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Neutral Lipids and Phospholipids of Free-Living and Bacteroid Forms of Two Strains of *Rhizobium* Infective on *Lotus pedunculatus*

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The neutral lipids and phospholipids of two strains of rhizobia in their free-living state and in symbiosis with a host plant are described. The principal lipid classes found were the polymer poly-β-hydroxybutyrate, phospholipids, free fatty acids, glycerides, methyl esters, aliphatic alcohols, and hydrocarbons. The lipids include unusual unsaturated methyl-branched and saturated methoxy-branched fatty acids. Most components were found to be common to both forms of both strains, although the proportions varied. A number of strain differences could be discerned.

Rhizobium is an aerobe, existing as a free-living bacterium in soil or as a partially anaerobic symbiont in the root nodules of leguminous plants. It is in the symbiotic form (bacteroid), which is frequently morphogenetically different, that nitrogen fixation takes place. Because of their nitrogen-fixing ability rhizobia are used as legume inoculants in agriculture.

Little is known about the lipids of rhizobia. The phospholipids of two strains of *Rhizobium japonicum* in the free-living form were described by Bunn et al. (7) and were found to differ considerably in composition. The composition of other lipids in free-living rhizobia appear to be unknown except for the presence of poly-β-hydroxybutyrate (PHB) (16). Recently Gerson et al. (10) characterized a group of unusual fatty acids isolated from cultured *Rhizobium* cells. These are distinguished by the presence of methyl- and methoxy-branched chains and are probably derived from lactobacillic acid.

We are not aware of any study of bacteroid lipids except on the metabolism of PHB by Wong and Evans (22), who demonstrated that the amounts present in root nodules depend on the photosynthetic products made available by the host plant. The lack of knowledge of the composition of bacteroid lipids has thus far made it impossible to establish the extent to which they differ from those of free-living bacteria.

A more precise knowledge of the lipids may provide useful criteria in the taxonomy of *Rhizobium* (20). The use of lipids in this connection has been discussed by Kates (16) and more recently by Shaw (20). Furthermore, differences as revealed by lipid composition may provide a useful guide in the identification of known rhizobial strains. It is for these reasons that we have undertaken a detailed comparison of the lipids of two strains of *Rhizobium* in both the bacterial and bacteroid states. We have taken care to ensure that they were grown under identical conditions (15, 16; J. A. Croom and J. J. McNeill, Bacteriol. Proc., p. 170, 1961) and in the same host plant.

MATERIALS AND METHODS

Cultures. The strains used were *Rhizobium* NZP 2037 and *Rhizobium* CC 814s. These were donated by R. M. Greenwood of this laboratory. In culture, NZP 2037 requires 4 to 5 h per cell division, whereas CC 814s requires 6 to 8 h. Both produced bacteroid forms in root nodules and actively fix nitrogen while in symbiosis with the leguminous plant *Lotus pedunculatus*. They differ, however, in that NZP 2037 belongs to the acid-producing group of rhizobia, whereas CC 814s does not. These two groups are further distinguished by the composition of their extracellular polysaccharides (2).

The free-living cells were grown at 25 C in 1-liter flasks containing 500 ml of culture in a shaker revolving at 150 rpm. The medium used was yeast-mannitol broth (21) at pH 6.5 to 7.0 without $CaCO_3$. The yeast extract was defatted by extraction with chloroform-methanol. The cells were harvested after 8 days of incubation by centrifuging the culture at 4 C for 20 min at 10,000 × *g*, followed by one wash with distilled water.

Preparation of bacteroids. Plants of the species of *L. pedunculatus* were grown in a glasshouse at 25 C

under aseptic conditions using surface-sterilized seeds in stainless-steel troughs containing sterilized pumice. They were provided with nitrogen-free Hoaglands medium (11) and inoculated at the time of planting. After 3 months of growth the nodules were harvested by hand, care being taken to avoid root fibers. The bacteroids were then obtained from the nodules by the procedure described by Bergersen (4) and stored at −10 C prior to lipid extraction. The dry weights of the cells were determined from freeze-dried aliquots of the cell suspension.

