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Complex lipids of a lipolytic and general-fatty-acid-requiring *Butyrivibrio* sp. isolated from the ovine rumen

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The complex lipids of the naturally-occurring general-fatty-acid auxotroph *Butyrivibrio* S2 [Hazlewood & Dawson (1979) *J. Gen. Microbiol.* **112**, 15–27] grown with palmitic acid as sole fatty-acid supplement have been investigated and some have been isolated in a state of purity and analysed. The majority are phospholipids (84%) and many contain galactose. They typically possess few esterified long-chain fatty-acid residues ($C_{16:0}$), but are rich in esterified butyric acid and C_{16}-alkenyl groups. Most of the phosphorus-containing lipids, including the two major lipids of the organism, contain esterified diabolic acid, a long-chain vicinal dimethyl-substituted dicarboxylic acid [Klein, Hazlewood, Kemp & Dawson (1979) *Biochem. J.* **183**, 691–700] in definite stoichiometric relationship to phosphorus. No phosphatidylglycerol was present, but its monobutyroyl ester was detected as a minor component. Galactofuranosyldiacylglycerol (plasmalogen) and its monobutyroyl ester, cetyl alcohol and diacylglycerol were also identified.

An anaerobic Gram-negative curved rod with the characteristics of the genus *Butyrivibrio* has been isolated from the ovine rumen (Hazlewood & Dawson, 1979). This organism is a general-fatty-acid auxotroph that grows in culture when supplied with straight-chain saturated (C_{13}–C_{18}) or monoenoic fatty acids in spite of the absence of fatty-acid-desaturation systems. It possesses very active lipolytic enzymes that can deacylate the plant galactolipids, phospholipids and sulpholipids present in the diet of the sheep to provide sufficient fatty acid for growth. Any linolenic acid and linoleic acid released is rapidly hydrogenated to *trans*-octadec-11-enoic acid which can act as an excellent growth promotor for the bacterium and is incorporated into its complex lipids.

Previous studies showed that, whereas the growth-promoting fatty acid is incorporated into bacterial lipids, either unchanged or as an ether-linked alkenyl grouping, part also is incorporated in the form of a new type of hydrophobic moiety quite different from the fatty acid itself or the long-chain aldehyde formed from the fatty acid (Hazlewood & Dawson, 1979). Often the proportion of the new moiety is substantial, e.g. when ^{14}C-labelled palmitic acid is used as growth supplement, it accounts for over 40% of the radioactivity recovered from the bacterium. The dimethyl ester of this substance has been isolated, after methanolic HCl hydrolysis of the complex lipid fraction of the organism, and its structure ascertained (Klein *et al.*, 1979). Depending on the fatty-acid supplement, a whole series of dimethyl-substituted long-chain dicarboxylic acids, the diabolic acids, can be produced, which chemically can be regarded as condensation products between two fatty acid molecules with a dehydrogenation linkage of the penultimate carbon atoms. Thus the diabolic acid isolated after palmitic acid supplementation is in the form of the dimethyl ester of 15,16-dimethyltriacontane-1,30-dioic acid (Hauser *et al.*, 1979). It is believed that the vicinal dimethyl branching plays a major part in maintaining the fluidity of the membrane lipids in the complete absence of unsaturation in the hydrophobic chains (Hauser *et al.*, 1979).

In the present paper, we examine the number and range of complex lipids when the auxotroph is grown with a palmitic acid supplement. It has been established that nearly all the phospholipids contain esterified diabolic acid, whereas very few of the non-phospholipids contain this substance. In addition no diabolic acid could be detected in a free non-esterified form.

