Changes in Membrane Lipid Composition of *Clostridium acetobutylicum* during Acetone–Butanol Fermentation: Effects of Solvents, Growth Temperature and pH

By C. LEPAGE, F. FAYOLLE, M. HERMANN AND
J.-P. VANDECASTEELE*

*Direction de Recherche Biotechnologie et Environnement, Institut Français du Pétrole,
1 et 4, avenue de Bois-Préau, 92506 Rueil-Malmaison Cédex, France*

(Received 11 March 1986; revised 24 July 1986)

Changes in membrane lipid composition of *Clostridium acetobutylicum* were studied during acetone–butanol fermentation. Large changes were found in phospholipid composition and in fatty acid composition, the latter characterized mainly by a decrease in the unsaturated/saturated fatty acid (U/S) ratio. The effects of the addition of alcohols (ethanol, butanol, hexanol and octanol) and of acetone were also studied. In all cases, large changes were observed in the U/S ratio but with differences which were related to the chain lengths of the alcohols. The effect of solvents appears to account for a large part of the changes in lipid composition observed during the fermentation. The pH was also important, a decrease in pH resulting in a decrease in the U/S ratio and in an increase in cyclopropane fatty acids. The effect of increasing temperature was mainly to increase fatty acid chain lengths.

INTRODUCTION

It is generally accepted that the limitation at about 16–20 g l⁻¹ in total solvent concentration observed in acetone–butanol fermentation is due to the toxicity of the butanol produced (Moreira *et al.*, 1981). This end product can affect a variety of target sites in cells leading to growth inhibition. Although the exact mechanism by which alcohols inhibit bacterial growth is not known, they have been shown to affect the membrane physiology of many micro-organisms by partitioning in lipid bilayers and then interfering with lipid–lipid and lipid–protein interactions (Jain & Wu, 1977; Jain *et al.*, 1978). These effects are largely interpreted in terms of membrane viscosity which is decreased by the presence of alcohols (Ingram, 1976; Vollherbst-Schneck *et al.*, 1984).

In the present work we studied the variations in lipid composition of membranes of *Clostridium acetobutylicum* during acetone–butanol fermentation, with the aim of correlating the variation observed with the effects of the solvents produced. We thus examined the effects on membrane fatty acid composition of acetone and the alcohols ethanol, hexanol, octanol and butanol. The effect of pH, an important parameter in acetone–butanol fermentation, was also studied. Because of the well documented effect of growth temperature on membrane lipid composition (Khuller & Goldfine, 1974; Russell, 1984) we also examined the response of *C. acetobutylicum* to different temperatures.

METHODS

Microbiological methods. *C. acetobutylicum* (IFP 903; ATCC 39057) was grown under strict anaerobiosis, using an anaerobic glove box (Lacalhe, France) and a gassing manifold as described by Balch *et al.* (1979). The culture

Abbreviations: CL, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; N-PE, *N*-monomethylphosphatidylethanolamine; PX, unidentified phospholipid; U/S ratio, unsaturated/saturated fatty acid ratio.

medium contained (l^{-1}): 30 or 60 g glucose; 4 g yeast extract (Difco); 2.5 g CH_3COOH ; 0.5 g KH_2PO_4 ; 0.1 g $MgSO_4 \cdot 7H_2O$; 10 mg $FeSO_4 \cdot 7H_2O$; 10 mg $MnSO_4 \cdot H_2O$. The pH was adjusted to 6.0 by addition of ammonia.

Minimum inhibitory concentration (MIC), defined as the highest concentration of a given solvent allowing measurable growth, was determined in 18×150 mm tubes containing 5 ml of medium with 30 g glucose l^{-1} . The tubes were inoculated with 5% (v/v) of a 16 h culture grown on the same medium and incubated at 35 °C under static conditions. Growth was estimated by measurement of OD_{600} over a period of 24 h.

