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ETHANOL-INDUCED CHANGES IN THE MEMBRANE LIPID COMPOSITION OF *CLOSTRIDIUM THERMOCELLUM*

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When ethanol is added to the growth medium of *Clostridium thermocellum* ATCC 27405 and C9, a different membrane composition is observed after the period of growth arrest. Changes in fatty acid composition and some unsaturated, branched hydrocarbons have been monitored by GLC-MS. There is a marked increase in normal and anteiso-branched fatty acids at the expense of isobranched fatty acids and an increase in short and unsaturated fatty acids. Thus, an adaptive response to growth in the presence of ethanol induces a membrane containing fatty acids with lower melting points and produces a more 'fluid' membrane. The suggestion is made that these membrane changes may be maladaptive to the performance of *C. thermocellum*.

Introduction

Ethanol and other end products of anaerobic metabolism are small amphiphilic molecules with low partition coefficients in lipid/water systems. This property allows them to partition and interact in cellular structures in a hydrophobic or a hydrophilic manner. These end-products can perturb a variety of target sites in cells and often lead to growth inhibition.

Clostridium thermocellum is an anaerobic, thermophilic, and chemotrophic bacterium that can be used for direct conversion of cellulose to ethanol and other end-products. Recent interest in this organism stems from its possession of a cellulase enzyme [1,2]. However, wild type *C. thermocellum* is relatively sensitive to its end-products; 50% growth inhibition is reached with 5 g/l ethanol. The ethanol-tolerant mutant, C9, requires 20 g/l ethanol for 50% growth inhibition [3]. The inhibited growth of *n*-alkanol-challenged cultures of *C. thermocellum* is characterized by three phases: a short phase of little or no inhibition (I), a period of growth arrest (L), and a phase of inhibited but sustained exponential growth (II) [3]. This contrasts with ethanol inhibition of yeast, where an immediate reduction in growth is observed [4].

The exact mechanism by which *n*-alkanols inhibit bacterial growth is not known. It has been demonstrated, however, that nonionized compounds such as *n*-alkanols can affect membrane physiology by partitioning in lipid bilayers and interfering with lipid-lipid and lipid-protein interaction [5–7]. The effect of such compounds may be due to physical perturbation of the bilayer or more direct interactions with specific permease systems (or other membrane proteins). When *C. thermocellum* growth rates are measured in phase II in response to alkanols, the predicted membrane concentrations of each alkanol necessary for 50%

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growth inhibition are essentially the same [3]. This suggests that (i) the target for growth inhibition is hydrophobic; (ii) the target is the same for all *n*-alkanols, and (iii) growth inhibition is directly proportional to the actual number of perturbing molecules that have partitioned into the hydrophobic milieu. The most obvious target with these characteristics is the cell membrane, although a cytoplasmic hydrophobic site must also be considered.

If the cell membrane is involved, what is the alteration in the mutant that allows higher *n*-alkanol tolerance, and what are membrane adaptations in phase II? In higher organisms, ethanol tolerance is associated with an increase in the specific activity of liver alcohol dehydrogenase [8]. In microorganisms solvents such as ethanol elicit a variety of membrane function responses [9–11], although the primary growth limiting targets are a matter of controversy. For yeast, *Escherichia coli, Bacillus subtillis*, and *Lactobacillus homohiochii*, solvent-resistant mutants or adaptation of growth to ethanol causes membrane lipid alterations. The magnitude and direction of fatty acid changes depend on the organism. For *E. coli*, Ingram and coworkers [12,13] suggest that two condensing enzymes of fatty acid synthesis are the site of ethanol action. This alters fatty acid composition of the membrane and hence, according to Ingram [12,13] a more suitable membrane is produced to sustain growth in the presence of ethanol.

We have examined the lipid composition of *C. thermocellum* wild type and mutant C9 grown in the absence of ethanol and in the ethanol-adapted phase by mass spectrometry and a variety of chromatographic and spectroscopic techniques. When ethanol is added to *C. thermocellum* an altered membrane composition is observed after the period of growth arrest. Fatty acid substitutions are in the direction of fluidizing the membrane. The ethanol tolerant mutant C9 shows similar alterations upon addition of inhibiting concentrations of ethanol. Such changes are discussed in view of the response of other bacterial cells to ethanol.

