Bacterial triterpenoids of the hopane series as biomarkers for the chemotaxonomy of *Burkholderia*, *Pseudomonas* and *Ralstonia* spp.

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Abstract

Hopanoid fingerprints allowed to differentiate bacteria formerly connected to the genus *Pseudomonas*. Whereas all strains related to *Pseudomonas* and *Ralstonia* were devoid of any detectable hopanoid, these pentacyclic triterpenoids were found in the *Burkholderia* species and in related soil isolates, which contained as main hopanoid a bacteriohopanetetrol carbapseudopentose ether, accompanied by significant amounts of its novel Δ9 unsaturated homologue. Unsaturated hopanoids represent an extremely rare feature in soil bacteria and the only known indication for a catabolism of this pentacyclic carbon skeleton in bacteria. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Chemotaxonomy; Hopanoid; Triterpenoid; *Burkholderia*; *Ralstonia*; *Pseudomonas*

1. Introduction

Bacterial triterpenoids of the hopane series are typical biomarkers for eubacteria. Even if no definitive conclusions can be drawn from their known distribution, hopanoids were regularly found in some phylogenetically related species [1]: e.g. in Rhodospirillaceae, obligate methanotrophs, acetic acid bacteria or cyanobacteria. First investigations on the hopanoid content of species formerly related to the genus *Pseudomonas* [1,2] revealed a striking lack of homogeneity of this genus. Whereas most strains did not produce any detectable hopanoids, these pentacyclic triterpenoids were present in some of the phytopathogenic strains [2]. Recent taxonomic changes modified the classification of this bacteria and separated the new genera *Burkholderia* and *Ralstonia* from the genus *Pseudomonas* [3,4]. It appeared therefore interesting to revisit the hopanoid distribution in these species in order to check whether these triterpenoids are useful markers, like fatty acid derivatives [5], for a chemotaxonomic approach.
2.2. Analytical methods

Analytical procedures and spectroscopic identifications were as previously described [7]. The relative amounts of the hopanoids were determined after conversion into the acetates of primary alcohols by side chain cleavage by integration of the gas chromatography (GC) peak areas [1]. Amounts of bacteriohopanepolyols were evaluated by GC, by comparison of the hopanoid peak areas with that of n-dotriacontane, which served as an internal standard. GC was performed on a Carlo Erba Strumentazione Fractovap Series 4160 chromatograph on a DB5 column (30 m × 0.25 mm, 0.1-μm film thickness). All GC-mass spectrometry (MS) spectra were recorded on a Fisons Instruments MD 800 spectrometer. 1H-nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC 200 spectrometer or on a Bruker WP 400 spectrometer in [2H]chloroform solution at 300 K.

2.3. Identification of hopanoids

Lyophilised cells were extracted under reflux with CHCl3/MeOH (2:1, v/v, 3 × 50 ml). An aliquot was treated by the H5IO6/NaBH4 derivatisation method for cleavage of the hopanoid side chains [1]. The remaining part of the crude extract was acetylated (Ac2O/Py, 1:1) overnight at room temperature. The heptaacetate of bacteriohopanetetrol carbapseudopentose ether (6a) from B. caryophylli (8.3 mg from a 8-l culture) and from B. gladioli (11.6 mg from a 8-l culture) or the mixture of the heptaacetates of (6a) and (6b) from strains G-21019, G-21027 and G-22034 (11.5, 10.5 and 16.8 mg, respectively, from 18-l cultures) were obtained after thin layer chromatography (TLC) separation (ethyl acetate/cyclohexane, 7:3, v/v, Rf = 0.38). Free bacteriohopane-32R,33R,34S,35-tetrol (5) tetraacetate was isolated from strain G-22037 (2.8 mg from a 18-l culture) after three successive TLC separations (ethyl acetate/cyclohexane, 7:3, v/v, two migrations, Rf = 0.98; ethyl acetate/cyclohexane, 1:9, v/v, Rf = 0.11; ethyl acetate/cyclohexane, 2:8, v/v, two migrations, Rf = 0.41). All hopanoids were identified by comparison of their 1H-NMR spectra with published data [8–11].