Experimental

Growth of organism

Butyrivibrio S2 was cultured in fatty-acid-free liquid medium with galactose (4g/litre), essentially as described previously (medium 3; Hazlewood &

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Dawson, 1979). The palmitic acid (final concentration 30mg/litre plus 6.25μCi of [1-^{14}C]palmitic acid/litre) required to promote growth was dispersed by ultrasonication (KB 80/1 ultrasonic bath; Kerry's Ltd., Basildon, Essex, U.K.) in a small volume of preheated (70°C) sodium taurocholate (final concentration in medium 0.3g/litre) solution, before addition to the remaining medium for autoclaving (115°C, 20min). Freshly prepared medium (4 litres) was inoculated (5% by vol.) with a 24h culture of *Butyrivibrio* S2 grown in the same medium. All cultures were incubated at 39°C for 16h and cells were harvested by centrifugation (21000*g*, 6°C, MSE continuous flow rotor).

Extraction of lipids

The bacterial pellet from 12 litres of culture medium was successively extracted at room temperature twice with 100ml of methanol/water (19:1, v/v), with 100ml of chloroform/methanol (2:1, v/v), followed by 100ml of chloroform/methanol (2:1, v/v) at 45°C. The residue remaining still contained about 10–30% additional radioactivity above that already extracted. More vigorous extraction with chloroform/methanol/conc. HCl (266:133:1, by vol.) showed this to be largely free palmitic acid that had, presumably, not been taken up by the bacteria. A small percentage of complex ^{14}C-labelled lipid extracted by the acid solvent, although partially decomposed, was essentially similar to that extracted by the neutral solvents. The residual fraction was not investigated further.

The combined neutral-solvent extracts were taken to dryness in a rotary evaporator, dissolved in 10ml of chloroform/methanol (2:1, v/v) and then shaken with 2.5ml of 0.9% NaCl containing 0.01M-NH_3. The lower phase was washed twice with the upper phase of a similar solvent partitioning: the ammonia present helped to remove unesterified short-chain fatty acid. The lower phase containing the lipids (6mg of P) was taken to dryness and re-dissolved in 2.5ml of chloroform/methanol (9:1, v/v).

Separation of lipids

To the above solution was added 12.5ml of diethyl ether and 4ml of acetone and the mixture was kept at −10°C for 48h. The precipitate containing virtually all of the phospholipids was collected by centrifugation at low temperature and re-dissolved in chloroform/methanol (4:1, v/v) for storage. The supernatant contained the bulk of the non-phosphorus-containing lipids, although some of the latter did contaminate the phospholipid fraction. Subsequent fractionation was entirely by preparative t.l.c. on silica-gel plates (F_{254}; Merck). In general, acidic solvents could not be used for the runs, because of the high content of acid-labile aldehydogenic groups in nearly all of the lipids. It was usually found better to make all the solvents alkaline with NH_3. The running strip of individual

Table 1. *Solvents used for t.l.c. purifications of complex lipids*

The extracted lipids were first separated either in chloroform/methanol/conc. NH_3 (100:12:1.2, by vol.) (lipid spots 1, 2, 3, 5, 6 and 12 in Fig. 1) or in chloroform/methanol/conc. NH_3 (110:50:13, by vol.) (all other lipids). The isolated fractions were then purified by re-running in the same solvent or in one of the solvents shown below. Although all t.l.c. runs were made in saturation chambers, better separations were often achieved by allowing the plate to protrude (0.5cm) from the top of the chamber, so that evaporation from an open top tank effectively 'lengthened' the chromatographic run. The overun time used is given in the final column.

Lipid spot no. (see Fig. 1)	Solvent composition (parts by volume)				Overun time (h)
	Chloroform	Methanol	Conc. NH_3	Water	
2, 3*	100	2	0.25	—	—
5	100	10	1.0	—	—
6	100	2	0.2	—	—
12	50	10	1.0	—	—
4	100	10	1	—	0.75
9, 10, 18	100	20	2	—	1.0
11, 13	100	37	0.6	6.0	1.5
14	100	30	3	—	0.25
15, 16, 17	100	20	0.25	0.75	2.0
19, 20	100	34	0.5	5.0	2.0
22	100	40	3	—	0.75
24, 25, 26, 27	50	31	2	5	1.0
28	100	62	2	13	1.0
29	100	62	2	13	3.25

* For a subsequent separation a solvent consisting of light petroleum (b.p. 40–60°C)/diethyl ether/acetic acid (50:10:1, by vol.) was used, since these lipids contained no aldehydogenic groups.

lipids could be accurately located by autoradiography of the plates, after which the silica gel was scraped off, inserted into a short column with a sintered glass plug and successively eluted with chloroform, chloroform/methanol (2:1, v/v) and finally methanol. After evaporation to dryness the lipids were dissolved in chloroform/methanol (9:1, v/v) and stored for 24h at −20°C, when any precipitated silicic acid was removed by centrifuging.

