JOURNAL OF BACTERIOLOGY, June 1983, p. 1291–1300
0021-9193/83/061291-10$02.00/0

Vol. 154, No. 3

Lipid Composition of *Zymomonas mobilis*: Effects of Ethanol and Glucose†

V. C. CAREY AND L. O. INGRAM*

Department of Microbiology and Cell Science and Department of Immunology and Medical Microbiology, University of Florida, Gainesville, Florida 32611

Received 13 December 1982/Accepted 1 April 1983

Zymomonas mobilis is an alcohol-tolerant microorganism which is potentially useful for the commercial production of ethanol. This organism was found to contain cardiolipin, phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylcholine as major phospholipids. Vaccenic acid was the most abundant fatty acid, with lesser amounts of myristic, palmitic, and palmitoleic acids. No branched-chain or cyclopropane fatty acids were found. Previous studies in our laboratory have shown that ethanol induces the synthesis of phospholipids enriched in vaccenic acid in *Escherichia coli* (L. O. Ingram, J. Bacteriol. **125**:670–678, 1976). The fatty acid composition of *Z. mobilis*, an obligately ethanol-producing microorganism, represents an extreme of the trend observed in *E. coli*. In *Z. mobilis*, vaccenic acid represents over 75% of the acyl chains in the polar membrane lipids. Glucose and ethanol had no major effect on the fatty acid composition of *Z. mobilis*. However, both glucose and ethanol caused a decrease in phosphatidylethanolamine and phosphatidylglycerol and an increase in cardiolipin and phosphatidylcholine. Ethanol also caused a dose-dependent reduction in the lipid-to-protein ratios of crude membranes. The lipid composition of *Z. mobilis* may represent an evolutionary adaptation for survival in the presence of ethanol.

Zymomonas mobilis is a gram-negative microorganism of uncertain taxonomic position (7, 10, 41–44). This organism is obligately fementative, utilizing glucose by the Entner-Doudoroff pathway (8, 13). *Z. mobilis* is commonly found as a spoilage organism in ciders and beers (7). It is present in ripening honey (36) and is used in the fermentation of cacao beans to make cocoa and chocolate (31). *Zymomonas* is used in the fermentation of palm wines (30) and plant saps (7, 42) to produce alcoholic beverages and has yet to be exploited for large-scale commercial alcohol production (34, 42). *Z. mobilis* is capable of producing up to 1.9 mol of ethanol per mol of glucose fermented (12, 13, 24, 42). It is reported to exhibit higher ethanol tolerance (42), higher glucose tolerance (33, 34, 42), and more rapid fermentation than *Saccharomyces cerevisiae* (26, 33, 34), the organism used for ethanol production in the United States.

Growth in the presence of ethanol has been shown to cause changes in the lipid composition of many organisms. In *Escherichia coli* (15–18, 20) and in yeasts (3, 35), alcohol induces an increase in the proportion of 18:1 fatty acid at the expense of 16:0. This change has been proposed as being adaptive for growth and survival in the presence of alcohol (3, 15). Other alcohol-induced changes in *E. coli* include an increase in the proportion of acidic phospholipids (16) and a decrease in the lipid-to-protein ratio (16). Unlike *E. coli*, *Z. mobilis* is capable of growth in high concentrations of glucose and of producing high concentrations of ethanol. Thus, *Z. mobilis* may have evolved specialized features to allow survival under these environmental stresses.

In a recent report by Tornabene et al. (47), the major lipids of *Z. mobilis* were identified. In this study, we have independently confirmed their identification of the major lipids and have examined the effects of ethanol and glucose concentrations on the membrane composition of *Z. mobilis*.

(A preliminary account of this work has appeared [V. C. Carey, and L. O. Ingram, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, K165, p. 248].)

MATERIALS AND METHODS

Bacterial strains, medium, and growth conditions. *Z. mobilis* ATCC 10988 (ZMI) (25) was the primary organism used in this study and was obtained from the American Type Culture Collection. Experiments were also conducted to examine the lipid composition of

† Florida Agricultural Experiment Station publication no. 4377.

five other strains of *Z. mobilis*. These were strains NRRL B-4576, NRRL B-14022, NRRL B-14023, ATCC 29191, and NRRL 4286. The first four of these strains have been designated by other workers (40) as Ag11, ZM3, ZM4, and ZM6, respectively. These five strains were generously supplied by Arie Ben-Basat of the Cetus Corporation (Berkeley, Calif.). Cultures were grown in the medium described previously by Gibbs and DeMoss (13) containing (per liter): 10 g each of glucose, tryptone, and yeast extract; 5 g of KH_2PO_4; and 20 ml of salt mixture (per 100 ml: 0.8 g of $MgSO_4 \cdot 7H_2O$, 0.16 g of $MnSO_4 \cdot H_2O$, and 0.04 g each of NaCl and $FeSO_4 \cdot 7H_2O$). Solid medium was prepared for culture maintenance by adding 2% agar. Glucose concentrations were varied in the medium as indicated. When appropriate, ethanol was added to sterile medium after cooling and immediately before inoculation.