GC-MS data of hopanoid derivatives obtained after side chain cleavage. Acetate of bis-homohop-6-en-32-ol (3b) from strain G-22009: m/z = 496 (M‡, 2%), 481 (M‡-CH3, 0.3%), 367 (M‡-side chain, 1%), 277 (ring B cleavage, 6%), 189 (ring B cleavage, 100%), 119 (C9H11, 40%).

Acetate of homohop-6-en-31-ol (4b) from strain G-22034: m/z = 482 (M‡, 2%), 467 (M‡-CH3, 0.6%), 367 (M‡-side chain, 1%), 263 (ring B cleavage, 4%), 189 (ring B cleavage, 100%), 119 (42%).

NMR data of the mixture of the heptaacetates of bacteriohopanetetrol aminocyclitol ether (6a) and v6-bacteriohopanetetrol ether (6b) from strain G-21027. Spectroscopic data labelled with a degree symbol (‡) correspond to the spectrum of heptaacetate of (6a), and those labelled with an asterisk (*) to the spectrum of its unsaturated homologue (6b). The data without superscript are common to the spectra of both hopanoids. 1H-NMR (400...
**Bacterial strain**  
**Culture**  

<table>
<thead>
<tr>
<th>Bacteriohopane-32-ol</th>
<th>Bacteriohopane-31-ol</th>
<th>Homohopane-31-ol</th>
<th>Homohopane-6-en-31-ol</th>
<th>Biohopanetetrol carbapentose ether</th>
<th>Biohopa-6-enetetrol carbapentose ether</th>
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<tr>
<td>(1a) diploptene, (2a) diplopterol, (5a) bacteriohopane-32R,33R,34S,35-tetrol, (6a) bacteriohopanetetrol carbapentose ether, (6b) bacteriohop-6-enetetrol carbapentose ether</td>
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<td>Obtained after side chain degradation: (3a) bis-homohopan-32-ol, (3b) bis-homohopan-6-en-32-ol, (4a) homohopan-31-ol, (4b) homohopan-6-en-31-ol.</td>
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3. Results

The type strains of two *Burkholderia* species and several slow growing strains isolated from oligotrophic soils and, according to their 16S rRNA sequences, phylogenetically related to the genus *Burkholderia* were analysed for their hopanoid content and compared to *Pseudomonas* and *Ralstonia* species. Whereas no hopanoids were found in all but one *Pseudomonas* and *Ralstonia* spp., they were present in all strains related to *Burkholderia* (Table 1).

In a first approach by GC and GC-MS analysis after derivatisation of the hopanoids by side chain cleavage (Table 2) [1], *bis*-homohopan-32-ol (3a) (Fig. 1) was the major hopanoid derivative in all strains. It was accompanied by significant amounts of its unsaturated analogue with a double bond at C-6 (2) (Table 2). Homohopan-31-ol (4a) and homohopan-6-en-31-ol (4b) were identified in much lower concentrations than those of their C32 homologues in nearly all strains (Table 2). Diploptene (1a) and diplopterol (2a) were both present in small amounts in most hopanoid producing strains (Table 2).

