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Lipid shape as a determinant of lipid composition in *Clostridium butyricum*. The effects of incorporation of various fatty acids on the ratios of the major ether lipids

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The lipid composition of *Clostridium butyricum* is strongly influenced by the aliphatic chain compositions of the membrane lipids. Growth on *cis*-monounsaturated fatty acids in the absence of biotin was shown to affect the relative proportions of phosphatidylethanolamine, plasmenylethanolamine, and the glycerol acetal of plasmenylethanolamine most strongly, with smaller effects on the acidic lipids, phosphatidylglycerol and cardiolipin. The ratio of the glycerol acetal of plasmenylethanolamine to total phosphatidylethanolamine in cells grown on a series of fatty acids is shown to decrease in the following order; *cis*-vaccenic acid ≥ oleic acid = C_{19}-cyclopropane fatty acid > linoleic acid > petroselinic acid > elaidic acid > 14-methylhexadecanoic acid (anteiso-C_{17}) > 12-methyltridecanoic acid (iso-C_{14}). All fatty acids were extensively incorporated into the lipid acyl, alkenyl, and alkyl chains. There was considerable chain-elongation of the iso-C_{14} to iso-C_{16}. The results are consistent with the hypothesis that the membrane lipid composition is strongly influenced by lipid shape and that the observed changes in lipid composition serve to stabilize the bilayer arrangement of the cell membrane.

Introduction

The control of the lipid composition of biological membranes is poorly understood at the present time. It is undoubtedly significant that most membranes contain mixtures of amphipathic lipids of varying molecular shape and charge [1], and that several of the most common lipids of biological membranes do not form bilayers when hydrated at physiological temperatures. Among these are unsaturated phosphatidylethanolamine, its plasmalogen (plasmenylethanolamine), and monoglucosyl- and monogalactosyldiacylglycerol (for general references see Refs. 1–3). Our studies have shown marked variation in the lipid class composition of *Clostridium butyricum*, which is dependent on the aliphatic chain compositions of the membrane lipids [4–6]. The major phospholipids in these cells are phosphatidylethanolamine, largely present in its plasmalogen form, a glycerol acetal of this plasmalogen, phosphatidylglycerol,

Abbreviations PE, phosphatidylethanolamine, PlaE, plasmenylethanolamine, MGDG, monoglucosyldiacylglycerol, DGDG, diglucosyldiacylglycerol, PC, phosphatidylcholine, DPPC, dipalmitoylphosphatidylcholine, DHPC, diheptadecanoylphosphatidylcholine, DAHPC, dianteisoheptadecanoylphosphatidylcholine, DIHPC, diisoheptadecanoylphosphatidylcholine, DOPC, dioleoylphosphatidylcholine

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and cardiolipin. The proportion of the latter two lipids in the plasmalogen form varies somewhat, but is usually less than forty percent [4–6].

We have recently shown that the ratio of the glycerol acetal of plasmenylethanolamine to phosphatidylethanolamine plus plasmenylethanolamine (which we shall refer to as total PE) varies with the content of unsaturated plus cyclopropane hydrocarbon chains in the cellular lipids. As the degree of unsaturation increases, the relative amount of the glycerol acetal of plasmenylethanolamine increases, with a concomitant decline in total PE. When the membranes are highly enriched in unsaturated plus cyclopropane chains, the ratio of the glycerol acetal lipid to total PE was approx. 2.0, as compared to a ratio of 0.7 when the lipids contained roughly equal proportions of saturated and unsaturated chains [6]. ^{31}P-NMR and X-ray diffraction studies of the phase behavior of the total PE and the glycerol acetal lipid from cells grown on oleic acid have shown that the total PE highly enriched with 18.1 and C_{19} cyclopropane chains was incapable of forming a lamellar phase at temperatures above 2°C. In contrast, the glycerol acetal of plasmenylethanolamine with over 92% unsaturated chains formed a lamellar phase at temperatures up to 50°C. This unusual lipid was capable of stabilizing the lamellar phase when mixed with egg phosphatidylethanolamine, dioleoylphosphatidylethanolamine and oleate- and C_{19}-cyclopropane-enriched total PE from *C butyricum* [7].