Lipid analyses were made on bacteria grown in fermenters. Two successive preculture steps before inoculation of the fermenters were used. A flask containing 10 ml of medium with 30 g glucose l^{-1} was incubated at 35 °C overnight, and used to inoculate a flask containing 200 ml of the same medium. This flask was used, after about 6 h incubation at 35 °C, to inoculate a fermenter (Biolafitte) containing 4 l (final vol.) of medium containing 60 g glucose l^{-1} . The fermenter contents were incubated at 35 °C with slow stirring; the pH was regulated at 6.0 by addition of 6 M-ammonia. Gas production was measured continuously using a gas counter (Flicon) equipped with a recording device. Samples were taken for measurements of growth (OD_{600}), glucose (enzymic analysis with the Beckman Analyser 2), solvents and acids, the latter being measured by gas chromatography (GC). A 3.2 mm $\times$ 2 m column containing 15% free fatty acid phase (FFAP) on 80/100 Chromosorb WAW, operated at 90 °C was used for solvents; for acids a 3.2 mm $\times$ 2 m column packed with 25% (w/w) neoglycopolyacid (NGPA) on 100/120 Chromosorb WAW, operated at 135 °C, was used. Four samples of cells were taken at various times for analysis of lipid composition. For the study of the effects of the presence of solvents and of the action of pH and temperature the same general fermentation conditions were used but the final fermentation volume was 1.8 l. Also, an additional transfer step was done: 200 ml portions of the culture growing in the fermenter were used after about 5 h (OD_{600} about 4) to inoculate other fermenters prepared as described above, each having the parameter in question (alcohol concentration, temperature or regulated pH) set at the required value. After 5 h of growth in these conditions, the bacteria were harvested for lipid analysis.

Lipid analysis. Cells were harvested by centrifugation (20 min, 48000 g, 0 °C). Pellets were washed twice with distilled water and stored at -20 °C. Lipids were extracted by a modification of the method of Bligh & Dyer (1959) using a single extraction operation with a solvent/cell paste ratio of 60:1 (v/w), as recommended by Nelson (1975). The lipids were purified from non-lipid contaminants by extraction with 0.74% KCl as described by Folch *et al.* (1957). The proportions of methanol, chloroform and water in the total mixture at the moment of phase partition were respectively 2, 2 and 0.95 (by vol.). Lipid extracts were finally dissolved in N_2 -flushed chloroform/methanol (2:1, v/v) mixtures to 1% (w/v) final concentrations. They were stored at -20 °C.

Thin-layer chromatography (TLC) on silica gel plates with a 4 cm concentration zone (Merck 13794) was used to separate the different lipid classes. Phospholipids were first separated from neutral lipids by migration in acetone (Gentner *et al.*, 1981), and were separated further into phospholipid classes by migration in chloroform/methanol/acetic acid (13:5:2, by vol.) in the same dimension. The two-dimensional technique described by Herrero *et al.* (1982) was also used. The detection reagents used were iodine vapour and the spray reagents (Sigma) molybdenum blue, orcinol (Bial's reagent) and ninhydrin. Lipid phosphorus was determined by the method of Rouser *et al.* (1970). Plasmalogen analyses were made on the separated phospholipids by the method of Viswanathan *et al.* (1968).

Fatty acid methyl esters were prepared by transesterification of the extracts with 0.5 M-sodium methoxide (Alltech) by the method of Pelick & Mahadeyan (1975). Transesterification with 14% (w/v) BF_3 /methanol, as described by Morrison & Smith (1964), was used to prepare fatty acid methyl esters and dimethylacetals from plasmalogens. These two classes of constituents were then separated by TLC on silica gel G plates (Merck 11845) by two successive migration steps in toluene; the corresponding spots were scraped off and eluted for further analyses. The fatty acid and aldehyde composition of individual phospholipid classes was determined according to Nelson (1975).

The quantitative analysis of fatty acid methyl esters was done by GC using a Packard 438 apparatus with flame ionization detection. A 3 m $\times$ 3.2 mm column packed with 15% (w/w) diethylene glycol succinate on 80/100 Chromosorb WHP was used; it was operated at 170 °C with N_2 as carrier gas. Chromatograms were recorded and analysed with a Packard 604 integrator. For identification purposes, fatty acid esters and dimethylacetals were also analysed by GC coupled with mass spectrometry (MS) on a Kratos MS 80 with the same GC conditions.

RESULTS

Characterization of membrane lipids

Acetone-butanol fermentation usually represents the first stage of growth and acid production followed by the second stage where solvents are produced with partial re-utilization of the acids (Walton & Martin, 1979). This pattern is illustrated in Fig. 1 for cell samples taken at various stages of the fermentation. The pH was maintained at 6.0 by ammonia addition during

Fig. 1. Acetone-butanol fermentation by *C. acetobutylicum* 903. Culture conditions are indicated in Methods. ■, OD₆₀₀; ▼, glucose concn; △, gas production; ○, solvent concn; ●, acetic acid concn; ▲, butyric acid concn; □, pH. Vertical arrows indicate the times when samples of the culture were harvested for study of the lipid composition of the bacteria.

growth and acid production but increased to 6.3 during solvent production because of acid consumption.