Extraction of lipids. The cells were disintegrated for 5 min at 0 C in a 100-W ultrasonic disintegrater in 5 ml of aqueous suspension and then extracted twice with 5 volumes of (acid-free) chloroform-methanol (2:1, vol/vol) (analytical grade). The combined chloroform extracts were evaporated to dryness, and the residue was re-dissolved in chloroform, filtered, and evaporated under a stream of nitrogen at 50 C. All samples were stored under N_2 at 0 C.

Analytical procedures. (i) Determination of PHB. The lipids were re-dissolved in a minimum quantity of chloroform and PHB precipitated with 10 volumes of diethylether. After precipitation and washing with ether, the PHB was dissolved in a known volume of chloroform, and an appropriate aliquot was used for determination by the method of Law and Slepecky (18). The ether-soluble lipids were recovered for further analysis.

(ii) Conversion to methyl esters for gas chromatography. Phospholipids were transesterified to methyl esters with BCl_3-methanol complex, which does not form artifacts with unsaturated and cyclic fatty acids (6, 17). Free fatty acids were esterified with freshly prepared diazomethane.

(iii) Hydrogenation. Methyl esters were hydrogenated by the micro-method of Appelquist (1), which does not destroy the cyclopropane ring of lactobacillic acid.

(iv) Phosphorus. Thin-layer chromatographic (TLC) plates on which the phospholipids had been separated were sprayed with 50% H_2SO_4 and charred. The spots were removed, and their phosphorus content was determined by the Fiske-Subbarow procedure as modified by Bartlett (3) and Boettcher et al. (5).

(v) TLC. Analyses by TLC of lipids other than PHB were carried out on glass plates (20 by 20 cm), coated with 0.30-mm Silica Gel G (Merck; for TLC) with the exception of phospholipids, which were resolved on plates (4.5 by 4.5 cm) coated with 0.25-mm Silica Gel H (Merck, for TLC).

Identification and isolation of lipid classes by TLC. For the identification of lipid classes by two-dimensional TLC the solvent systems used were chloroform in the first dimension and hexane-ether-acetic acid (85:15:1) in the second. The identity of the components was further established by running the sample against standards on two plates, first with hexane, which moved the hydrocarbons to the top of the plates. The other components remained at or near the origin. After ruling the hydrocarbon spot off, the plates were re-run either with hexane-ether-acetic acid (85:15:1) or with hexane-ether-ammonia (85:15:1). Free fatty acids were retained at the origin with ammonia. The system hexane/hexane-ether-acetic acid was also used for preparative TLC of the lipid classes. Twenty to 30 mg in 100 μl of chloroform was applied. They were washed from the Silica Gel G scrapings with chloroform-methanol-water (50:50:5). Lipids other than phospholipids and PHB were determined by weighing.

For phospholipid identification and determination, the solvent systems used were chloroform-methanol-ammonia (65:35:3) in the first dimension and chloroform-methanol-acetone-water (50:35:15:5) in the second, after drying for 1 h under vacuum. Phospholipids were identified by the use of pure internal standards.

Identification and determination of hydrocarbon, alcohol, and fatty acid compositions by gas-liquid chromatography. The equipment used was an F & M model 5750 gas chromatograph fitted with dual flame ionization detectors. Stainless-steel columns (2 m by 2.3 mm ID) were used throughout. Nitrogen was used as carrier gas.

Methyl esters were analyzed on 5% (wt/wt) EGSSX and 3% APL coated onto 100- to 120-mesh Chromosorb G (acid washed, silanized) at 185 C. Hydrocarbons were analyzed using 3% SE 30 on 100- to 120-mesh Chromosorb W (acid washed, silanized), programmed from 180 to 250 C at 1°/min. Alcohols were analyzed on 3% JXR coated onto Gas-Chrom Q (100 to 120 mesh) programmed at 180 C for 10 min and then increasing at 3°/min to 280 C.

