Bacterial hopanoids from pink-pigmented facultative methylotrophs (PPFMs) and from green plant surfaces

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Six strains of pink-pigmented facultatively methylotrophic bacteria (PPFMs) isolated from phylloplane surfaces of different plants were analysed for the presence of triterpenoids of the hopane series. All of the cultures produced hopanoids in abundant quantities and contained the same compounds as the type strain of *Methylobacterium organophilum*: diplopterol, 2 β -methylidiplopterol, bacteriohopanetetrol, a tetrol glycoside and two tetrol ethers. The presence of a guanidinium group on the carbapseudopentose moiety of one of these ethers and/or of 2 β -methylidiplopterol seems to be restricted to the genus *Methylobacterium*. Small amounts of bacteriohopanepolyols were detected in three of seven plants studied. Since no bacterial C₃₅ hopanoids have been reported in eukaryotes, we believe they are probably derived from eubacterial epibionts present on the phylloplane surfaces, the most numerous of which are *Methylobacterium* spp.

Keywords: hopanoids, isoprenoids, *Methylobacterium*, PPFMs, phylloplane

INTRODUCTION

Triterpenoids of the hopane series were first revealed by the wide distribution of their molecular fossils (Ourisson *et al.*, 1979) and they are considered important constituents of prokaryotes. They have been found in Gram-negative and Gram-positive bacteria scattered through numerous eubacterial taxonomic groups (Rohmer *et al.*, 1984). The ability to synthesize hopanoids may be interesting for taxonomic reasons, but the number of strains analysed to date is too restricted to draw any definitive conclusions about the importance of hopanoids in bacterial taxonomy. Typical triterpenoids of this class are bacteriohopanepolyols. Their structure derives from the hopane skeleton linked to a C₅ n-alkyl polyhydroxylated side-chain derived from a D-pentose (Flesch & Rohmer, 1988; Rohmer *et al.*, 1989).

The pink-pigmented facultative methylotrophs (PPFMs) are ubiquitous Gram-negative bacteria, widely distributed in nature and regularly isolated from plant surfaces (Corpe, 1985). They have been assigned to species of the genus *Methylobacterium* (Green *et al.*, 1988). PPFMs grow on a variety of carbon sources (Green & Bousfield, 1982; Corpe & Basile, 1982) and possess great metabolic versatility. This has proved useful for the incorporation of

¹³C-labelled precursors in order to elucidate a novel non-mevalonate pathway for isoprenoid biosynthesis in eubacteria (Flesch & Rohmer, 1988; Rohmer *et al.*, 1993). In the course of our hopanoid screening, it appeared interesting to examine PPFMs isolated from plant sources and to compare their hopanoids for qualitative similarity with those from *Methylobacterium organophilum* (Renoux & Rohmer, 1985) and from whole-plant material, thus providing a basis for a specific chemical signature of bacterial epibionts.

METHODS

PPFM strains and cultivation. The PPFMs SAL (isolated from an aquatic fern and identified as *Methylobacterium fujisawaense* by P. Green on the base of phenotype analysis), CONO (from a liverwort), MESO (*Methylobacterium mesophilicum*, ATCC 29983, from rye grass), MIM-2 (from a mimosa), CM (from a liverwort) and PC 1 (from white clover) have been described in previous papers (Corpe & Basile, 1982; Corpe *et al.*, 1986; Corpe & Rheem, 1989). In the present work, PPFMs were usually grown on a modification of the medium of Hestrin & Schramm (1954) in 2 litre Erlenmeyer flasks for 30 h at 30 °C on rotatory tables. The SAL and CONO strains were also grown for biosynthetic studies on a synthetic ammonium/mineral salts medium supplemented with vitamins and using acetate or glucose as sole carbon source (Rohmer *et al.*, 1993).

Plant material. Two samples of the aerial parts of the moss *Abietinella abietina* (Hedm.) Fleisch. were examined: the first one

Abbreviation: PPFM, pink-pigmented facultative methylotroph.