These findings indicate that *C. butyricum* is capable of regulating its membrane polar lipid composition in a manner which promotes bilayer stability. As membranes are enriched with *cis*-unsaturated aliphathic chains, the tendency of PE and PlaE to form non-lamellar phases is increased and this is counterbalanced by the conversion of PlaE to its glycerol acetal [8]. Since the tendency to form non-lamellar phases is largely determined by the packing properties of the lipid molecules, which in part derive from the lengths and volumes occupied by the hydrocarbon chains [9,10], we have postulated that parameters, which we refer to as lipid shape, largely determine lipid composition in this organism. In order to test this hypothesis further, we have grown *C butyricum* under conditions of fatty acid auxotrophy on monounsaturated, diunsaturated, cyclopropane, iso- and anteiso-branched chain fatty acids and have studied the resulting lipid class composition. Our findings presented here are consistent with this hypothesis.

Experimental procedures

Cells and culture conditions *C. butyricum* ATCC 19398 was grown in 500 to 4000 ml cultures, anaerobically, with Casamino acids and fatty acids in the absence of biotin as described [6,11]. After overnight growth, the cells were harvested by centrifugation at 3500 to 5000 × *g* for 10 min and washed by resuspension in potassium phosphate buffer, 0.05 M, pH 7.2.

Lipid extraction and analysis Cells were extracted with chloroform/methanol (2.1, v/v), and the washed lipid extracts were separated into neutral and phospholipid fractions by silicic acid column chromatography as described [12] Phospholipid analyses were done in triplicate by thin layer chromatography on silica gel 60 plates (E. Merck, Darmstadt, F.R.G.), in the solvent chloroform/methanol/7 M ammonia (60 : 35 : 5, v/v). The lipids were located by brief exposure to iodine vapor, which was then allowed to evaporate before scraping the individual lipid spots into acid-cleaned test tubes for digestion and phosphate analysis as described [12]. Blank areas were scraped from adjacent areas and digested in parallel, and the experimental values were corrected for the blank values. These lipids have been characterized [4,5,7], and standards were run alongside the lipids extracted from these cells. The acyl, alkenyl, and alkyl chain compositions of the total phospholipids from each culture were analyzed in duplicate as described [12]. Standards were available for most of the fatty acid methyl esters, for comparison on gas chromatography with compounds isolated from cellular phospholipids. For the branched fatty acids, formed by chain elongation of those fed to the cells, identification was based on plots of the log retention time vs. chain length on a polar phase, diethyleneglycol succinate, and a non-polar phase, SE-30, of known branched fatty acids of the same structure, i e. iso or anteiso. In the analyses of aldehydes, for which only 16:0 and 16:1 standards were available, plots of the al-

dehyde retention times (R.T.) vs. chain length were parallel to those obtained with the fatty acid methyl esters, thus providing a constant factor (R.T. aldehyde/R.T. fatty acid methyl ester = 0.85) on diethyleneglycol succinate at 165 °C.

cis-Unsaturated fatty acids were obtained from either Sigma Chemical Co. (St. Louis, MO) or Nu-chek Prep (Elysian, MN). Branched-chain fatty acids were obtained from Analytical Standards (Kungsbacka, Sweden). The branched chain fatty acid methyl ester standard mixture (BC-1) was obtained from Applied Sciences Laboratories (State College, PA). The C_{19}-cyclopropane fatty acid, *cis*-9,10-methylene octadecanoic acid, was generously provided by Dr. R.N. McElhaney.

Results

Lipid class compositions

The lipids that were found to undergo the greatest changes in concentration as a function of lipid aliphatic chain composition in *C. butyricum* were total PE and the glycerol acetal of PlaE (GAPlaE). Over 80% of these lipids were found in the outer monolayer of the cell membrane in cells grown in the absence of biotin with mixtures of palmitic and oleic acids or on oleic acid alone [6]. In extending this work to a variety of single fatty acids, we observed even larger fluctuations in the GAPlaE/total PE ratio (Table I). These two lipids represented from 52 to 76% of total extractable lipid phosphorus in all groups of cells except those grown on petroselinic acid (*cis*-6-18:1) or *i*-C_{17}, in which they represented 46 and 43% of lipid phosphorus, respectively.