In order to identify the intact biohopanoids, six strains were selected amongst the best hopanoid producers, the two type strains for *B. caryophylli* and *B. gladioli* and...
soil isolates phylogenetically related to *Burkholderia cepacia* (G-21019, G-21027, G-22034) and to *Burkholderia pseudomallei* (G-22037). The bacteriohopanetetrol carboxypentose ether (6a) was isolated as heptaacetate from *B. caryophyllii, B. gladioli* as well as from all other strains, with the exception of the strain G-22037. The stereochemistry of the carboxypentose moiety was identical with that of the same carboxypentose previously identified from *Methylbacterium organophilum* [9], *Rhodospseudomonas acidophila* [12] and *Zymomonas mobilis* [9,13] and distinct from that found in the hopanoids from the cyanobacterium *Anacystis montana* [13] or in the prenyl lipids from *Sulfolobus acidocaldarius* [14]. In the case of strains G-21019, G-21027 and G-22034, the saturated hopanoid (6a) was accompanied by significant amounts of the unsaturated bacteriohopanetetrol carboxypentose ether (6b) (Table 2). This novel hopanoid was characterised by the presence in the $^1$H-NMR spectrum of two doublets at 5.46 and 5.61 ppm, respectively, corresponding to the 6-H and 7-H vinylic protons and by the characteristic chemical shifts of the hopane methyl singlets [10]. Confirmation of the presence of a $\Delta^6$ double bond was obtained by MS from the presence of the $m/z$ 119 fragment in the mass spectra of the acetates of bis-homohop-6-en-32-ol (3b) and homohop-6-en-31-ol (4b) obtained after side chain cleavage [10].

Free bacteriohopanetetrol (5a) was only isolated from strain G-22037, a bacterium related to *B. pseudomallei*. According to the $^1$H-NMR spectrum [11], the stereochemistry is the most common one, corresponding to a side chain derived from d-ribose linked via the C-5 carbon atom to the hopane isopropyl group [15].

The hopanoids with a C$_{31}$ skeleton (4a) and (4b) obtained after H$_2$IO$_6$/NaBH$_4$ treatment most likely arose from bacteriohopanepentol derivatives, which could not be characterised. Bacteriohopanepentol derivatives, including carboxypentose ether, were previously only fully characterised from the cyanobacterium *Nostoc* sp. [16], from *Azotobacter vinelandii* [17] and from *Acetobacter* spp. [18,19].

4. Discussion

Hopanoids proved interesting biomarkers for bacterial species formerly related to the genus *Pseudomonas*. Their distribution was in accordance with the recent taxonomic revisions [3,4]. They were only present in the two investigated *Burkholderia* type strains and in several related soil isolates (Tables 1 and 2). In contrast, they were found neither in the *Pseudomonas* nor in the *Ralstonia* species. The hopanoid fingerprints were quite homogeneous in the *Burkholderia* species. All but one related to *B. pseudomallei*, which only contained free bacteriohopanetetrol (5a), were characterised by the same mixture of bacteriohopanetetrol carboxypentose ethers: the saturated hopanoid (6a) was accompanied by its $\Delta^6$ unsaturated analogue (6b), which was never reported before from bacteria. The presence of unsaturated hopanoids is an extremely rare feature outside the group of the acetic acid bacteria [8,15]. Only $\Delta^{11}$-hopanoids have been reported from obligate methanotrophs belonging to the genus *Methylocalidum* [20]. Introduction of double bonds is the only known oxidative modification of this pentacyclic triterpenic skeleton in bacteria and might represent a first step of a hopanoid catabolism, which is yet completely unknown [21]. $\Delta^6$-Hopanoids were repeatedly reported from sediments of diverse origins [22–25], suggesting that such unsaturated hopanoids might be more widespread amongst eubacteria than expected from their known distribution.

Several strains were grown using either the same culture conditions or even different culture media. Significant changes were observed in the hopanoid distribution without obvious explanation (Table 2). Such changes depending on the growth conditions were previously observed for *Methylbacterium* species depending on the carbon sources [9,26].

The absence of any detectable hopanoids in *Pseudomonas* and *Ralstonia* species has to be reinvestigated more closely. This absence might just be the consequence of a lack of expression of this biosynthetic pathway in the culture conditions we used. Curiously, hopanoids were detected in low amounts in strain G-21032, which was correlated with *Pseudomonas chlororaphis/Pseudomonas amygdali* (Table 2), pointing to the necessity for a closer look on the systematic position of these bacteria and/or on the expression of the hopanoid biosynthetic pathway.

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