Fig. 1. Hopanoids from PPFMs.

(9 g, dry weight) was collected near Osenbach, the second one (2.5 g) near Village Neuf (both from Haut-Rhin, France). Leaves of the following plants were collected where indicated: *Trifolium repens* L. (white clover, 17 g), *Viola odorata* (violet, 5.6 g), *Fragaria vesca* L. (wild strawberry, 9.5 g) and *Solanum lycopersicum* L. (tomato, 14 g) in gardens in Mulhouse (Haut-Rhin, France), and *Vitis vinifera* L. leaves (vine, 6 g) at the research station of the Institut National de la Recherche Agronomique (Colmar, Haut-Rhin, France). *Amaryllis* sp. leaves (10 g) came from an indoor-grown plant. All plant tissues were harvested at the end of August 1990 and were freeze-dried before extraction; they were healthy and showed no signs of rotting.

Analytical methods and isolation of hopanoids. Hopanoid extraction and derivatization, qualitative GLC, quantitative GLC, GLC/MS, HPLC, ^1H - and ^{13}C -NMR were performed as previously described (Peiseler & Rohmer, 1991; Stampf *et al.*, 1991). As the amounts of hopanoids found during these investigations were expected to be low in the plant material examined, we utilized the most sensitive method, i.e. side-chain $\text{H}_3\text{IO}_6/\text{NaBH}_4$ degradation of the bacteriohopanepolyols and derivatization for GLC analysis (Rohmer *et al.*, 1984); the detection limit of this method for hopanoids lies around 0.1 μg for each analysed fraction (Jürgens *et al.*, 1992).

In order to obtain hopanoids with intact side-chains the crude chloroform/methanol extract was acetylated overnight at room temperature with acetic anhydride/pyridine (1:1, v/v) (Renoux & Rohmer, 1985). After removal of excess reagents under vacuum, the residue was thoroughly washed with toluene in order to selectively dissolve the acetylated hopanoids from poly- β -hydroxybutyric acid, which is not soluble in this solvent. The toluene extract containing the peracetylated triterpenoids was separated by preparative TLC (hand-made Merck 7744 silica gel plates, 1 mm thickness, cyclohexane/ethyl acetate, 2:8, v/v) giving less polar compounds ($R_F > 0.80$), octa-acetate of the bacteriohopanetetrol ether V ($R_F = 0.70$) and a fraction ($R_F = 0.60$) which was further separated by TLC (cyclohexane/ethyl acetate, 4:6, v/v) giving the hepta-acetate of bacteriohopanetetrol ether V with a free tertiary hydroxy group ($R_F = 0.20$) and the hepta-acetate of *O*- β -D-glucosaminyl-

bacteriohopanetetrol VII ($R_F = 0.25$). The less polar compounds ($R_F > 0.80$) were further separated by TLC (methylene chloride) into tetra-acetate of bacteriohopanetetrol IV ($R_F = 0.32$) and a fraction containing diploptene II and 2 β -methyl-diploptene III ($R_F = 0.44$). The two latter compounds could be separated from one another by reverse-phase HPLC on a Dupont Zorbax ODS column (250 $\times$ 3.9 mm, methanol/water, 96:4, v/v, 1 ml min $^{-1}$). Pure octa-acetate of the other bacteriohopanetetrol ether V could be obtained after a similar reverse-phase HPLC (methanol/water, 98:2, v/v, 1.2 ml min $^{-1}$).

All hopanoids were identified by comparison of their ^1H - and ^{13}C -NMR spectra and/or GLC or HPLC retention times with those of reference compounds previously synthesized or isolated in this laboratory from *Methylobacterium organophilum* and *Zymomonas mobilis* (Renoux & Rohmer, 1985; Bissleret *et al.*, 1985; Flesch & Rohmer, 1986).