Methods

Bacterial growth

Clostridium thermocellum ATCC 27405 and its ethanol-resistant derivative, C9, were grown under anaerobic conditions at 60°C in CM4-Cb medium. This medium, a modification of the CM3-medium reported by Weimer and Zeikus [14], contains (in g/l): KH_2PO_4, 1.5; K_2HPO_4, 2.9; $(NH_4)_2SO_4$, 1.3; $MgCl_2 \cdot 6H_2O$, 0.75; NaCl, 1.0; $CaCl_2 \cdot 2H_2O$, 0.013; sodium thioglycollate, 0.5; $FeSO_4 \cdot 7H_2O$, 1.25 mg/l; 1% resazurin, 1.0 ml/l; yeast extract, 10.0; carbon and energy source, 6.0; pH 7.4. The carbon and energy sources added were either cellobiose (Sigma) or cellulose. This latter was used in two forms; amorphous cellulose Solka Floc SW_{40} (Brown Co.), or crystalline α-cellulose (Sigma). Cell growth was initiated with inoculum sizes that were always above 5% v/v. Manipulation of cells was always done in an anaerobic chamber (Coy Laboratory Products) of controlled atmosphere (3% v/v, 5% v/v CO_2 and N_2 to balance). Cell mass of *C. thermocellum* strains was quantified by measuring the optical density at 660 nm and the dry cell weight.

Extraction of total lipids

Total lipids of *C. thermocellum* ATCC 27405 grown in CM4Cb medium both in the presence and absence of ethanol [3], were extracted by a procedure based on the methods of Ames [15], and of Langworthy et al. [16]. 1.5 liters of growing cultures with A_{660} from 0.5 to 0.7 were centrifuged at $7000 \times g$ for 15 min and washed once with distilled water. The cell pellet was extracted with 100 ml of $CHCl_3/CH_3OH$ for 2 h at room temperature (extract I). The slurry was filtered and the cell debris extracted again with 50 ml of $CHCl_3/CH_3OH$ (2 : 1, v/v) for 0.5 h (extract II). To remove non-lipid contaminants, extracts I and II were pooled and washed with 100 ml of saturated KCl (this salt precipitated into the organic phase). The upper aqueous phase was re-extracted with 200 ml of $CHCl_3/CH_3OH$ (1 : 1, v/v) and then discarded; no lipid could be detected in this phase. The two organic phases from this purification step were pooled, filtered to remove the salt, and evaporated at 60°C under vacuum and with a continuous N_2-gas stream to a final volume of 50 ml. Anhydrous Na_2SO_4 (10 g) was added to the lipid solution and left overnight at −20°C under N_2 atmosphere. After this drying time, the lipid solution was concentrated under vacuum to con-

stant weight using a continuous N_2-gas stream at 40–50°C; the amount of solvent-free lipids was then determined gravimetrically.

Analysis of total lipids

Lipids, 1–2 mg, were applied onto silica gel plates (250 μm E. Merck pre-coated silica gel 60 TLC plates without fluorescent indicator) that had been activated for 1 h at 105°C. Thin-layer chromatography of these plates was run at room temperature in two dimensions with the following solvent systems: (i) System I $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v); (ii) System II_G $CHCl_3/(CH_3)_2CO/CH_3OH/CH_3COOH/H_2O$ (50 : 20 : 10 : 10 : 5, v/v).

Lipids were visualized on TLC plates by exposure to I_2. Phospholipids were identified by spraying with Dittmer's reagent [17] obtained from Applied Science Laboratories. The presence of glycolipids was confirmed by the Bial's-Orcinol reagent (Applied Science Labs., Inc.): plates were heated at 80°C for 1.5 h inside a closed chamber with a HCl atmosphere, sprayed with the orcinol reagent, and heated for 15 min at 80°C. Free NH_2-groups were assayed with the ninhydrin reagent [18].

Analysis of the lipid fatty acid composition

Fatty acids methyl esters were prepared both from whole cells and from extracted lipids according to procedure 'B' described by Moss et al. [19]. *C. thermocellum* C9 strain is stored in ethanol-containing broth. Therefore, when fatty acid methyl esters from this mutant were studied, 10 transfers in the absence of ethanol (= 30 generations) were given to this strain. The effect of ethanol on lipid composition of C9 was then studied by adding this compound to the growth media.