Total lipids accounted for 1–1.5% (w/w) of the wet cell wt (5–6%, w/w, of the dry cell wt). Spectrophotometric examination (200–800 nm) did not reveal the presence of pigments in the lipid extracts dissolved in methanol.

After separation by TLC in one dimension, six lipid spots containing phosphorus, as revealed by the molybdenum blue reagent, were detected. Two other spots were revealed with general lipid reagents, but not by the glycolipid-specific orcinol reagent. No further identification was attempted on these two spots. With our two-dimensional technique of lipid separation, we could still separate only six spots corresponding to phospholipids.

The phospholipids were tentatively identified, by migration with their respective standards, as phosphatidic acid (traces), cardiolipin (CL), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), *N*-monomethylphosphatidylethanolamine (*N*-PE) and an unidentified phospholipid (PX) which, like PE and *N*-PE, was ninhydrin-positive. The results for the fractions PE and *N*-PE are given together, under the name PE, as the sum of the two fractions, as the two compounds were not always completely resolved. Analysis of the plasmalogen composition of the phospholipids showed that the PX fraction was only in plasmalogen form as it gave, after acidic hydrolysis, a single spot which migrated exactly like lyso-PE.

Fatty acids were analysed using the basic transesterification technique which is specific for esterified fatty acids. Ten fatty acids, 12:0, 14:0, 14:1, 16:0, 16:1, 17△ (*C*₁₇ cyclopropane), 18:0, 18:1, 18:2 and 19△ were identified both by comparison of retention times with those of standard samples in GC and by GC/MS. The alk-1-enyl chains of plasmalogens were identified after acidic transesterification and contained the same carbon chains as fatty acids, except for the absence of *C*_{12:0} and *C*_{18:2}.

The possible presence of hopanoids in the membrane lipids of *C. acetobutylicum* was also investigated in collaboration with Professor M. Rohmer (ENSCM, Mulhouse, France). Neither bacterial triterpenes such as diplotene, diplopterol and squalene nor bacteriohopanes were detected.

Lipid variation during acetone-butanol fermentation

The results obtained are summarized in Tables 1 and 2. Regarding phospholipids (Table 1), PE and PG decreased during the fermentation whereas CL and PX increased; the ratio of acidic to neutral phospholipids (CL + PG)/(PE + PX) increased slightly. Although ten different fatty acids were identified (Table 2), three of them, 16:0, 16:1 and 18:1, accounted for 90% of the total. Two cyclopropane fatty acids (17△ and 19△), which originate respectively from the 16:1 and 18:1 fatty acids, were also present. During solvent production, saturated and cyclopropane fatty acids increased, whereas unsaturated fatty acids decreased. These changes took place for the most part early in solvent production whereas phospholipid changes were more gradual.

Table 1. *Membrane phospholipid composition of C. acetobutylicum during acetone-butanol fermentation*The results are means of at least three experiments $\pm$ SD.

Solvents (g l ⁻¹)	Phospholipids*			
	CL	PG	PE	PX†
0	15 $\pm$ 0.7	29.1 $\pm$ 1.4	46.2 $\pm$ 1.8	9.5 $\pm$ 0.9
6.3 $\pm$ 0.3	26.5 $\pm$ 0.6	18.3 $\pm$ 0.7	40.8 $\pm$ 2	14.3 $\pm$ 1.1
9.6 $\pm$ 0.6	38.7 $\pm$ 1.5	11.1 $\pm$ 1	36 $\pm$ 1.3	14.3 $\pm$ 0.9
13.5 $\pm$ 0.5	39 $\pm$ 1.5	8.5 $\pm$ 1.5	34 $\pm$ 1.6	18.2 $\pm$ 2.1

* Expressed as a percentage of the total phosphorus of phospholipids.

† Unknown phospholipid (see text).

Table 2. *Membrane fatty acid composition of C. acetobutylicum during acetone-butanol fermentation*The results are means of at least three experiments $\pm$ SD.