Overnight cultures were grown at 30°C with gentle agitation in a shaking water bath and diluted 1:100 into fresh medium containing various concentrations of glucose and ethanol. Growth was measured as optical density at 550 nm over time, using a Spectronic 70 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.).

Determination of ethanol concentration in broth cultures. Gas samples were collected from the headspace area (21) of broth cultures. Ethanol concentrations were measured by using gas liquid chromatography on a diethylene glycol succinate (DEGS) column at 80°C against a standard curve of known ethanol concentrations in water.

Lipid extraction. Overnight cultures were diluted 1:100 into serum bottles containing 100 ml of medium and sealed with rubber serum stoppers. A sterile tuberculin needle was inserted into each stopper for releasing excess pressure caused by CO_2 produced during growth of the cultures. The outer opening of the tuberculin needle was protected by a 13-mm culture tube cap. Cells were harvested in exponential phase at an optical density of 0.6 ($\sim 10^8$ CFU/ml), in stationary phase (48-h-old cultures), or at various times as ethanol accumulated in the medium. Cells were inactivated by the addition of trichloroacetic acid (5% final concentration) and harvested by centrifugation (6,000 × *g*, 10 min). Lipids were extracted in chloroform-methanol (22) and used for phospholipid or fatty acid analyses. Neutral and polar lipids were separated with a Pasteur pipette column containing 0.7 g of silicic acid (Unisil silicic acid; Clarkson Chemical Company, Inc., Williamsport, Pa.). The lipid sample was suspended in 4 ml of chloroform and applied to the column. The column was rinsed with 8 ml of chloroform to elute the neutral lipid fraction. The polar lipid fraction was then eluted with 4 ml of chloroform-methanol (1:1) followed by 4 ml of methanol. These two fractions were used for fatty acid analyses.

Fatty acid analyses. The washed chloroform extracts of total lipids or lipid fractions were evaporated in vacuo. For fatty acid analysis, methyl esters were prepared by transesterification in 2% H_2SO_4 in methanol (39), extracted into pentane, and concentrated under N_2 before analysis. The various methyl esters were separated on a gas chromatograph (model 560; Tracor Instruments, Austin, Tex.) equipped with a flame ionization detector, using a glass column (2 mm by 180 cm) packed with 10% SP2330-coated Gas-Chrom Q (Supelco, Bellefonte, Pa.). Analyses were run isothermally at 155°C with a carrier gas (N_2) flow of 30 ml/min. Fatty acids were identified by using authentic standards. Fatty acid composition was expressed as a percentage of total peak area. Peak areas were calculated by a Minigrator digital integrator (Spectra-Physics, Mountain View, Calif.). To estimate the proportion of neutral and polar lipids, samples of fatty acid methyl esters of both polar and neutral fractions were adjusted to equal volumes before injection, and total peak areas were determined for each.

Argentation chromatography. Silica gel G plates (Rediplates; Fisher Scientific Co., Orlando, Fla.) were impregnated with silver nitrate by submersion in acetonitrile containing 10% silver nitrate (6). These were dried and activated for 1 h at 130°C. Fatty acid methyl esters of lipid samples and standards were spotted onto cooled plates. These were developed twice in toluene at −20°C. To visualize lipids, plates were sprayed with fluorescein and observed under UV illumination.

Phospholipid analyses. For the identification of phospholipids, lipid extracts were separated by thin-layer chromatography. Silica gel G plates were developed in chloroform-methanol-acetic acid (65:25:8) for one-dimensional analyses and with chloroform-methanol-water (65:25:4) as the second solvent in two-dimensional analyses (1). Lipids were visualized with iodine vapor (50), phosphate spray reagent (49), ninhydrin reagent for amino groups (50), Dragendorff reagent for choline (4), and α-naphthol reagent for glycolipids (38).

For the quantitative analysis of phospholipid composition, cells were uniformly labeled with $^{32}PO_4^{3-}$ (0.1 μCi/ml) under various growth conditions. Lipids were extracted in chloroform-methanol and separated by one-dimensional thin-layer chromatography. Autoradiograms of the phospholipids were prepared by exposing the thin-layer chromatography plates to X-ray film (Kodak type NS-2T) for 2 to 7 days. Spots containing radioactive lipids were scraped into scintillation vials and counted with a liquid scintillation counter (LS8000 series; Beckman Instruments, Inc., Fullerton, Calif.). In scraping the plates, the spots for cardiolipin and phosphatidic acid were combined, and the spots corresponding to the unknown and to lysophosphatidylethanolamine were combined. The phosphatidylglycerol, phosphatidylethanolamine, and phosphatidylcholine spots were each scraped separately.