The preliminary separation of the less polar lipids was in chloroform/methanol/conc. NH_3 (100:12:1.2, by vol.). Purification was achieved by re-running the isolated lipid in the same solvent or alternatively the solvents denoted in Table 1. The more polar lipids were separated in chloroform/methanol/conc. NH_3 (110:50:13, by vol.) and further purified by re-running in the same solvent or one of the solvents given in Table 1. The silicic acid was removed from solutions of the purified lipids as indicated above and they were then checked for purity by t.l.c. and autoradiography and stored to await analysis (−20°C).

Analysis

Distribution and quantification of long-chain hydrophobic moieties. A sample of the lipid was treated with anhydrous methanolic HCl (0.55M; 2ml) in a sealed tube at 90°C for 2.5h. After cooling the contents of the tube were neutralized with anhydrous Na_2CO_3, 2ml of water was added and the mixture was extracted thrice with 4ml of diethyl ether. The combined diethyl ether extracts were analysed by t.l.c. on silica gel F_{254} plates (Merck), with light petroleum (b.p. 40–60°C)/diethyl ether/acetic acid (100:12:1.2, by vol.) as solvent. Autoradiography was used to locate the following spots: the methyl esters of long-chain fatty acids, the dimethyl acetals formed from aldehydogenic groups, dimethyl esters of diabolic acids and long-chain alcohols if present (Hazlewood & Dawson, 1979). The radioactive areas were scraped from the plates into scintillation vials, 10ml of scintillation fluid was added and the radioactivity was determined in a scintillation counter.

Since the synthesis of long-chain hydrophobic groupings from non-radioactive sources was extremely small (Hazlewood & Dawson, 1979; Hauser *et al*,. 1979) the distribution of radioactivity gave a direct assessment of the amount of each grouping in the individual lipid. The absolute amount of each type of hydrophobic grouping was determined by using the known specific radioactivity of the palmitic acid after allowance had been made for the non-radioactive fatty acid added with the inoculum culture. The validity of this approach was shown by also measuring the specific radioactivity of isolated diabolic acid after mass determination by titration or weighing: it agreed with that expected from the [^{14}C]palmitic acid precursor.

Lipid hexose. Initial determinations were made by the method of Spiro (1966), but in later work a modified version of the more sensitive method of Roughan & Batt (1968) was used. To the lipid was added 0.5ml of 5% (w/v) phenol in acetic acid/water (1:2, v/v). The mixture was warmed to 70°C and 2ml of H_2SO_4 at 70°C was added down the side of the tube. Some spluttering resulted but this was decreased by the inclusion of acetic acid and the reproducibility of the method was much improved. Since no unsaturated fatty acids were present, neither method suffered appreciably through false colours being produced.

Volatile fatty acid. Many of the complex lipids contained esterified short-chain fatty acid (identified as being exclusively butyric acid), which was estimated by g.l.c. with propionic acid as internal marker as described by Clarke *et al.* (1976).

Glycerol. The lipid was hydrolysed in a sealed tube in 2ml of 1M-HCl for 96h at 100°C. The hydrolysate was washed with light petroleum and 2M-NaOH added to neutralize the hydrolysate and the mixture was buffered with 0.1M-triethanolamine/HCl, pH 7.6, before estimation of glycerol by the enzymic method of Garland & Randle (1962). The enzymes glycerol kinase (EC 2.7.1.30), pyruvate kinase (EC 2.7.1.40)) and lactate dehydrogenase (EC 1.1.1.27) were obtained from Boehringer (Mannheim). Concentrations of glycerol up to 0.1μmol gave a linear response.