For the whole cell fatty acid methyl ester preparation, 120 ml (twelve Hungate's tubes) of *C. thermocellum* cultures were centrifuged at 10 000 × *g* for 10 min and the cells pooled and washed once with fresh broth. 5 ml of a 5% NaOH solution in 50% aqueous methanol were added to the cell pellets in teflon-lined, screw-capped glass tubes; this mixture was heated for 15 s in a boiling water bath. Upon cooling, HCl was used to bring the pH to 2.0. Methylating reagent, 4 ml of 14% BF_3 in methanol (Sigma Chemical Co.), was added to the sample which was boiled for 5 min in the same teflon-lined glass tube. Saturated NaCl (10 ml) was added and the fatty acid methyl esters extracted twice with one volume of $CHCl_3$/hexane (1 : 4, v/v). The extracts were pooled and the solvent evaporated by vacuum. When a volume of 5–10 ml was reached, anhydrous Na_2SO_4 (1 g) was added and the mixture dried overnight at −20°C. After this step, the salt was removed by filtration and fatty acid methyl esters were concentrated to dryness by an N_2-gas stream in a small vial. The solvent-free fatty acid methyl esters were redissolved in hexane to suitable concentrations for gas liquid chromatography and mass spectrometry.

The same procedure was used for the methanolysis of purified *C. thermocellum* lipids.

Identification of fatty acid methyl esters

Routine analyses of fatty acid methyl esters were performed in a Hewlett-Packard 5830 A gas chromatograph using a 6 ft. 1/8 inch outer diameter stainless steel column packed with 10% Silar 10 C on 100/120 Gas Chrom Q (Applied Science Labs., Inc.). Alternatively, a 3% SP-2100-DOH column of the same dimensions was used (Supelco Inc.). Fatty acid methyl esters were identified by comparison of their retention times (RT) with those of known standards.

An experimental sample of unknown composition was run either after or before the standard mixtures at exactly the same chromatographic conditions. A fatty acid methyl ester was considered positively identified when its estimated number of C atoms did not differ from an integer number in more than 0.10, i.e. $N_x \pm 0.10$. Results from the two columns and at the different temperatures agreed extremely well. This is significant because Silar 10 C and SP 2100-DOH are two stationary phases of different polarities and therefore have different specificities for the fatty acid methyl ester series; the order of elution from the Silar 10 C column for methyl esters with an equal N_x is iso-, anteiso, normal and unsaturated, whereas in the SP 2100-DOH the order of elution is unsaturated-, iso-, anteiso-, and normal-fatty acid methyl esters.

Isothermal runs were used also for quantitation of the percentage of each fatty acid methyl ester

present in *C. thermocellum* membranes. Temperature programming (110°C–175°C initial and final temperatures at 2 K/min) gave excellent results with the two columns used.

Determination of fatty acid composition of yeast extract

Difco Yeast Extract (No. 666512) (20 g) was extracted by the method of Bligh and Dyer [20]. The total lipid content was gravimetrically determined and then subjected to methanolysis. The extracted Yeast-Extract was used to grow *C. thermocellum* ATCC 27405 and to study its fatty acid composition by GLC-MS.

Identification of C. thermocellum fatty acid methyl esters by GLC-MS

C. thermocellum ATCC 27405 was grown both in the presence and absence of ethanol and in extracted Yeast Extract-CM4-Cb medium. Fatty acid methyl esters were injected in a Varian 3700 analytical chromatograph which was coupled to a Finnigan MAT-212 mass spectrometer. The chromatography column was a fused-silica, 25 ft. capillary column with SE-52 as stationary phase (J & W Scientific).

Temperature programming from 110°C to 180°C at 3 K min was used. Helium carrier gas pressure at the inlet port was adjusted at 13 lbs/inch^2.

Studies on membrane compositional changes by ^{1}H-NMR

Total lipids were extracted into organic solvent and washed with 10 mM EDTA at pH 7.4 in saturated KCl. (Samples were dissolved in C^2HCl_3 at 30 mg/ml concentration.) ^{1}H-NMR spectra of the dried lipid extracts were obtained on a Bruker 250 spectrometer coupled to an Aspect 2000 computer.

Results

Lipid composition and overall changes induced by ethanol

Total lipids account for 5–6% w/w of the *C. thermocellum* (wild type and cells grown in ethanol) dry cell weight. TLC analysis of *C. thermocellum* extracted lipids indicates that the lipid composition of this bacterium is rather complex (Fig. 1). Out of 33 resolved lipid spots, 20 were phospholipids, 12 were glycolipids and 6 were phosphoglycolipids. In addition, a yellow glycolipid could be detected moving with the solvent front in both systems. This non-polar glycolipid may be part of the chemical structure that gives rise to the yellow appearance of the cells. The redox-indicator resazurin was carried over during the extraction procedure. Lipid TLC patterns from cells grown in the presence of ethanol appeared very similar to those of control cells. No further attempts were made to quantify changes that take place in the individual phospholipid classes as a consequence of cell exposure to ethanol.