Solvents (g l ⁻¹)	Fatty acids*				
	12:0	14:0	14:1	16:0	16:1
0	0.4 $\pm$ 0.05	3.5 $\pm$ 0.1	0.5 $\pm$ 0.08	45.2 $\pm$ 0.8	31.8 $\pm$ 0.7
6.3 $\pm$ 0.3	0.8 $\pm$ 0.1	4.6 $\pm$ 0.2	0.4 $\pm$ 0.05	48.8 $\pm$ 1	19.2 $\pm$ 0.6
9.6 $\pm$ 0.5	0.9 $\pm$ 0.05	5 $\pm$ 0.5	0.3 $\pm$ 0.05	49.8 $\pm$ 1.1	17.1 $\pm$ 0.8
13.5 $\pm$ 0.5	1.2 $\pm$ 0.2	5.2 $\pm$ 0.4	0.3 $\pm$ 0.1	49.5 $\pm$ 1	17.3 $\pm$ 0.8

Solvents (g l ⁻¹)	Fatty acids*					U/S†
	17 Δ	18:0	18:1	18:2	19 Δ	
0	2 $\pm$ 0.2	1 $\pm$ 0.1	13.5 $\pm$ 0.4	0.4 $\pm$ 0.05	1.1 $\pm$ 0.2	0.98
6.3 $\pm$ 0.3	10.4 $\pm$ 1.2	1.9 $\pm$ 0.3	6.7 $\pm$ 1.5	0.7 $\pm$ 0.3	6.1 $\pm$ 0.6	0.77
9.6 $\pm$ 0.5	10.2 $\pm$ 1.2	2.6 $\pm$ 0.4	6 $\pm$ 0.9	0.8 $\pm$ 0.1	6.9 $\pm$ 0.8	0.70
13.5 $\pm$ 0.5	10.3 $\pm$ 1	2.8 $\pm$ 0.5	5.7 $\pm$ 0.6	0.7 $\pm$ 0.05	6.8 $\pm$ 0.6	0.69

* Expressed as a percentage of the total fatty acids.

† Ratio of unsaturated/saturated fatty acids. Cyclopropane fatty acids (17 Δ and 19 Δ) are counted with the unsaturated fatty acids.*Effects of solvents on fatty acid composition*

These are summarized in Table 3, along with the corresponding measured MIC value. For alcohols, MIC values rapidly decreased as the chain lengths of alcohols increased. Regarding the effects on fatty acid composition, in the case of acetone there was a decrease in 16:1 and an increase in 16:0. Similar results were obtained with ethanol, except that 18:1 also increased. A decrease in 16:1 and an increase in 16:0 were also observed in the case of butanol but in addition, 18:1 decreased and 14:0 increased. With longer-chain alcohols a different pattern was observed as the shorter-chain fatty acids 12:0 and 14:0 increased whereas 16:0 remained constant. There was a large decrease in 16:1 and a slight increase in 17 Δ and 19 Δ . Thus for these alcohols, decreases in the unsaturated/saturated (U/S) ratio and a shortening of the fatty acid chain length were observed.

Effect of growth temperature on fatty acid composition

When the fermentation temperature was decreased from 38 to 25 °C, there was an increase in 16:1 and a decrease in 18:1 as well as of 17 Δ and 19 Δ (Table 4). The U/S ratio remained constant, the main effect being on fatty acid chain length which decreased when temperature decreased, as shown by the variations in the chain-length ratio.

Table 3. *Effects of some n-alcohols and acetone on the membrane fatty acid composition of C. acetobutylicum*The results are means of three experiments $\pm$ SD, except in the case of octanol, for which a single experiment was done.