Phosphorus. This was determined by the procedure of Bartlett (1959).

Detection of reactive groups of lipids on thin-layer chromatograms. Phospholipids were detected as described by Vaskovsky *et al.* (1975). Aldehydogenic lipids gave yellow spots on spraying the plate with 30% (v/v) H_2SO_4 and after 5min with a reagent containing 2,4-dinitrophenylhydrazine (1.2g), methanol (260ml), conc. HCl (40ml) and chloroform (20ml). Glycolipids were detected by spraying the plate with 10% (w/v) resorcinol in water and then kept in an oven at 150°C for 12min. The hot plate was quickly sprayed with conc. H_2SO_4. Hexose-containing lipids gave deep-red spots, and many other lipids gave yellow spots. Amino lipids were located by spraying with 0.25% (w/v) ninhydrin in acetone and warming at 100°C for 3–5min. The general detection of all lipids was achieved by autoradiography or exposure to I_2 vapour or by charring.

Results and discussion

The results of a two-dimensional separation of the complex lipids of *Butyrivibrio* S2 grown in the presence of [1-^{14}C]palmitate are shown in the

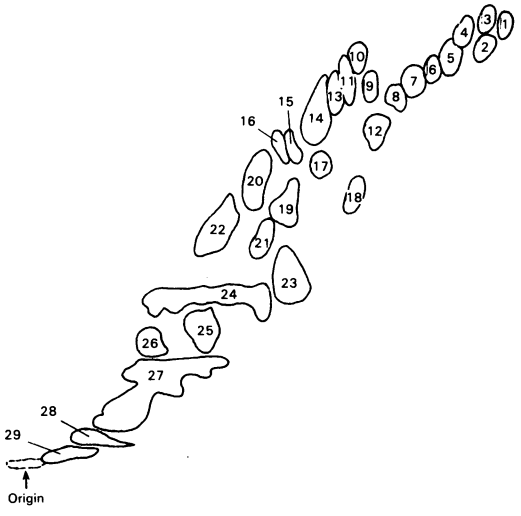

Fig. 1. *Drawing of autoradiograph of the t.l.c. separation of the complex lipids of Butyrivibrio S2 grown in the presence of* [1-^{14}C]*palmitic acid*

Solvents: vertical, chloroform/methanol/conc. NH_3 (20:10:1, by vol.); horizontal, chloroform/methanol/water/conc. NH_3 (100:37:6:0.6, by vol.).

autoradiograph presented in Fig. 1. The separation obtained is acceptable considering the limitations imposed by the need to use alkaline solvents to prevent decomposition of the plasmalogens present. The reaction of the radioactive spots to various spray reagents is given in Table 2.

The complex lipids consist of phosphoglycolipids (59% of incorporated radioactivity), phospholipids (25%) and glycolipids (13%). This can be calculated from the results on the distribution of radioactivity presented in Table 2, which ignores the radioactivity present in spot 23 (Fig. 1); the latter is unesterified fatty acid and is presumed to represent palmitic acid not taken up by the organism during growth. Nearly all the complex lipids contain appreciable amounts of acid-labile aldehydogenic groups (Tables 2 and 3), which is in line with the general pattern established for anaerobic bacteria, particularly those derived from the rumen (Kamio *et al.*, 1969, 1970; Verkley *et al.*, 1975). The long-chain aldehyde liberated proved to be almost entirely palmitaldehyde (Hauser *et al.*, 1979).

Many of the isolated lipids contained esterified volatile fatty acid, which had been observed previously with lipids obtained from other *Butyrivibrio* species (Clarke *et al.*, 1976). On analysis by g.l.c. such short-chain fatty acids proved to be exclusively butyric acid. The esterified long-chain acid was almost exclusively palmitic acid (Hauser *et al.*, 1979).