Changes in the lipid species occur upon cell challenges with ethanol. This can be rapidly assessed by ^{1}H-NMR spectra of total lipids extracted from ethanol-exposed and control cells. Fig. 2 shows ^{1}H-NMR spectra of lipids extracted from *C. thermocellum* ATCC 27405 grown in the absence (A) and presence (B) of ethanol. Adap-

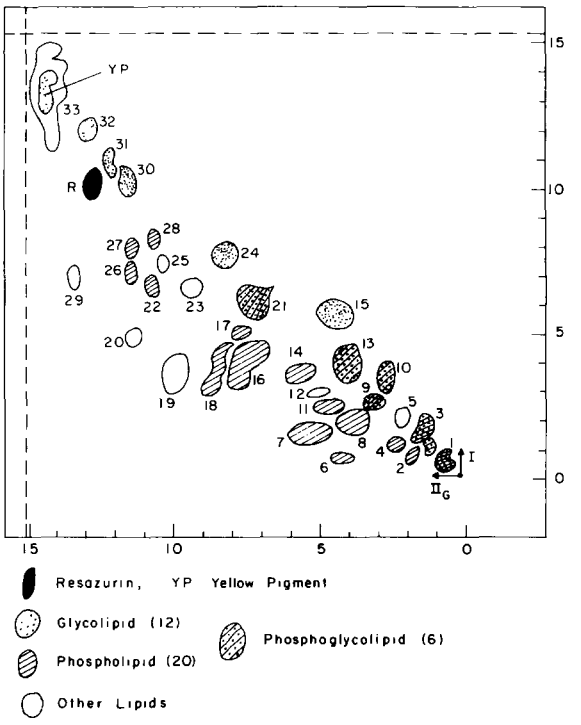

Fig. 1. TLC of lipids extracted from *C. thermocellum* ATCC 27405 grown in the absence of ethanol. Solvent systems are given in Materials and Methods.

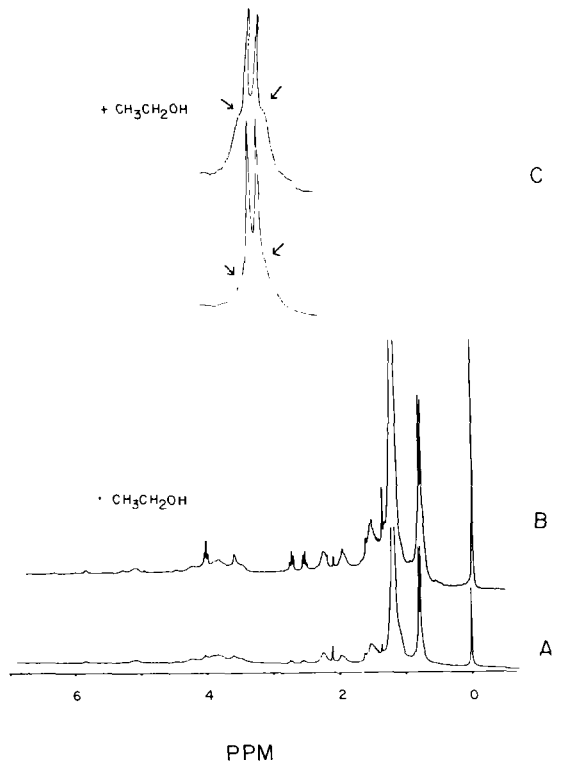

Fig. 2. ^{1}H-NMR spectrum (250 MHz) of lipids (in C^2HCl_3) extracted from *C. thermocellum* ATCC 27405. (A) Grown without ethanol. (B) Grown in the presence of 5 g/l ethanol. The methyl regions of both spectra are expanded in (C).

tation to growth in ethanol causes a relatively small increase in olefinic protons (5–6 ppm from TMS) and a larger increase in methylene groups adjacent to double bonds (these signals are resolved in the 2.5–2.8 ppm region as a doublet of triplets). The most detectable alteration in membrane composition of *C. thermocellum* ATCC 27405 induced by ethanol is the increase in normal and anteiso-branched fatty acids at the expense of iso-branched fatty acids. The methyl groups at an iso-branched point are indicated by a doublet coupling pattern centered at 0.9 ppm; the methyl group of a normal chain is seen as a triplet also centered at around 0.9 ppm. The overlap of the two resonances gives a characteristic pattern where the triplet's wings appear as shoulders on the more dominant iso-branched methyl doublet (Fig. 2C). An enlargement of this methyl region shows that upon growth in ethanol there is a large increase in triplet signal (indicated by the arrows) at the expense of doublet signal (the center line is not easy to detect, given the large doublet), revealing a relatively large increase in normal and anteiso-branched chains and a decrease in iso-branched chains when cells adapt to ethanol.