The highest ratios of GAPlaE/total PE were found in cells grown on oleic, *cis*-vaccenic, and dihydrosterculic (C_{19}-cyclopropane) acids. A slightly lower ratio was observed in cells grown on linoleic or petroselinic acids. The lowest ratios were observed in cells grown on elaidic acid (*trans*-9-18:1), which was somewhat higher than those found in cells grown on the saturated, branched-chain fatty acids, *a*-C_{17} and *i*-C_{14}.

Incorporation of exogenous fatty acids

Previous work with *C. butyricum* grown without biotin has shown that exogenous fatty acids are extensively incorporated into the membrane phospholipids. Over 90% of the acyl and 99% of the alkenyl plus alkyl chains were 18:1 in cells grown on elaidic acid. The absence of cyclopropane ring formation and the high gel to liquid crystalline phase transition temperature of the PE indicated that the chains were almost entirely *trans*-18:1 [4]. Similarly in cells grown on oleic acid, the major lipid aliphatic chains were 18:1 and C_{19}-cyclopropane [4,7]. The principal phospholipid aliphatic chains in cells grown on unsaturated and cyclopropane fatty acids in the present experiments are given in Table II. In all cultures, 84% or more of the acyl and ether-linked chains were apparently derived from the fed fatty acids. In cells grown on dihydrosterculic acid, 7.7% of the acyl chains and 8.4% of the ether-linked chains were 18:1, probably derived from the inoculum used in these experiments. Thus 18:1 plus C_{19}-cyclopropane represented over 93% of the aliphatic chains in the phospholipids in these cells.

The aliphatic chain compositions of cells grown on branched fatty acids are presented in Table III. In those grown on the iso-C_{14} and anteiso-C_{17} fatty acids, the principal acyl chains are either the fed fatty acid (*a*-C_{17}) or a combination of the fed

TABLE I

RATIO OF THE GLYCEROL ACETAL OF PLASMENYLETHANOLAMINE TO TOTAL PHOSPHATIDYLETHANOLAMINE IN *CLOSTRIDIUM BUTYRICUM* GROWN ON VARIOUS LONG-CHAIN FATTY ACIDS IN THE ABSENCE OF BIOTIN

The cells were grown at 37 °C in the absence of biotin in media containing 20 mg/l of a fatty acid *i*, iso, *a*, anteiso, C_n, number of carbon atoms. The results are given as the molar ratio of the glycerol acetal of plasmenylethanolamine to total phosphatidylethanolamine (diacyl PE + plasmenylethanolamine) ± S E The number of independent experiments is given in parenthesis

Fatty acid supplement	GAPlaE/PE
C_{19}-cyclopropane (2)	2.88 ± 0 86
Oleic (2)	2.93 ± 0 18
cis-Vaccenic (*cis*-11-18:1) (2)	3 1 ±0.1
Linoleic (2)	2 2 ±0.2
Petroselinic (*cis*-6-18.1) (3)	1 77 ± 0 17
Elaidic (*trans*-9-18 1)	0 82 ± 0 28 [a]
a-C_{17} (2)	0 64 ± 0 03
i-C_{14} (2)	0 44 ± 0 04

[a] Unpublished data from Ref 4

TABLE II

PRINCIPAL ACYL, ALKENYL, AND ALKYL CHAIN COMPOSITIONS OF THE TOTAL PHOSPHOLIPIDS OF *C BUTYRICUM* GROWN ON STRAIGHT-CHAIN FATTY ACIDS [a]

Cells were grown at 37 °C in the absence of biotin in the presence of 20 mg/l of fatty acid The alkenyl chains derived from the plasmalogens and the alkyl chains derived from the glycerol acetal of plasmenylethanolamine were analyzed together as aldehydes released on acetic acid hydrolysis. Abbreviations number before colon, the number of carbon atoms, number after the colon, the number of double bonds, cyc, cyclopropane Data are the means from at least two independent cultures and are given as mol% of total acyl or alkenyl plus alkylchains