Individual methyl esters were identified by their carbon number (13, 14) before and after hydrogenation and bromination. Pure standards were used to identify branched and cyclopropane fatty acids as well as hydrocarbons and alcohols.

RESULTS

The overall composition of neutral and phospholipids of bacteria and bacteroids of both strains is summarized in Table 1.

PHB accounts for 80 to 90% of bacterial and 60 to 65% of bacteroid lipid (8).

The composition of phospholipids is shown in Table 2. The unidentified phospholipids are present in small amounts only, and the figures in Table 2 constitute the sum total of these. The absence of phosphatidyl serine in bacteroids is noteworthy and also the presence of phosphatidyl inositol. The latter was not found by Bunn et al. (7) in *R. japonicum*.

The phospholipid fatty acid composition is summarized in Table 3. The designation of the components listed in Tables 3 to 8 corresponds to that used by Hoffstetter et al. (13).

The unusual acids *cis*-11-methyl-Δ11-octadecenoic (11-Me-Δ11-18:1), 12-methoxy-11-methyl and 11-methyoxy-12-methyl-octadecanoic (12-OMe-11Me-18:0, 11-OMe-12Me-18:0), 11-methoxy and 13-methoxy-nonadecanoic (11-OMe-19:0 and 13-OMe-19:0) were identified by gas-liquid chromatography-mass spectrometry

TABLE 1. *Composition of the lipids of NZP 2037 and CC 814s*

Lipid composition	Bacteria		Bacteroids	
	NZP 2037	CC 814s	NZP 2037	CC 814s
Recovery (mg/g of dry cells)				
Total lipid	558	273	243	182
PHB	505	223	160	112
Lipids other than PHB	42	43	66	76
Composition of lipids other than PHB (% by weight)				
Aliphatic hydrocarbons	12.9	18.5	9.8	18.9
Aliphatic alcohols	2.6	13.2	16.5	16.2
Methyl esters	9.0	10.7	11.4	2.6
Glycerides	3.7	2.5	13.0	6.3
Free fatty acids	28.9	17.4	12.7	6.3
Phospholipids	42.9	37.7	36.6	49.7

TABLE 2. *Composition of phospholipids[a] of NZP 2037 and CC 814s*

Phospholipid	Bacteria		Bacteroids	
	NZP 2037	CC 814s	NZP 2037	CC 814s
Cardiolipin	15.9	20.5	16.0	26.8
Phosphatidyl ethanolamine	33.1	17.6	29.6	22.4
Phosphatidyl choline	51.0	21.4	32.7	28.5
Phosphatidyl serine	Tr[b]	9.7	—[c]	—
Phosphatidyl inositol	Tr	17.8	7.2	10.2
Unidentified	Tr	13.0	14.5	12.1

[a] Expressed as percent by weight.
[b] Tr, Trace.
[c] —, Not detected.

(10), as were 12-methyl-tetradecanoic (15:anteiso), *cis*-vaccenic (18:1), and lactobacillic (19:cyclopropane) acids.

The composition of the free fatty acids and those of glycerides and methyl esters are shown in Tables 4 to 6. Glyceride fatty acids (Table 5) and methyl ester fatty acids (Table 6) have distinctive features. The former contain 20 to 25% methyl-branched acids in the bacteria, whereas the methyl esters are virtually devoid of monoenes and 28% of their saturated straight chain acids are odd numbered.

The composition of aliphatic alcohols and hydrocarbons is shown in Tables 7 and 8, respectively. They were found to be present as homologous series. It is of interest that the composition calculated from average values of both strains for each chain length adhered closely to a normal distribution. This had a mean of 22 carbons (standard deviation ± 2.5) for the alcohols and a mean chain length of 25 carbons (standard deviation ± 3.3) for the hydrocarbons.