RESULTS AND DISCUSSION

Hopanoid content of the PPFMs

Quantitative GLC analysis of the fractions arising from the chloroform/methanol extract, treated according to our side-chain cleavage procedure (Rohmer *et al.*, 1984) showed that all the PPFMs contained appreciable amounts of hopanoids: diploptene I, diploptene II, 2 β -methyl-diploptene III (Fig. 1) and bacteriohopanepolyol derivatives as indicated by the presence of the acetate of the C_{32} bis-homohopanol (Table 1). From the acetylated chloroform/methanol extract hopanoids with intact side-chains could be isolated. Whereas the bacteriohopanetetrol derivative content of the SAL, CONO and CM strains (bacteriohopanetetrol IV, tetrol ethers V and VI and tetrol glycoside VII) was qualitatively identical with that reported from *Methylobacterium organophilum* (Renoux & Rohmer, 1985), only tetrol IV could be detected in the MIM-2 and MESO strains (Fig. 1). Furthermore if the

Table 1. Hopanoid content ($\mu\text{g g}^{-1}$, freeze-dried material) in PPFMs and plants as determined by weighing (II to VII) or GLC (I and C₃₂ acetate obtained after H₅IO₅/NaBH₄ treatment of the bacteriohopanepolyols)

Biological material	C ₃₀		C ₃₅				Acetate C ₃₂ alcohol
	Diploptene I	Diplopterol II and 2β-methyl diplopterol III	Tetrol IV	Ether V	Ether VI	Glycoside VII	
PPFMs							
SAL	200	4300 (80 %)*	2300	3600	900	500	5600
CONO	700	3900 (85 %)*	1600	2600	300	200	3600
CM	500	2400 (90 %)*	1500	1800	200	200	2300
MIM	700	3600 (55 %)*	1000	ND	ND	ND	2100
MESO	500	2800 (60 %)*	1000	ND	ND	ND	1900
PC1†	200	600					900
Plants							
<i>Abietinella abietina</i>	6	+‡					6
<i>Trifolium repens</i>	17	ND					0.2
<i>Viola odorata</i>	6	ND					6
<i>Solanum lycopersicum</i>	ND	ND					ND
<i>Vitis vinifera</i>	ND	ND					ND
<i>Fragaria vesca</i>	ND	ND					ND
<i>Amaryllis</i> sp.	ND	ND					ND

ND, Investigated but not detected.

* Relative amount of 2 β -methyl diplopterol in the mixture of diplopterol and 2 β -methyl diplopterol as determined by HPLC.

† Composite hopanoids of the PC1 strain have not been investigated.

‡ Diplopterol and methyl diplopterol were detected by GLC/MS in the Osenbach sample only.

quantitative data obtained by the two methods of determination (GLC determination after H₅IO₅/NaBH₄ side-chain derivatization or weighing of the intact acetylated bacteriohopanepolyol fractions) fit well together for the three former strains, isolation of intact acetylated hopanoids yields an apparent underestimation compared with the side-chain cleavage method (Table 1). This discrepancy between the values was reproducible. Even when larger amounts of cells (15 g instead of 7 g dry weight for the MESO strain; 6 g instead of 2 g for the MIM-2 strain) were utilized for a second determination in order to minimize experimental errors, the same concentrations of diploptene, diplopterol, 2 β -methyl diplopterol and bacteriohopanetetrol were found. This might point to the possible presence of hopanoids which are not extracted and/or not detected by our methods. Such forms, insoluble in organic solvents, are for instance known for carotenoids which form complexes with proteins (Thirkell & Hunter, 1969; Schwencker *et al.*, 1974) and hopanoids (Herrmann & Rohmer, unpublished results). A bacteriohopanetetrol ether similar to ether VI could not be directly extracted from freeze-dried cells of an *Acetobacter* species, even after repeated treatment with chloroform/methanol (2:1) under reflux. Finally in PPFMs, as already observed for other bacteria, bacteriohopanepolyol content is largely dependent on the growth conditions

and the composition of the culture medium (Renoux & Rohmer, 1985; Bissleret *et al.*, 1985; Zundel & Rohmer, 1985; Rohmer & Ourisson, 1986). After ten transfers, during three weeks, of the SAL and CONO strains from a complex to a minimal medium, the bacteriohopanepolyols IV, V, VI and VII could no longer be detected, the slightly higher diplopterol concentration (5.7 instead of 4.3 mg per g dry weight) not compensating the loss of the composite hopanoids. Further transfer from minimal to complex medium restored the initial hopanoid composition where all bacteriohopane derivatives were present, showing that these changes were fully reversible.