Solvent concn (g l ⁻¹)	MIC of solvent (g l ⁻¹)	Fatty acids*										U/St
		12:0	14:0	14:1	16:0	16:1	17Δ	18:0	18:1	18:2	19Δ	
Ethanol												
0	40	0.4 ± 0.08	3.9 ± 0.1	0.5 ± 0.03	44 ± 0.5	32.7 ± 0.8	3.5 ± 1.1	1.2 ± 0.1	13.1 ± 0.4	0.2 ± 0.05	1 ± 0.1	1.03
10		0.5 ± 0.1	4.2 ± 0.1	0.4 ± 0.05	46.9 ± 0.6	28.9 ± 0.4	4.3 ± 0.3	1.1 ± 0.2	11 ± 0.2	0.5 ± 0.06	1.6 ± 0.2	0.88
20		0.3 ± 0.05	3.2 ± 0.2	0.3 ± 0.05	47.2 ± 0.8	25.7 ± 1.2	3.5 ± 0.2	1.1 ± 0.05	15.1 ± 0.9	1.5 ± 0.1	1.7 ± 0.2	0.92
Butanol												
0	10	0.5 ± 0.05	3.6 ± 0.3	0.5 ± 0.05	44.7 ± 0.7	32.6 ± 0.3	1.7 ± 0.6	1 ± 0.05	13.7 ± 0.6	0.4 ± 0.3	0.9 ± 0.1	0.99
4		0.6 ± 0.16	5.6 ± 0.3	0.5	45.1 ± 0.2	28.4 ± 0.2	4.5 ± 0.7	1.7 ± 0.2	11 ± 0.6	0.5 ± 0.1	1.5 ± 0.3	0.87
8		0.9 ± 0.1	5.6 ± 0.3	0.4 ± 0.06	47.2 ± 0.3	25 ± 0.6	4.4 ± 0.3	2.6 ± 0.3	11 ± 0.7	0.7 ± 0.2	2.2 ± 0.4	0.77
Hexanol												
0	1.4	0.5 ± 0.05	3.6 ± 0.05	0.5 ± 0.03	44.7 ± 0.4	32.1 ± 0.6	2.2 ± 0.2	1 ± 0.17	13.4 ± 0.4	0.4 ± 0.05	1.2 ± 0.2	0.99
0.5		1.2 ± 0.2	10 ± 0.6	0.4 ± 0.05	46.6 ± 0.2	23.8 ± 0.3	3.3 ± 0.4	2.3 ± 0.2	9.3 ± 0.4	0.6 ± 0.1	2.1 ± 0.2	0.65
1		1.2 ± 0.2	9.4 ± 0.2	0.2 ± 0.03	45.8 ± 0.3	18.2 ± 0.3	3.2 ± 0.3	6 ± 0.7	12.5 ± 0.7	0.6 ± 0.08	2.9 ± 0.1	0.60
Octanol												
0	0.4		3.9	0.4	43.9	31.9	3.8	1.2	13.2	0.3	1.2	1.03
0.2		1.25	6.75	0.4	50.1	24.1	1.6	1.9	8.6	0.5	1.7	0.66
Acetone												
0	40	0.4 ± 0.05	3.7 ± 0.3	0.5 ± 0.1	45.2 ± 1.2	31.9 ± 0.7	2.2 ± 0.4	0.9 ± 0.05	13.4 ± 0.5	0.4 ± 0.03	1 ± 0.1	0.98
10		0.3 ± 0.1	3.5 ± 0.4	0.4 ± 0.05	49.2 ± 0.8	27.3 ± 0.7	2.6 ± 0.6	1.5 ± 0.2	13.4 ± 0.6	0.3 ± 0.05	1.1 ± 0.2	0.82
20		0.4 ± 0.1	3.7 ± 0.3	0.2 ± 0.06	51.4 ± 0.9	23.9 ± 0.6	2.3 ± 0.6	2.4 ± 0.3	13.4 ± 0.4	0.7 ± 0.16	1.2 ± 0.2	0.72

*, † See Table 2.

Table 4. *Effect of growth temperature on the membrane fatty acid composition of C. acetobutylicum*The results are means of four experiments $\pm$ SD.