Fatty acid supplement	Acyl chains [b]				Alkenyl plus alkyl chains [b]		
	14 0	18 1	19-cyc	18·2	18 1	19-cyc	18 2
C_{19}-cyc		7 7	85		8 4	85	
cis-Vaccenic		80	15		63	30	
Linoleic				93			96
Petroselinic	8 9	84			97		
Elaidic	5 4	90			99		

[a] Data for oleic acid-supplemented cells are in Refs 6 and 7 The acyl and ether-linked chain compositions were not analyzed for the present experiments

[b] For the major (≥ 10 mol%) acyl and ether-linked chains, the interculture variability ranged from 1 to 5% of the mean except for cells grown on *cis*-vaccenic acid in which there was variable conversion of 18 1 to 19-cyc However, the sum of 18 1 plus 19-cyc acyl or ether-linked chains was consistent (±4%)

fatty acid and an elongation product in the iso-C_{14} grown cells The ether-linked chains in cells grown on anteiso-C_{17} and in one culture (A) grown on iso-C_{14} were also highly enriched in branched compounds In another culture (B) grown on iso-C_{14}, 57% of the ether-linked chains were 18·1 or 19:cyc, presumably derived from the inoculum It is interesting to note that the ratio of GAPlaE/PE in the two iso-C_{14} cultures were similar, 0.48 and 0.40, respectively. Although this result may seem anomalous, it should be noted that in these, as in most of the cultures examined, only about 50% of the phospholipids were either plasmalogens or the glycerol acetal of plasmenylethanolamine (data not shown). Thus, there are three acyl chains for every ether-linked chain in the phospholipids of these cells, and it is presumably the effects of the acyl chains, that predominate.

TABLE III

ACYL, ALKENYL AND ALKYL CHAIN COMPOSITION OF THE PHOSPHOLIPIDS OF *C BUTYRICUM* GROWN ON BRANCHED-CHAIN FATTY ACIDS

The cells were grown as described in Table I *i*, iso, *a*, anteiso, cyc, cyclepropane, C_n number of carbon atoms Data are given as mol%

Fatty acid supplement	Aliphatic chain composition						
	14 0	*i*-C_{14}	16 0	*i*-C_{16}	*a*-C_{17}	18.1	19-cyc
Acyl chains							
i-C_{14} A		71 5		25 5		2 2	
B		68 1		23 1		7 0	
a-C_{17}	4 5		2 5		86 5 [d]	5 4	
Alkenyl + alkyl chains							
i-C_{14} A	2 8	3 5		75 8		17 0	
B				38 7		39 8	17.4
a-C_{17}			2 1		83 3 [d]	8.6	4 5

[d] The interculture variability of the *a*-C_{17} content was ±11% for the acyl chains and ±8% for the alkenyl plus alkyl chains

Discussion

The lipid class composition of prokaryotic cell membranes has been observed to undergo considerable variation as a function of pH, growth temperature, phosphorus or magnesium limitation or the stage of growth of batch cultures [13,14]. The physical significance and the physiological mechanisms underlying these variations are generally poorly understood. Fatty acid auxotrophy has also been found to result in significant alterations in lipid class composition in three groups of organisms [8]. The best studied of these is *Acholeplasma laidlawii* strain A, in which marked variation in lipid composition was observed to occur as a function of the membrane lipid saturated/unsaturated acyl chain composition As the degree of unsaturation was increased by manipulating the composition of exogenous fatty acids, a marked increase in the ratio of diglucosyldiacylglycerol (DGDG) to monoglucosyldiacylglycerol (MGDG) was observed. The addition of cholesterol to cells grown on oleate resulted in an even higher DGDG/MGDG ratio [15,16]. These changes have been postulated to occur as a result of changes in the shapes of the lipid molecules. As the lipids become more unsaturated they become more wedge-shaped so that they cannot pack closely in a lamellar structure; this increases the tendency of MGDG to form non-lamellar phases. The changes in lipid class composition have been postulated to serve to stabilize the membrane bilayer organization.