GLC analysis of fatty acid composition

Fatty acid methyl esters were prepared from whole cells and analyzed by GLC. Table I summarizes the results obtained in both *C. thermocellum* strains ATCC 27405 and C9. The C16 species are the single largest components of wild-type and C9 lipids. When both strains are grown in the presence of ethanol, the *n*C16 : 0 species decrease 2-fold (31% to 15% for wild type and 38% to 22% for C9). In table II is a more quantitative trend analysis showing that fatty acid composition after growth in the presence of ethanol is shifted to species that could provide a higher membrane fluidity (i.e., the fatty acids in such cultures have lower melting points). First of all, there are large increases in short chain fatty acids ($< C14$) in cultures grown in the presence of ethanol. This change is slightly larger in the mutant strain (9% to 30%) than in the wild type (11% to 24%). Changes also occur in unsaturation and chain branching. We can quantify them by considering that for a given chain length, the melting points of the fatty acid isomers decrease in the order normal-(even) – iso-(even) > *trans*-monounsaturated > anteiso-(odd) > *cis*-monounsaturated > di- and poly-unsaturated species [21,22]. Therefore, for fatty acids greater than 14 carbons, an increase in the quantity (anteiso + monounsaturated)/(saturated) should reflect net 'fluidization' of the membrane. Both wild-type and C9 fatty acids do increase anteiso-branching and unsaturation upon growth in ethanol (Table II). A more specific change is indicated in Table II for C18 species: unsaturation increases at the expense of normal and iso-branched chains. The changes agree with the results from ^{1}H-NMR where the olefinic protons increase slightly as do anteiso-branched and normal chain (presumably shorter chain) methyl protons at the expense of iso-branched methyl protons.

Mass spectrometry of extracted lipids

Fatty acid methyl esters were obtained from *C.*

TABLE I

FATTY ACIDS OF *C. THERMOCELLUM* STRAINS GROWN IN CM4-Cb

RT is the observed retention time in an isothermal (155°C) chromatogram at 25 ml/min gas flow rate, using a SILAR 10-C (8 ft., 1/8 inch, stainless steel) column. Ethanol concentrations are those required to effect 50% inhibition of the growth rate in each strain. Abbreviations used for fatty acids: br, branched; *n*, normal; i, isobranched; a, anteisobranched; u.i., unidentified. Tr, trace, fatty acid present in less than 0.5% w/w.

Fatty acid	RT (min)	Percentage (w/w)			
		ATCC 27405		C9	
		Control	Ethanol (4.5 g/l)	Control	Ethanol (20 g/l)
br C10:0	1.22	7.4	10.1	5.0	8.6
n C11:0	1.77	–	Tr	Tr	0.6
br C12:0	2.48	Tr	Tr	Tr	0.6
n C12:0	2.75	Tr	5.0	Tr	1.8
u.i.	3.01	–	Tr	–	3.3
br C13:0	2.66	2.6	Tr	–	4.7
n C13:0	3.92	–	3.9	3.7	3.3
i C14:0	4.16	2.5	2.7	2.0	3.3
n C14:0	4.95	1.0	3.5	0.8	2.5
a C15:0 +C14:1	5.69	2.4	7.0	2.0	2.9
n C15:0	–	Tr	Tr	Tr	Tr
i C16:0	7.70	31.4	15.0	38.0	21.8
n C16:0	9.08	22.3	9.1	21.9	21.1
a C17:0 +C16:1	10.49	24.6	14.0+ 12.3	20.1	21.0
i C18:0	14.45	4.2	0.7	4.5	1.2
n C18:0	17.06	1.2	2.3	1.0	1.3
C18:1	20.21	0.3	4.5	1.0	2.0
Total		99.0	100.1	100.0	100

TABLE II

TREND ANALYSIS IN FATTY ACID PROFILES OF ETHANOL-CHALLENGED CULTURES OF *C. THERMOCELLUM*

Ethanol concentrations are those required to exert a 50% inhibition in each strain.