Temp- erature (°C)	Fatty acids*										U/S†	S/L‡
	12:0	14:0	14:1	16:0	16:1	17 Δ	18:0	18:1	18:2	19 Δ		
38	0.4 $\pm$ 0.2	3.5 $\pm$ 0.4	0.5 $\pm$ 0.1	43.7 $\pm$ 0.6	28.7 $\pm$ 2.0	2.7 $\pm$ 0.7	2.1 $\pm$ 0.4	16 $\pm$ 1.3	0.7 $\pm$ 0.1	1.7 $\pm$ 0.7	1.01	3.87
35	0.4 $\pm$ 0.15	4.1 $\pm$ 0.2	0.5 $\pm$ 0.1	45.6 $\pm$ 0.3	31.7 $\pm$ 1.3	3.3 $\pm$ 0.5	1 $\pm$ 0.05	11.6 $\pm$ 1.2	0.5 $\pm$ 0.1	1.1 $\pm$ 0.3	0.94	6.1
30	0.6 $\pm$ 0.2	3.5 $\pm$ 0.6	0.7 $\pm$ 0.2	44.9 $\pm$ 2.2	34.3 $\pm$ 2.1	2.4 $\pm$ 0.8	0.7 $\pm$ 0.16	11.2 $\pm$ 1.5	0.6 $\pm$ 0.15	0.6 $\pm$ 0.4	1	6.5
25	1.2 $\pm$ 0.5	3.3 $\pm$ 0.2	0.9 $\pm$ 0.2	43.6 $\pm$ 1.5	36.8 $\pm$ 1.3	2 $\pm$ 0.2	0.8 $\pm$ 0.15	10 $\pm$ 0.6	0.6 $\pm$ 0.1	0.3 $\pm$ 0.1	1.03	7.4

*, † See Table 2.

‡ Ratio of short-chain ($< C_{18}$) to long-chain ($\geq C_{18}$) fatty acids.Table 5. *Effect of pH on the membrane fatty acid composition of C. acetobutylicum*The results are means of three experiments $\pm$ SD.

pH	Fatty acids*										U/S†
	12:0	14:0	14:1	16:0	16:1	17 Δ	18:0	18:1	18:2	19 Δ	
6	0.4 $\pm$ 0.05	3.6 $\pm$ 0.1	0.5 $\pm$ 0.1	44.4 $\pm$ 0.5	31.8 $\pm$ 0.3	2.8 $\pm$ 0.1	1 $\pm$ 0.1	13.4 $\pm$ 0.6	0.4 $\pm$ 0.05	1.2 $\pm$ 0.2	1.01
5.5	0.4 $\pm$ 0.1	3.7 $\pm$ 0.2	0.4 $\pm$ 0.05	46.7 $\pm$ 0.6	26.6 $\pm$ 0.6	6.1 $\pm$ 0.4	1.1 $\pm$ 0.15	10.8 $\pm$ 0.9	0.4 $\pm$ 0.1	3.4 $\pm$ 0.5	0.91
5	0.6 $\pm$ 0.2	4 $\pm$ 0.2	0.4 $\pm$ 0.08	47.5 $\pm$ 0.8	22.3 $\pm$ 0.7	8 $\pm$ 0.6	1.2 $\pm$ 0.12	8.8 $\pm$ 0.4	0.8 $\pm$ 0.2	6 $\pm$ 0.4	0.87

*, † See Table 2.

Effect of pH on fatty acid composition

The main effects were a decrease in 16:1 and 18:1 and an increase in 17 Δ and 19 Δ when the pH was decreased from 6 to 5 (Table 5).

DISCUSSION

Few studies have been made on the lipid composition of *C. acetobutylicum* (Johnston & Goldfine, 1983; Vollherbst-Schneck *et al.*, 1984), but more detailed investigations have been made of closely related species such as *Clostridium saccharoperbutylacetonicum* (Ogata *et al.*, 1982) and *Clostridium butyricum* (Goldfine & Bloch, 1961; Baumann *et al.*, 1965; Khuller & Goldfine, 1974; Johnston & Goldfine, 1983; Koga & Goldfine, 1984). Johnston & Goldfine (1983) reported for *C. acetobutylicum* ATCC 824 the same fatty acids and alk-1-enyl chains as we observed with strain 903, except that the 18:2 acid was not found. However, the quantitative values for individual fatty acids were quite different from ours. It is likely, as shown by the results of the present study, that culture conditions largely account for these differences. The fatty acid composition of *C. acetobutylicum* ATCC 824 obtained by Vollherbst-Schneck *et al.* (1984) was also similar to ours except for the absence of the 12:0, 17 Δ , 19 Δ and 18:2 acids which were not mentioned. The presence of the 18:2 acid as a minor constituent in our preparations was unexpected since polyunsaturated fatty acids are not known to be synthesized in anaerobic micro-organisms. It is thus probable that this acid, whose identity was confirmed by MS, originated from the culture medium.