In the butyric acid-producing clostridia, *Clostridium beijerinckii* [11,17] and *C. butyricum* [6], lipid class composition was also observed to vary with lipid aliphatic chain composition. In these clostridia, as in *A. laidlawii*, when the degree of unsaturation of the membrane lipids was increased, unsaturated lipids that do not form a lamellar phase upon hydration at physiological temperatures, PE and PlaE, are replaced by a lipid that stabilizes the lamellar phase, the glycerol acetal of plasmenylethanolamine (GAPlaE), [7,18]. In previous work with these clostridia, we studied the effects of substituting *cis* or *trans* mono-unsaturated fatty acids for endogenously synthesized mixtures of saturated, monounsaturated and cyclopropane aliphatic chains on the phospholipid composition [6,11,17]. In the present work we have extended these studies to poly-unsaturated, cyclopropane and saturated iso- and anteiso-branched fatty acids. We have shown that *C. butyricum* can grow on fatty acids of diverse structures in the absence of biotin. In cells grown on linoleic acid the predominant acyl and ether-linked chains were 18:2, resulting in highly unsaturated membranes, whereas cells grown on iso-C_{14} and anteiso-C_{17} had low levels of 18:1 and 19:cyc in the acyl chains and variable levels of unsaturation in the ether-linked chains ranging from 13 to 57% (Tables II and III). As noted above, there are approximately three times as many acyl as ether-linked chains in the phospholipids of cells grown on iso-C_{14} and anteiso-C_{17} as a result of the presence of approximately 50% acylphospholipids. Thus the acyl chains, which are largely saturated and branched, predominate in these cells.

The lipid class compositions of cells grown on exogenous fatty acids can be compared to that found in cells grown with biotin, which synthesize mixtures of saturated, mono-unsaturated and cyclopropane chains, and cells grown on mixtures of palmitic and oleic acids in the absence of biotin in which the ratio of fed palmitic to oleic is greater than 40 : 60 w/w [5,6]. In these cells the ratio of GAPlaE to total PE was in the range 0.60 to 0.86 In cells grown on *cis*-mono-unsaturated, cyclopropane, and *cis*-diunsaturated fatty acids the ratio was markedly higher, ranging from 1.8 to 3.1 (Table I). Essentially identical ratios were found in cells grown on 19:cyc, *cis*-11-18:1 (*cis*-vaccenic cid) and *cis*-9-18:1 (oleic acid). A somewhat lower ratio was found in cells grown on *cis*-6-18:1 (petroselinic acid). However, these cells contained greatly increased levels of a normally minor phospholipid, which is structurally related to the plasmalogen glycerol acetal. The identification of this compound will be reported separately. Only in cells grown on iso-C_{14} was the ratio of GAPlaE to PE lower than that observed in biotin-grown cells. The ratio in cells grown on anteiso-C_{17} was similar to the lowest values observed in biotin-grown cells (Table I).

We postulate that the changes observed are related to the tendency of PE to undergo lamellar to non-lamellar phase transitions, which is a function of the shapes of these lipid molecules [1,10].

DioleoylPE undergoes this transition at 5 to 10 °C (T_{BH}) [19], dilinoleoylPE at −15 to −25 °C, and dielaidoylPE at 60 to 63 °C [1]. For a semisynthetic PE with oleic *sn*-2 acyl chains and 80% saturated *sn*-1 acyl chains T_{BH} was 68 °C, whereas T_{BH} for PlaE of similar chain composition was 30 °C [3]. In contrast to the phase behavior of dioleoylPE, and oleate-enriched PE/PlaE (approx. 50:50) from *C butyricum*, which underwent a lamellar to reversed hexagonal phase transition near 0 °C, GAPlaE highly enriched with oleate chains formed a lamellar phase in excess water between 16 and 50 °C [7].

Hydrated diisopalmitoylPE appears to be stable in the lamellar phase up to at least 98 °C [20]. non-lamellar phases of PE with pure anteiso branched chains have not been studied; however, consideration of this lipid isolated from *Bacillus megaterium* is instructive. When isolated from strain Ft R32, a facultatively thermophilic strain, grown at 20 °C, PE had 88.5% branched chains and the iso/anteiso ratio was 0.30. A lamellar liquid crystalline phase was formed at temperatures up to 50 °C with 3 mol H_2O/mol lipid and a pure cubic phase was observed at 58 °C at this water content. PE isolated from strain Ot 32, an obligate thermophile grown at 55 °C had 93% branched chains, iso/anteiso = 3.2. This lipid formed a lamellar phase at least up to 65 °C both below and above the maximum hydration capacity of the lipid [21]. Thus, whether enriched with iso or anteiso chains, saturated branched PE tends to form relatively stable lamellar phases, but the lipid containing the less bulky iso chains forms a more stable lamellar phase.