DISCUSSION

The lipids of the two strains of lotus rhizobia described here are unusual in having a relatively high proportion of naturally occurring methyl esters. These have been previously reported as naturally occurring lipids (9, 19). There has, however, always been some doubt about the validity of such reports, since methyl esters can be formed in small amounts as artifacts during the extraction with chloroform-methanol. In the present case we were satisfied that they were indeed naturally occurring bacterial lipids only after we found that their fatty acid composition differed greatly from that of the other lipids.

The phospholipids found in the two strains described here differ in one major respect from those shown by Bunn et al. (7) in *R. japonicum*.

TABLE 3. *Composition of phospholipid fatty acids[a] of NZP 2037 and CC 814s*

Fatty acid[b]	Bacteria		Bacteroids	
	NZP 2037	CC 814s	NZP 2037	CC 814s
<15:0	1.2	2.3	2.7	1.0
15:aiso	0.6	Tr	Tr	Tr
15:0	0.7	1.9	Tr	0.3
16:0	15.3	13.1	15.2	8.5
16:1	6.1	1.2	2.9	6.1
17:0	1.1	4.6	Tr	Tr
18:Br	Tr	Tr	Tr	Tr
18:0	6.0	6.9	1.9	0.6
11-methyl-Δ11-18:1 (*cis*)	25.2	10.0	35.6	13.1
Δ11-18:1 (*cis*-vaccenic)	9.6	45.8	34.5	62.8
19:0	3.2	3.9	1.8	Tr
19:cpr (lactobacillic)	9.2	3.2	4.1	7.6
11-methoxy-12-methyl-18:0 and 12-methoxy-11-methyl-18:0	0.5	2.4	Tr	Tr
11-methoxy-19:0 and 13-methoxy-19:0	21.4	4.7	1.3	Tr
Total				
n-Saturated	27.5	32.7	21.6	10.4
n-Monoenes	15.9	47.0	37.4	68.9
Cyclic	9.2	3.2	4.1	7.6
Branched saturated	22.5	7.1	1.3	Tr
Branched monoenes	25.2	10.0	35.6	13.1

[a] Expressed as percent by weight.
[b] Abbreviations: aiso, anteiso; Br, branched; cpr, cyclopropane; Tr, trace.

TABLE 4. *Composition of free fatty acids[a] of NZP 2037 and CC 814s*

Fatty acid[b]	Bacteria		Bacteroids	
	NZP 2037	CC 814s	NZP 2037	CC 814s
<15:0	5.6	1.5	1.1	2.4
15:aiso	0.4	Tr	0.9	2.0
15:0	0.5	0.8	0.3	1.8
16:0	8.1	19.2	7.7	25.7
16:1	1.2	4.4	2.9	5.1
17:Br	3.3	Tr	1.7	5.4
17:0	0.6	Tr	0.5	1.8
18:Br	1.9	Tr	1.3	4.3
18:0	6.1	2.6	3.5	12.5
11-methyl-Δ11-18:1 (*cis*)	10.4	16.0	50.3	4.1
Δ11-18:1 (*cis*-vaccenic)	6.9	50.4	24.6	30.1
19:0	Tr	1.5	1.5	2.0
19:cpr (lactobacillic)	52.4	2.6	3.0	2.9
11-methoxy-12-methyl-18:0 and 12-methoxy-11-methyl-18:0	1.4	Tr	Tr	Tr
11-methoxy-19:0 and 13-methoxy-19:0	1.2	1.0	0.7	0.8
Total				
n-Saturated	20.9	25.6	14.6	46.2
n-Monoenes	8.1	54.8	27.5	35.2
Cyclic	52.4	2.6	3.0	2.0
Branched saturated	8.2	1.0	4.6	12.5
Branched monoenes	10.4	16.0	50.3	4.1

[a] Expressed as percent by weight.
[b] See Table 3 for abbreviations.