Ratio of fatty acid species	Wild Type		C9	
	control	+4.5 g/l ethanol	control	+20 g/l ethanol
Short/long [a]	0.11	0.23	0.10	0.30
$\frac{\text{Ante+Mu}}{\text{Saturated}}$ [b]	0.44	0.87	0.34	0.51
C18:1/C18:0 [c]	0.06	1.50	0.18	0.80

[a] Short/Long = (% weight fatty acid with C < 14)/(% weight fatty acid with C > 14).

[b] $\frac{\text{Ante+Mu}}{\text{Saturated}}$ = (% weight fatty acids with anteiso-odd and monounsaturated chains)/(% weight fatty acid with saturated (both *n*- and i-) chains longer than 14 carbons).

[c] $\frac{C18:1}{C18:0}$ = (% weight C18:1)/(% weight *n*- and i- C18:0).

thermocellum ATCC 27405 grown on yeast extract that was delipidated by organic solvent extraction. This latter procedure was introduced to ascertain if *C. thermocellum* could synthesize all fatty acids (in particular, unsaturated fatty acids) present in Table I. Total lipids in Difco Yeast Extract amount to 0.6% w/w of its dry weight. The composition of extracted lipids is very rich in unsaturated fatty acids (~ 60% w/w). Mass spectra of fatty acid methyl esters from *C. thermocellum* ATCC 27405 grown in extracted yeast-extract medium confirmed the presence in this strain of all fatty acids described in Table I (> C13), including unsaturated fatty acids (Dr. C. Costello, personal communication) in both *C. thermocellum* ATCC 27405 and C9.

An analysis of *C. thermocellum* whole-cell fatty acid methyl ester preparations from cells grown in extracted yeast-extract-CM4-Cb medium, shows that this organism has unsaturated, branched hydrocarbons in its membrane. The characteristics of these hydrocarbons and the effects that ethanol has on their content are shown in Table III. These three hydrocarbons were not present in a blank prepared by running a control fatty acid methyl ester preparation and extraction (10-fold higher volumes of all reagents) in which *C. thermocellum* cells had been omitted. Therefore, these hydrocarbons are not contaminant products of any reagent or solvent used during the methyl ester preparation procedure. The relative content of these unsaturated hydrocarbons increases when ethanol is introduced in the growing medium. The quantitation relies on peak areas in the GLC-MS chromatogram, and is only semiquantitative because of the characteristics of the capillary column. The increase in concentration when cells have been exposed to ethanol, however, is very apparent. A mass spectrum of hydrocarbon 'C' is given in Fig. 3. Possible structures of this compound are indicated on the figure.

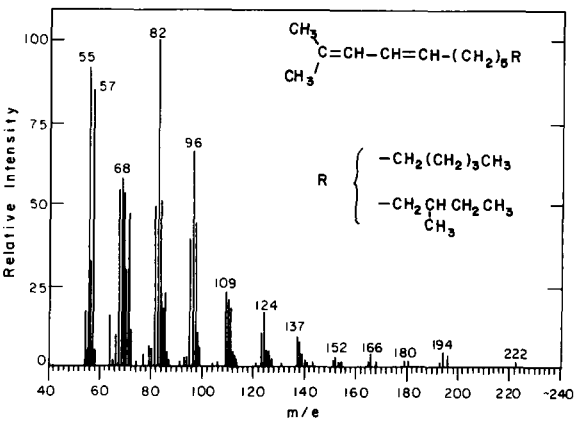

Fig. 3. Mass-spectrum of the branched, unsaturated hydrocarbon 'C'; two possible structures of this compound consistent with fragments are shown.

In addition, mass spectrometry has revealed the presence of a series of compounds that give rise to a characteristic fragment of $m/e = 75$ (Fig. 4). A possible structure giving rise to such a fragment is also indicated. These compounds may appear from methanolysis of a plasmalogen. Plasmalogens are typical in Clostridia and other anaerobic bacteria

TABLE III

UNSATURATED BRANCHED HYDROCARBONS PRESENT IN *C. THERMOCELLUM*

UBH [a]	Elution [b] order	Molecular weight	% w/w of C16:0 [c]		Empirical formula
			Control	+ Ethanol [d]	
A	1	?	Tr	6.0	?
B	2	194	26.0	67.0	$C_{14}H_{26}$
C	3	222	10.0	26.0	$C_{16}H_{30}$
D	4	?	3.0	8.0	?