The available literature also provides hints as to the nature of phospholipid PX which was detected in our preparations. Ogata *et al.* (1982), working on *C. saccharoperbutylacetonicum*, also separated on TLC an unknown phospholipid in conditions quite similar to ours. Johnston & Goldfine (1983) identified in *C. acetobutylicum* an unusual phospholipid, previously identified in *C. butyricum* (Matsumoto *et al.*, 1971), as the glycerol acetal of ethanolamine plasmalogen. The following points lead us to tentatively identify PX with this compound: PX was ninhydrin positive; it did not present a diacyl form in plasmalogen analysis; it migrated exactly as did lyso-PE; analysis of fatty acid composition by acidic transesterification showed that PX contains over 30% of dimethylacetals.

Large changes in lipid composition of *C. acetobutylicum* were brought about by addition of solvents. Alcohols of various chain lengths, and acetone, caused a decrease in the U/S ratio. This effect can be interpreted as an adaptive response to the interactions of solvents with membrane lipids. As insertion of alcohols into the membrane lipid bilayer would tend to increase the fluidity of the membrane, it would be counterbalanced by the opposite effect of the decrease of the U/S ratio (Ingram, 1976). Longer-chain alcohols, however, have also been found to shorten the fatty acid chain lengths and this additional effect cannot be interpreted in general terms of membrane fluidity. A suitable explanation would probably have to take into account the complex structure of the membrane and the possibility of specific interactions of the alcohols with individual lipid components.

The influence of growth temperature on membrane lipid composition has been extensively studied. Since the work of Marr & Ingraham (1962) with *Escherichia coli*, much evidence has been accumulated to support the interpretation summarized by Sinensky (1974) in the concept of 'homeoviscous adaptation'. Our results are in line with this interpretation but *C. acetobutylicum* reacts to an increase in temperature mainly by shortening the fatty acid chain length, in a way similar to that described for *C. butyricum* (Khuller & Goldfine, 1974). It should also be noted that in *C. acetobutylicum*, an increase in membrane fluidity is brought about in different ways: the presence of solvents decreases the degree of unsaturation whereas a temperature decrease shortens the chain lengths of the fatty acids.

The overall changes in lipid composition brought about during acetone-butanol fermentation were largely accounted for by solvent production. It thus seems puzzling that *C. acetobutylicum* has a lipid composition which is little different from the non-solvent-producing species *C. butyricum*. A possible reason could be that acids such as butyric acid produced by the latter interact with the membrane in a way related to that of solvents. Solvent production, however, cannot account for all the variations in lipid composition observed in *C. acetobutylicum*. The

presence of cyclopropane fatty acids in particular has been found to be strongly influenced by pH. In addition, these acids appear to be synthesized during acetone–butanol fermentation in a way which, as reported by Rock & Cronan (1982) for *E. coli*, appears related to changes from the exponential to the stationary phase of growth. Explanations for such changes in fatty acid composition would require some understanding of the specific role of cyclopropane fatty acids in membrane structure.