By varying the composition of the alkaline solvents used for preparative t.l.c. and because of the precise non-destructive location of bands by autoradiography, it was possible to isolate at least the main complex lipids present in a state of purity. Analysis of these indicated acceptable stoichiometry between the molecular entities present (Table 3). The long-chain dimethyl-substituted dicarboxylic acid, diabolic acid, existed as an esterified component of nearly all of the phosphorus-containing lipids no free diabolic acid could be detected.

Apart from the lipids containing diabolic acid denoted in Table 3, analysis showed that this dicarboxylic acid was also present in lipid spots 4, 7, 9, 10, 13, 17, 18, 25, 26, 27, 28 and 29 (Fig. 1). Thus it can be calculated from the results presented in Table 2 that a minimum of 77% of the radioactivity from [1-^{14}C]palmitic acid is incorporated into lipids containing diabolic acid. Only two minor non-phosphorus containing glycolipids (spots 4 and 7) contain diabolic acid. Although other types of high-molecular-weight dicarboxylic acids are rarely found in bacteria, certain strains of mycobacteria contain dicarboxylic acids of the mycolic acid series. However, such mycolic acids exist in nature either in the unesterified form or esterified simply to sugars or in a triacylglycerol-type structure (Asselineau, 1966; Alshamaony *et al.*, 1976).

Presumably, therefore, diabolic acid must exist as an intrinsic part of the phospholipids of the plasma membrane of *Butyrivibrio* S2 and cannot be regarded in any way as a component part of a fatty coat used to protect the organism in a hostile environment. The question must arise as to whether such hydrophobic moieties containing a continuous chain of 28 carbon atoms with polar moieties either end-span the plasma membrane of the organism or whether the chain is bent so that both polar regions of the molecule are oriented to either the intracellular or extracellular environment.

The lipid occurring in highest concentration is a phosphoglycolipid (spot 14), which is unusual not only because it contains esterified diabolic acid, but also because it possesses little esterified palmitic acid (Table 3). There are approximately two long alkenyl groups per phosphorus atom. The lipid contains esterified butyric acid groups, as do many of the other complex lipids investigated (Table 3). Such volatile fatty acid groupings seem to be characteristic of the *Butyrivibrio* species in general (Clarke *et al.*, 1976), and this may be related to the ease with which the genus can form short-chain fatty acids, especially butyric acid, by the fermentation of hexose. Analysis of the hexose present in this main phosphoglycolipid by methods previously described (Clarke *et al.*, 1976) indicate that this is entirely galactose and this is true of all the other glycolipids of the organism so far investigated.

Table 2. *Distribution of radioactivity and reactions of the complex lipids of Butyrivibrio S2 grown with [1-^{14}C]palmitate*

Lipid spot no. (see Fig. 1)	Radioactivity in lipid extract (%)	Reactions on t.l.c. plate			
		P	NH_2	Glycolipids	Aldehyde
1	0.2	–	–	–	–
2	1.4	–	–	–	–
3	1.4	–	–	–	–
4	2.0	–	–	+	+
5	7.1	–	–	+	+
6	0.2	–	–	–	–
7	1.0	–	–	+	+
8	0.4	–	–	+	+
9	0.5	–	–	+	+
10	0.8	+	–	+	+
11	2.7	+	–	+	+
12	1.6	–	–	+	+
13	2.6	+	–	+	+
14	21.9	+	–	+	+
15	3.0	+	–	–	+
16	1.1	+	–	–	+
17	0.9	+	–	+	+
18	0.8	–	–	+	+
19	5.1	+	–	+	+
20	5.6	+	–	–	+
21	1.2	+	–	+	+
22	12.0	+	–	–	+
23	(Fatty acid)	–	–	–	–
24 (area)	5.6	+	–	+	+
25	2.2	+	–	–	+
26	2.9	+	–	–	+
27 (area)	8.8	+ (all)	+ (some)	+	+
28	5.2	+ (all)	+ (some)	+	+
29	1.9	+ (all)	+ (some)	+	+

Table 3. *Analysis of the main complex lipids present in Butyrivibrio S2*

The results are expressed as the nearest-whole-number molar ratios when compared with the [^{14}C]palmitic acid chain (mol) incorporated into the lipid structure.