[a] Unsaturated branched hydrocarbon.
[b] Chromatographic conditions described under Methods.
[c] Amount of hydrocarbon relative to an equivalent amount of *n* C16:0.
[d] Cells grown in the presence of 5 g/l ethanol.

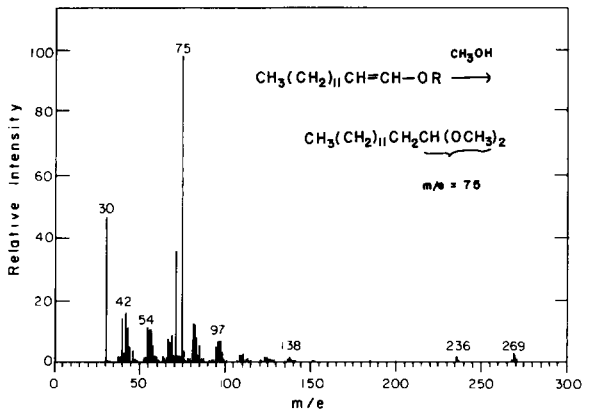

Fig. 4. Mass-spectrum of a type IV methanolysis product of *C. thermocellum* with $n = 11$. The fragment at $m/e = 75$ is suggestive of a plasmalogen.

[23]. The chain lengths of these unsaturated species are in the range from C14 to C18. The mass-spectrum of a type IV compound with $n = 11$ is given in Fig. 4. The total amount of these compounds could not be precisely determined because some of them eluted together with some fatty acid methyl esters in the mixture. However, an estimation based on peak areas from the mass spectrum gives a value of up to 20% w/w of the total fatty acid methyl ester content in the injected sample. These data strongly suggest the presence of plasmalogens in *C. thermocellum* ATCC 27405. These compounds are present both in control cells and in cells grown in the presence of ethanol.

Discussion

The lipids of *C. thermocellum* consist of a mixture of phospholipids, glycolipids, phosphoglycolipids and other neutral species. The presence of glycolipids may be very significant for *C. thermocellum* physiology. It is known that cellulose hydrolysis is a surface phenomenon; *C. thermocellum* cells attach to the cellulose fiber and thus are able to degrade it and utilize soluble sugars for growth. Glycolipids on the surface of *C. thermocellum* may serve for recognition and/or binding to the substrates; in addition the interaction of glycolipid sugar with cellulose may help in the process of breaking the crystalline structure of the cellulose fiber, a step that seems to be required for full enzyme attack [24].

Studies on the membrane compositions of *C. thermocellum* strains challenged with ethanol at the respective equipotency concentration (EPC^{50}), have revealed that phase L results in lipid alterations in both strains. Exponentially growing cultures actively involved in biosynthesis are more likely to adjust to environmental changes that require biosynthetic pathway redirection to alter membrane structure. *Lactobacillus* sp., *B. subtillis* and *E. coli* cultures also show a period of growth arrest as well as altered membrane fatty acid composition when ethanol is added. The duration of the period of growth arrest may reflect the time required for cells to adapt in response to membrane fluidity changes caused by ethanol addition [3]. Upon addition of ethanol to *B. subtillis* cultures there are major alterations in the phospholipid and fatty acid synthesis processes of these cells [25].

The fatty acid composition of *C. thermocellum* strains was similar to those described for thermophilic bacilli [26,27] and other thermophiles [28,29]. Branched fatty acids are commonly found in these species. Unsaturated, branched hydrocarbons such as those in *C. thermocellum* membranes have been found in other bacteria, yeasts, fungi and plants [30–32]. The estimate of unsaturated branched hydrocarbons in *C. thermocellum* agrees with data reported for other bacteria (e.g. *C. acidiurici* [32]). The types of branching that have been described in bacterial hydrocarbons include iso-, anteiso-, iso-iso (branch at the two ends) iso-anteiso and anteiso-anteiso [32]. For example, *Sarcina lutea* produces a mixture of singly and doubly iso- and anteiso-branched mono-olefins, similar to the unsaturated branched hydrocarbons in *C. thermocellum*, but of different chain lengths. Branched fatty acids appear to be intermediates in the synthesis of the hydrocarbons; after a decarboxylation step, head to head condensation reactions occur. Acetate is a common precursor of both fatty acids and unsaturated branched hydrocarbons [31]. Hydrocarbon synthesis using labelled acetate in *S. lutea* requires ATP, coenzyme A, Mg^{2+}, NADPH and either pyridoxal or pyridoxamine phosphate [31,32]. The synthesis of these hydrocarbons is also related to the metabolism of plasmalogens [32]

which we suggest exist in *C. thermocellum* ATCC 27405. Plasmalogens are typical lipids of anaerobic organisms and Clostridia in particular [33].