REFERENCES

- BALCH, W. E., FOX, G. E., MAGRUM, L. J., WOESE, C. R. & WOLPE, R. S. (1979). Methanogens: reevaluation of a unique biological group. *Microbiological Reviews* **43**, 260–296.
- BAUMANN, N. A., HAGEN, P. O. & GOLDFINE, H. (1965). Phospholipids of *Clostridium butyricum*. Studies on plasmalogen composition and biosynthesis. *Journal of Biological Chemistry* **240**, 1559–1567.
- BLIGH, E. G. & DYER, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**, 911–917.
- FOLCH, J., LEES, M. & SLOANE-STANLEY, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry* **226**, 497–509.
- GENTNER, P. R., BAUER, M. & DIETERICH, I. (1981). Separation of major phospholipids classes of milk without previous isolation from total lipid extracts. *Journal of Chromatography* **206**, 200–204.
- GOLDFINE, H. & BLOCH, K. (1961). On the origin of unsaturated fatty acids in *Clostridia*. *Journal of Biological Chemistry* **236**, 2596–2601.
- HERMANN, M., FAYOLLE, F., MARCHAL, R., PODVIN, L., SEBALD, M. & VANDECASTEELE, J. P. (1985). Isolation and characterization of butanol resistant mutants of *Clostridium acetobutylicum*. *Applied and Environmental Microbiology* **50**, 1238–1243.
- HERRERO, A. A., GOMEZ, R. F. & ROBERTS, M. F. (1982). Ethanol-induced changes in the membrane lipid composition of *Clostridium thermocellum*. *Biochimica et biophysica acta* **693**, 195–204.
- INGRAM, L. O. (1976). Adaptation of membrane lipids to alcohols. *Journal of Bacteriology* **125**, 670–678.
- JAIN, M. K. & WU, N. M. (1977). Effect of small molecules on the dipalmitoyl lecithin liposomal bilayer. III. Phase transition in lipid bilayer. *Journal of Membrane Biology* **34**, 157–201.
- JAIN, M. K., GLEESON, J., UPRETI, A. & UPRETI, G. G. (1978). Intrinsic perturbing ability of alkanols in lipid bilayers. *Biochimica et biophysica acta* **509**, 1–8.
- JOHNSTON, N. C. & GOLDFINE, H. (1983). Lipid composition in the classification of the butyric acid-producing clostridia. *Journal of General Microbiology* **129**, 1075–1081.
- KHULLER, G. K. & GOLDFINE, H. (1974). Phospholipids of *Clostridium butyricum*. V. Effects of growth temperature on fatty acid alk-1-enyl ether group, and phospholipid composition. *Journal of Lipid Research* **15**, 500–507.
- KOGA, Y. & GOLDFINE, H. (1984). Biosynthesis of phospholipids in *Clostridium butyricum*: kinetics of synthesis of plasmalogens and the glycerol acetal of ethanolamine plasmalogen. *Journal of Bacteriology* **159**, 597–604.
- MARR, A. G. & INGRAHAM, I. L. (1962). Effect of temperature on the composition of fatty acids in *Escherichia coli*. *Journal of Bacteriology* **84**, 1260–1267.
- MATSUMOTO, M., TAMIYA, K. & KOIZUMI, K. (1971). Neutral lipids and a new type of aldehydogenic ethanolamine phospholipid in *Clostridium butyricum*. *Journal of Biochemistry* **69**, 617–620.
- MOREIRA, A. R., ULMER, A. C. & LINDEN, J. C. (1981). Butanol toxicity in the butylic fermentation. *Biotechnology and Bioengineering Symposium* **11**, 567–579.
- MORRISON, W. R. & SMITH, L. M. (1964). Preparation of fatty acids methyl esters and dimethylacetals from lipids with boron fluoride-methanol. *Journal of Lipid Research* **5**, 600–608.
- NELSON, G. J. (1975). Isolation and purification of lipids from animal tissues. In *Analysis of Lipids and Lipoproteins*, pp. 1–22. Edited by E. G. Perkins. Champaign: American Oil Chemists' Society.
- OGATA, S., VOSNINO, S., OKUMA, Y. & HAYASHIDA, S. (1982). Chemical composition of autoplast membrane of *Clostridium saccharoperbutylacetonicum*. *Journal of General and Applied Microbiology* **28**, 293–301.
- PELICK, N. & MAHADEYAN, V. (1975). Lipids derivatives and gas liquid chromatography. In *Analysis of Lipids and Lipoproteins*, pp. 23–35. Edited by E. G. Perkins. Champaign: American Oil Chemists' Society.
- ROCK, C. O. & CRONAN, J. E., JR (1982). Regulation of bacterial membrane lipid synthesis. *Current Topics in Membranes and Transport* **17**, 207–233.
- ROUSER, G., FLEISCHER, S. & YAMAMOTO, A. (1970). Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* **5**, 494–496.
- RUSSELL, N. J. (1984). Mechanisms of thermal adaptation in bacteria: blueprints for survival. *Trends in Biochemical Sciences* **9**, 108–112.
- SINENSKY, M. (1974). Homeoviscous adaptation. A homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **71**, 522–525.
- VISWANATHAN, C. V., PHILLIPS, F. & LUNDBERG, W. O. (1968). Two-dimensional reaction thin-layer chromatography in the analysis of phosphatide plasmalogens. *Journal of Chromatography* **35**, 66–71.
- VOLLHERBST-SCHNECK, K., SAND, J. A. & MONTENECOURT, B. S. (1984). Effect of butanol on lipid composition and fluidity of *Clostridium acetobutylicum* ATCC 824. *Applied and Environmental Microbiology* **47**, 193–194.
- WALTON, V. & MARTIN, J. L. (1979). Production of butanol–acetone by fermentation. In *Microbial Technology*, vol. 1, pp. 187–209. Edited by H. J. Peppler & D. Perlman. London: Academic Press.