We found appreciable amounts of phosphatidyl serine only in bacteria of strain CC 814s, whereas phosphatidyl inositol, which was not found in *R. japonicum*, is an important constituent in all except the free-living NZP 2037.

Our results suggest that there are some differences in lipid composition of free-living bacteria and symbiotic bacteroids. Table 1 shows that more PHB was found in bacteria, whereas more of the lipids other than PHB were found in the bacteroids. Bacteria contained greater proportions of free fatty acids but less glyceride. It therefore appears that bacteria contain more of those lipids that are immediately available as carbon or energy sources than bacteroids. On the other hand, this may be due to differences in nutrient availability, reflecting two entirely different conditions of growth. A further distinction between them is the absence of phosphatidyl serine in bacteroids.

Certain strain differences are also apparent, most of which appear to be confined to bacteria, although some are common to both forms. Thus, both bacteria and bacteroids of NZP 2037 contain more PHB than those of CC 814s.

Phospholipid fatty acids reveal strain differences in that the proportions of 11-Me-Δ11-18:1 and Δ11-18:1 of both bacteria and bacteroids and the proportions of 11-OMe-19:0 and 13-OMe-19:0 of the bacteria are different. Free fatty acids show strain differences in the proportions of palmitic acid in bacteroids and vaccenic and lactobacillic acids in bacteria. Indeed the phospholipid and free fatty acids of CC 814s were found to be far richer in vaccenic acid than those of NZP 2037.

No strain differences were found in glyceride or methyl ester fatty acids, nor in alcohols or hydrocarbons.

It is noteworthy that the lipids described above have fatty acid spectra which are characteristic for each lipid. The greatest variation was found in the proportions of 11-Me-Δ11-18:1, Δ11-18:1 (vaccenic), 11-OMe-12Me-18:0, 12-OMe-11Me-18:0, 11-OMe-19:0, 13-OMe-19:0, and lactobacillic acids. The amount of vaccenic acid seems to vary inversely with one or more of these derivatives. It is known that lactobacillic acid is derived from *cis*-vaccenic (12), and 11-Me-Δ11-18:1, 11-OMe-12Me-18:0, 12-

TABLE 5. *Composition of glyceride fatty acids[a] of NZP 2037 and CC 814s*

Fatty acid[b]	Bacteria		Bacteroids	
	NZP 2037	CC 814s	NZP 2037	CC 814s
<15:0	7.7	5.5	3.1	5.2
15:aiso	5.6	5.2	2.6	3.2
15:0	1.2	Tr	Tr	0.8
16:0	16.5	11.0	18.6	27.5
16:1	2.3	2.6	0.2	4.4
17:Br	8.7	6.4	3.2	4.3
17:0	1.0	Tr	4.1	0.8
18:Br	6.9	13.5	Tr	4.4
18:0	14.1	9.6	8.7	12.5
11-methyl-Δ11-18:1 (*cis*)	5.7	1.4	12.2	1.9
Δ11-18:1 (*cis*-vaccenic)	17.8	26.3	23.6	24.3
19:0	2.3	Tr	Tr	0.5
19:cpr (lactobacillic)	5.6	9.5	9.3	7.2
11-methoxy-12-methyl-18:0 and 12-methoxy-11-methyl-18:0	0.8	1.2	7.7	0.7
11-methoxy-19:0 and 13-methoxy-19:0	3.8	7.6	6.7	2.3
Total				
n-Saturated	42.8	26.3	34.5	47.3
n-Monoene	20.1	28.9	23.8	28.7
Cyclic	5.6	9.5	9.3	7.2
Branched saturated	25.8	33.9	20.2	14.9
Branched monoene	5.7	1.4	12.2	1.9

[a] Expressed as percent by weight.
[b] See Table 3 for abbreviations.

