabilizers (Rohmer *et al.*, 1979; Simonin *et al.*, 1992). Our findings of additional hopanoids in *Frankia* strains, especially the moretan-29-ol, under nitrogen-replete conditions are highly suggestive that hopanoids might be implicated in other processes besides the oxygen protection of nitrogenase.

The total amount of hopanoids has been estimated at approximately 35 mg (g lyophilized cells)^{–1} (data not shown). All *Frankia* strains tested from three infectivity groups show a high hopanoids/lipids ratio, expanding the results obtained by Berry *et al.* (1991) on strains HFPArI3 and HFPCcI3. This ratio was found to be much higher than those of other hopanoid-producing bacteria, even higher than those of *Z. mobilis* (Hermans *et al.*, 1991) and *Al. acidocaldarius* (Poralla *et al.*, 1984), which were previously known to produce the highest amounts of hopanoids. This suggests that the large amount of hopanoids (20–87% of total lipids) is a general feature of the *Frankia* genus and could be used as a phenotypic characteristic of this genus among actino-

mycetes (Marechal *et al.*, 2000). Such high concentrations are probably not compatible with their commonly accepted role as membrane stabilizers, at least in the way described for sterols interacting with the phospholipid side chains.

The moretan-29-ol (IV) and the bacteriohopanetetrols (Ia) and (Ib), the most abundant hopanoids in *Frankia* cells, have been found in different ratios among the *Frankia* strains tested. Quantitative variations under the alternative nitrogen conditions were much less important in our experiments, since such variation represented only up to 20%. A similar result was found in another nitrogen-fixing bacterium, *Az. vinelandii*, where production of hopanoids was not stimulated under nitrogen-fixing conditions (Vilchèze *et al.*, 1994).

The consistently high proportion of hopanoids observed, even under nitrogen-replete conditions, indicates that in all tested *Frankia* strains, most of the hopanoids are synthesized independently of the nitrogen source of the culture. Nevertheless, hopanoids are the main lipid component of the vesicles (Kleemann *et al.*, 1994), which are essentially produced under nitrogen-fixing conditions. This suggests that hopanoids could be remobilized rather than neosynthesized under nitrogen-free culture conditions in order to build up the multi-lamellate envelope of the vesicles.

The structural diversity of hopanoids and their high proportion in all *Frankia* populations over different infectivity groups suggest that these compounds play important roles in *Frankia* cells. Their roles could be expressed in symbiotic interactions, i.e. in root nodules, where hopanoids were first reported (Berry *et al.*, 1991), and also under *in vitro* culture conditions, where they represent up to 87% of the total lipids detected. Hopanoids might have a fundamental function in *Frankia* ecology, and the variations observed in the hopanoid concentration between strains would be the expression of different adaptive capacities. This hypothesis might point to new functions for hopanoids that are currently being tested.

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