Since the aggregation properties of amphipathic lipids are related to the hydrocarbon chain lengths and volumes [1,10], we can expect PE or GAPlaE enriched with *cis*-vaccenic acid or 19:cyc to display similar phase behavior to the corresponding oleate-substituted compounds, and, as noted above, cells grown on these fatty acids have similar GAPlaE to PE ratios. The gel to liquid crystal phase transition temperature of di-*cis*-6-18:1 PC is higher than that of DOPC [22]. We can therefore assume that the bilayer packing of the former lipid is closer, resulting in increased van der Waals interactions Assuming that this effect also occurs in a PE bilayer, then it may be expected that T_{BH} for the di-*cis*-6-18:1 PE is higher than for DOPE. Consistent with this concept and the need to stabilize the lamellar phase, cells grown on petroselinic acid exhibit a lower GAPlaE to total PE ratio compared to cells grown on oleic acid (Table I). However, interpretation of this result is complicated by the presence of approximately 15 mol% of a novel acetal lipid of unknown phase behavior. In view of the low T_{BH} of dilinoleoylPE, the finding that the GAPlaE to total PE ratio was lower than that of cells grown on oleate or *cis*-vaccenate is not consistent with these arguments. The reason for this discrepancy is not apparent, emphasizing the need to study the amount of dilinoleoyl GAPlaE required to stabilize the lamellar phase when mixed with dilinoleoylPE.

The results obtained with the two branched fatty acids fully conform to the concept that the GAPlaE/PE ratio is related to the tendency of the PE to form non-lamellar phases. As observed by Rilfors et al [21], PE enriched with branched fatty acids of similar chain length to those we have used forms relatively stable lamellar phases, and the fatty acids can be expected to occupy volumes intermediate between those of saturated and unsaturated fatty acids. This was found to be the case for monolayers of di-iso-C_{17} (DIHPC) and di-anteiso-C_{17} (DAHPC) phosphatidylcholines spread at a surface pressure of 25 dyn/cm. At temperatures 10 K above their estimated or measured gel to liquid crystal phase transition, the molecular areas of the two branched-chain PCs were similar to that of dipalmitoylPC (DPPC). However, in the condensed phase, 10 K below T_c, DPPC occupied a smaller surface area (51 Å^2), than DIHPC (54 Å^2) and DAHPC (approx. 57 Å^2) [23]. DOPC molecules at similar surface pressures occupy 70 Å^2 [24]. These molecular area data indicate that branched chain PE molecules can adopt a close-packed lateral packing density which stabilizes the lamellar phase as opposed to the reverse hexagonal phase.

Our results with *C butyricum* can be compared to those obtained with *A. laidlawii* grown on unsaturated and branched fatty acids. The GAPlaE to total PE ratios in *C butyricum* and the DGDG to MGDG ratios in *A laidlawii* strain A [25] and strain B [26] are in the order *cis*-unsaturated > anteiso branched > iso branched. Although the

order is the same, the ratios for *M. laidlawii* strain A grown on the anteiso-C_{15} and anteiso-C_{17} were closer to that found in cells grown on oleate [25] than the ratios found in strain B or the GAPlaE/PE ratio in *C. butyricum* grown on anteiso-C_{17}. The difference between the two strains of *A. laidlawii* and between strain A and *C. butyricum* may reside in the ensemble of other lipids and the proteins interacting with the lipids most affected by aliphatic chain composition.

We conclude that there is a strong influence of lipid shape on the ratio of the two major lipids associated with each other in the outer monolayer of the cell membrane of *C. butyricum*. These changes probably serve to stabilize the bilayer arrangement of the cell membrane, by assuring optimal packing of the membrane lipids, possibly with a relatively constant and optimal intrinsic radius of curvature [19]. The regulatory mechanisms involved in these phenomena have not been explored and are clearly of considerable interest in understanding the assembly and dynamics of lipids in biological membranes

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