TABLE 6. *Composition of methyl ester fatty acids[a] of NZP 2037 and CC 814s*

Fatty acid[b]	Bacteria		Bacteroids	
	NZP 2037	CC 814s	NZP 2037	CC 814s
<15:0	11.8	5.0	3.7	10.3
15:0	10.2	8.5	7.7	11.5
16:0	9.1	13.0	6.7	15.3
17:Br	Tr	4.1	Tr	Tr
17:0	9.3	16.9	12.4	16.1
18:Br	1.9	Tr	—[c]	4.8
18:0	29.1	11.5	13.0	15.8
11-methyl-Δ11-18:1 (*cis*)	—	—	—	—
Δ11-18:1 (*cis*-vaccenic)	2.4	2.2	0.8	1.3
19:0	8.4	18.2	18.1	13.3
19:cpr (lactobacillic)	7.7	1.8	5.4	Tr
11-methoxy-12-methyl-18:0 and 12-methoxy-11-methyl-18:0	5.1	10.3	17.5	6.2
11-methoxy-19:0 and 13-methoxy-19:0	5.0	8.5	14.7	5.4
Total				
n-Saturated	77.9	73.1	61.6	82.3
n-Monoenes	2.4	2.2	0.8	1.3
Cyclic	7.7	1.8	5.4	Tr
Branched	12.0	22.9	32.2	16.4

[a] Expressed as percent by weight.
[b] See Table 3 for abbreviations.
[c] —, Not detected.

TABLE 7. *Composition of aliphatic alcohols[a] of NZP 2037 and CC 814s*

No. of C atoms	Bacteria		Bacteroids	
	NZP 2037	CC 814s	NZP 2037	CC 814s
<18	1.9	7.4	1.8	2.7
18	4.6	6.9	1.9	4.6
19	8.2	8.7	2.2	4.4
20	10.5	8.2	6.8	10.2
21	12.9	10.9	12.5	10.4
22	13.2	11.0	10.3	13.3
23	12.1	10.5	17.5	14.3
24	10.3	10.8	15.9	12.2
25	11.0	6.6	10.9	13.2
26	6.0	5.7	15.9	6.8
27	3.9	5.4	2.5	4.6
28	3.5	3.3	1.4	1.8
>28	1.8	4.8	0.4	1.5

[a] Expressed as percent by weight.

OMe-11Me-18:0, 11-OMe-19:0, and 13-OMe-19:0 are therefore likely to be biosynthetically related to vaccenic or lactobacillic acid. Vaccenic and 11-Me-Δ11-18:1 were previously established to be of the *cis* configuration (10).

Bunn et al. (7) reported an unknown fatty acid between 18:0 and 18:1. This is probably 11-Me-Δ11-18:1, identified in lotus rhizobia strain NZP 2037 (10).

TABLE 8. *Composition of aliphatic hydrocarbons[a] of NZP 2037 and CC 814s*

No. of C atoms	Bacteria		Bacteroids	
	NZP 2037	CC 814s	NZP 2037	CC 814s
<20	11.4	6.5	3.8	3.5
20	2.8	2.5	2.9	1.8
21	4.1	4.1	3.5	4.2
22	7.6	8.4	6.4	7.7
23	9.7	10.9	10.7	11.0
24	12.0	11.5	12.9	13.1
25	12.0	13.1	14.0	12.5
26	10.2	10.3	12.7	11.3
27	7.9	8.5	9.2	9.7
28	7.1	8.2	7.9	8.8
29	5.8	4.5	6.0	6.6
30	3.6	4.2	4.0	4.8
>30	5.7	7.3	6.0	5.0

[a] Expressed as percent by weight.

With very few exceptions the lipids and fatty acids listed above were present in the bacteria and bacteroids of both strains of lotus rhizobia, although major differences in the proportions were found. The phospholipid composition and the presence of unusual fatty acids may be useful criteria in the taxonomy of *Rhizobium*.

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