Taxonomic implications

The taxonomic position of the PPFMs was for a long time rather uncertain and confused. Generic assignment to the genus *Methylobacterium* was finally made on the basis of DNA:DNA homologies and phenotypic data (Green & Bousfield, 1982, 1983; Bousfield & Green, 1985; Hood *et al.*, 1987). Corpe & Jensen (1991) studied the distribution and localization of PPFMs on plant leaf surfaces. They used a specific antiserum prepared in rabbits against whole cells of the PPFM strain PC1 isolated from clover. Only cells of *Methylobacterium* strains isolated from diverse

sources developed an immunoreaction against this anti-serum. Representative bacteria of other genera did not produce such reactions.

Analysis of the other PPFM hopanoids and comparison with those of the type culture of *Methylobacterium organophilum* were in accord with the classification of PPFMs within the genus *Methylobacterium*. Indeed, *M. organophilum* and the other PPFMs were found to be good hopanoid producers. They represent the only bacteria found to date that contain high concentrations of diplopterol II and/or 2 β -methyldiplopterol III. The amounts found are usually around several mg per g dry weight. All other bacteria investigated show diplopterol concentrations of at least one order of magnitude lower (Rohmer *et al.*, 1984; M. Rohmer, unpublished results). Furthermore, although 2 β -methylbacteriohopanepolyols are common in cyanobacteria (Bisseret *et al.*, 1985; Simonin, 1993), the presence of 2 β -methyldiplopterol III seems to be restricted to *M. organophilum* and the other PPFMs. The bacteriohopanetetrol ether VI bearing a guanidium group on its carbapseudopentose moiety has been found only in *M. organophilum* and another three PPFMs. All results at the level of the triterpenic composition of these bacteria suggest they may be related, within or close to the genus *Methylobacterium*.

Bacterial hopanoids in plant samples

A major ecological niche of PPFMs is the phylloplane, i.e. the plant leaf surface. Leaves from several plant species and a moss were investigated for the presence of bacterial hopanoids. 3-Deoxyhopanoids have never been reported to occur in higher plants. Diploptene has been found in some mosses in amounts as high as 1 mg g⁻¹, as reported by Marsili *et al.* (1972), but not bacteriohopane derivatives. In our results (Table 1) bacterial hopanoids (diploptene and bacteriohopanepolyol derivatives) were detected in two higher plant species: *Trifolium repens*, which was already known to possess numerous PPFMs on its phylloplane (Corpe & Rheem, 1989), and *Viola odorata*. Two samples of the moss *Abietinella abietina*, which was already known to contain diploptene I (Marsili *et al.*, 1972), both contained bacterial hopanoids (Table 1). This is the first report of bacterial triterpenoids being isolated from eukaryotic plants, but most probably not synthesized by these plants. The detection of derivatives with the C₃₅ bacteriohopane skeleton characteristic for eubacteria, should represent the signature of symbiotic or epibiontic bacteria. In the other plants studied, no hopanoids could be detected. The hopanoid concentration might have been too low and/or their GLC and GLC/MS detection hampered by the large amounts of plant metabolites present in the hopanoid-containing fractions. Indeed the chromatograms of the fractions containing diplopterol or the bacteriohopanepolyols derivatives showed large peaks in the region corresponding to hopanoid retention times. We are left to conclude that hopanoid producers are simply absent or present in very small numbers in the samples investigated. At this stage of our knowledge of hopanoid distribution, the presence of bacterial hopanoids in plant extracts cannot be correlated with specific

bacterial epibionts, but this method might prove to be useful for the detection of such hopanoid-producing micro-organisms from plant tissues or from other natural habitats.

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