The alterations in lipid biochemistry triggered by the presence of ethanol result in the synthesis of fatty acids with lower melting points. This suggests that adaptation in *C. thermocellum* after exposure to ethanol produces a more fluid membrane. Anteiso fatty acids have a lower melting point than the corresponding iso-branched isomers of equivalent carbon number. The decrease in fatty acid chain length observed upon *C. thermocellum* exposure to ethanol would also result in membrane fluidization. These major changes will be further accentuated by the increased levels of unsaturated fatty acids and unsaturated branched hydrocarbons. These unsaturated compounds may promote changes in membrane fluidity, even if present in small amounts.

Based on physical studies of model bilayers, Jain and Wu [6] have proposed that the physical effect of ethanol at relatively high ethanol/lipid ratios is a tightening of the membrane. The significance of the bacterial response (fluidizing) would be to counteract the physical effect of ethanol [10]. This model has some exceptions; in *B. subtillis*, ethanol-induced changes in fatty acids decreased, rather than increased, membrane fluidity [25]. If the responses of *E. coli* (where fatty acids become more fluid) and *B. subtillis* were both adaptive, the physical effect of ethanol in the membranes would be in opposite directions (although phospholipid headgroup changes in *B. subtillis* occurring at the same time could result in an overall fluidization). Studies with fluorescence depolarization have shown that the in vivo tightening brought about by ethanol is small [34]. This suggests the possibility that *E. coli* cells overreact in response to ethanol mediated fluidity changes. The changes in fatty acid composition induced by ethanol are similar to changes induced by altering bacteria growth temperature. Similar mechanisms may be involved in these changes. Gill and Suisted [35] have examined the fatty acid composition of a large variety of organisms as a function of varying growth rate. The observed changes led them to suggest that variations in fatty acids may be the result of failures in feedback control of enzymatic systems responsible for conservation of constant fatty acid composition. Thus, lipid changes induced by different growth conditions may be fortuitous and not a direct response to physical perturbations of the membrane.

The extent of the 'fluidization' or increase in lower melting fatty acids observed in ethanol-inhibited cultures of wild-type and C9 *C. thermocellum* are of a comparable magnitude, at least judging by the fatty acid changes appearing in the ethanol exposed cultures. However, the ethanol concentrations needed to inhibit the two cultures ($-EPC^{50}$) were 4.5 g/l for the wild type and 20 g/l for the ethanol tolerant mutant strain C9. It has been shown in a variety of systems [10,11] that the extent of the fluidizing response promoted by *n*-alkanol challenges is also directly proportional to the number of molecules of *n*-alkanol present in the bacterial membrane. From the fatty acid changes in *C. thermocellum*, it appears that the ethanol tolerant mutant, strain C9, does not change its fatty acid composition upon addition of ethanol as dramatically as the wild type. When more ethanol molecules are present in C9, the extent of the change is similar to that observed in the wild type at five-times less ethanol concentration. This lessening of membrane changes per gram ethanol correlates with higher ethanol tolerances. A possible implication of this argument is that the homeoviscous response in *C. thermocellum* strains may be maladaptive. Perhaps a fluidity 'sensor' and/or 'effector' may be mutated in the strain C9 so that this organism either does not feel fluidity changes or does not counteract them. One possibility is that ethanol tolerant mutants may have altered their 'sensor' system to exhibit a degree of relaxation and do not accumulate nucleotide polyphosphates as a response to *n*-alkanol challenges [36]. Alternatively, a direct effect of ethanol on the fatty acid-producing systems may change the substrate specificity of these enzymes and hence cause random alterations in the reaction products. Such changes induced by ethanol could be detrimental for the overall cell performance. In fact, membrane energization and phosphate transport are slowed in *C. thermocellum* adapted to growth in ethanol (Herrero, Gomez and Roberts, unpublished results). This would suggest that the ethanol resistant mutants have then altered their fatty acid producing systems so that ethanol does not affect

their specificities to the same extent (or at the same dose) as in the wild-type organism.

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