Lipid spot no. (see Fig. 1)	Palmitic acid chains incorporated	Diabolic acid (two palmitic acid chains)	Palmitoyl groups	Long-chain aldehyogenic groups	P	Hexose (galactose)	Glycerol	Butyroyl groups
14	4.0	1.02	0.051	1.91 (1.96)	1.00	0.94	2.9	2.0
22	4.0	1.0	0.13	1.87 (2.0)	1.86	0.14	3.8	2.0
20	5.0	1.03	0.98	1.95 (2.93)	2.0	0.2	3.9	1.31
19	4.0	1.01	0.04	1.94 (1.98)	1.01	1.06	2.9	1.14
11	5.0	1.05	0.98	1.93 (2.91)	0.98	1.07	3.0	1.15
15	2.0	0	1.57	0.43 (2.0)	0.99	0	2.1	1.03
5	2.0	0	0.98	1.02 (2.0)	0	0.91	0.9	1.0
12	2.0	0	0.99	1.01 (2.0)	0	1.04	1.0	0

The main sugar-free phospholipid (spot 22) contains two phosphorus atoms and four glycerol molecules to every molecule of esterified diabolic acid. No traces could be found of any of the phospholipids usually found in the membranes of bacteria, such as phosphatidylethanolamine (and its *N*-methyl derivative), diphosphatidylglycerol and phosphatidylglycerol or its *O*-amino acid ester (Goldfine, 1972). The complete absence of diphosphatidylglycerol is consistent with the findings for other *Butyrivibrio* species, but that of phosphatidylglycerol is surprising, since it is generally assumed to be ubiquitous in the bacterial kingdom (Clarke *et al.*, 1976). Nevertheless the organism does contain a small amount (3%) of a substance (spot 15) analysed (Table 3) as a monobutyroyl ester of alkenylacylglycerophosphoglycerol. This phospholipid co-chromatographed with the phosphatidylglycerol monobutyroyl ester (plasmalogenic) previously isolated from *Butyrivibrio* LM8/1B (Clarke *et al.*, 1976) and on deacylation gave as expected a proportion of a lipid that co-chromatographed with alkenylglycerophosphoglycerol.

The main galactolipid (spot 5) analyses as a monobutyroylester of galactosyldiacylglycerol (plasmalogenic) (Table 3); again it showed the same properties as the equivalent substance that we have previously isolated from *Butyrivibrio* LM8/1B with the galactose being in the furanose form. The parent compound, galactofuranosyldiacylglycerol (plasmalogenic) was represented by the minor spot (12), the analysis for which is included in Table 3. Galactofuranosyldiacylglycerol has previously been described in *Bifidobacterium bifidum* (Veerkamp, 1972), in *Mycoplasma mycoides* (Plackett, 1967) and in other *Butyrivibrio* species (Clarke *et al.*, 1976).

Little further investigation of the non-phosphorus-containing lipids was carried out. The trace component (spot 6) was identified as cetyl alcohol from its chromatographic properties (t.l.c. and g.l.c.) and spot 3 appeared to be a true diacylglycerol that contained no aldehydogenic groups. The non-polar spot (2) was not decomposed in the presence of methanolic HCl and migrated slightly faster than cetyl alcohol on t.l.c. in alkaline solvents; its identity was not established.

The complex lipids of the general-fatty-acid auxotroph *Butyrivibrio* S2 grown in the presence of palmitic acid are certainly totally different from those of bacteria in general, and in many respects atypical of the genus itself. Perhaps such a composition may be related to the fact that the organism contains powerful phospholipases both of the A and C varieties and glycolipases (Hazlewood & Dawson, 1979). In addition, the preponderance of diabolic acid-containing lipids and esterified butyroyl groups could well be necessary to maintain membrane bilayer fluidity in the almost total absence of hydrocarbon chain unsaturation (Hauser *et al.*, 1979). The structure of these diabolic acid-containing phospholipids are considered in the following paper (Clarke *et al.*, 1980).

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