Purification of phosphatidylcholine. Phosphatidylcholine was purified from lipid extracts of *Z. mobilis* by column chromatography, using a modification of the procedure described previously by Gilmore et al. (14). Phospholipids were applied to a column (2.5 by 12 cm) containing 25 g of activated silicic acid (Unisil silicic acid; Clarkson Chemical Company, Inc.) as a chloroform solution. Phospholipids were eluted with the following solvents containing increasing concentrations of methanol in chloroform: 500 ml of 9%, 625 ml of 18%, 500 ml of 25%, 1,500 ml of 40%, and 625 ml of 100% methanol. Fractions of 100 ml each were collected and spotted onto thin-layer chromatography plates for visualization with iodine vapor to identify lipid peaks. Concentrated samples of fractions containing lipids were run on thin-layer chromatography plates to identify the fraction(s) containing phosphati-

TABLE 1. Fatty acid composition of *Z. mobilis* ATCC 10988

Fatty acid	Composition (% total)[a]			
	Log-phase cells			Stationary-phase cells
	Total lipids	Polar lipid fraction	Neutral lipid fraction	Total lipids
Lauric acid (12:0)	Tr	Tr	11	Tr
Myristic acid (14:0)	11	10	12	17
Myristoleic acid (14:1)	Tr	Tr	6	Tr
Palmitic acid (16:0)	12	10	15	15
Palmitoleic acid (16:1)	7	2	16	3
Stearic acid (18:0)	1	Tr	3	1
Vaccenic acid (18:1)	69	78	30	64
Unknown	Tr	Tr	7	Tr

[a] Cells were grown in 0.56 M glucose without added ethanol and harvested in log phase (optical density at 550 nm, 0.6) and in stationary phase at 48 h (optical density at 550 nm, >2.0). Average of two experiments.

dylcholine. Phosphatidylcholine was recovered in the 40% methanol fraction as previously reported by Gilmore et al. (14). Infrared spectra were obtained with a spectrophotometer (model 567; The Perkin-Elmer Corp., Norwalk, Conn.), confirming our identification of this compound in *Z. mobilis*.

Phospholipid/protein ratios of isolated membranes. Batch cultures grown in 0.56 M glucose and various ethanol concentrations were harvested in exponential phase at an optical density of 0.7 at 550 nm. The cells were washed once with 0.03 M Tris buffer (pH 8.1). The resulting pellet was resuspended in 9 ml of 0.03 M Tris buffer (pH 8.1), and 1 ml of fresh lysozyme solution was added (1 mg/ml in 0.1 M EDTA, pH 7.3). The cells were kept on ice and were broken with a Sonicator Cell Disruptor (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). The sonic disruptor was adjusted to a setting of 5, and samples were pulsed six times for 15 s each time, with 3-min intervals between each pulse to allow for cooling of the sample. After a low-speed centrifugation for 5 min at 3,000 × *g* to eliminate cell debris, the supernatant was diluted to a final volume of 54 ml with 3 mM EDTA (pH 7.3) and harvested in Oak Ridge-type centrifuge tubes at 100,000 × *g* for 1 h. These samples were used for the analysis of lipid-soluble phosphorus and protein. Total phospholipid was estimated (after digestion in perchloric acid [9]) by measuring the concentration of phosphorus extracted into chloroform and methanol (2). Total protein was measured by the method of Lowry et al. (27).

Chemicals. Radioactive phosphate (carrier-free) was obtained from the Amersham Corp. (Arlington Heights, Ill.) and was used for the quantitative analysis of phospholipids. Yeast extract, tryptone, and agar were obtained from Difco Laboratories (Detroit, Mich.). Reagent-quality absolute ethanol was obtained from U.S. Industrial Chemicals Co. (New York, N.Y.). Fatty acid and phospholipid standards were obtained from the Sigma Chemical Co. (Saint Louis, Mo.). Lipid extracts of *E. coli* were prepared in this laboratory and were also used as standards for comparison to *Z. mobilis*.

RESULTS

Identification of major lipids in *Z. mobilis*. The fatty acid composition of lipids from *Z. mobilis* was determined by gas chromatography (Table 1). Only saturated and monounsaturated fatty acids were found. No cyclopropane or branched-chain fatty acids were observed. High levels of the 18:1 fatty acid were present in log-phase and stationary-phase cultures, with lower levels of myristic (14:0), palmitic (16:0), and palmitoleic (16:1) acids. The 18:1 fatty acid was identified as vaccenic acid by argentation chromatography, by comparison with the methyl esters of authentic oleic and vaccenic acids as standards. Increases in myristic and palmitic acids and slight decreases in palmitoleic and vaccenic acids were observed in stationary-phase cells.

The fatty acid components of the neutral and polar lipid fractions were also examined (Table 1). The neutral fraction contained higher levels of lauric, myristoleic, palmitic, and palmitoleic acids and lower levels of vaccenic acid than either the polar or the total lipid fractions. The neutral fraction also contained an unknown with a longer retention time than vaccenic acid. The polar lipid fraction was enriched in vaccenic acid at the expense of shorter-chain fatty acids as compared with both the total and neutral lipid fractions. Based on the total peak areas of the fatty acid chains in each fraction, approximately 25 to 28% of the total fatty acids were present in the neutral lipid fraction.

Attempts were made to alter the fatty acid composition of *Z. mobilis* by adding fatty acid supplements during growth (20 μg/ml). However, no changes in fatty acid composition were observed after growth in the presence of vaccenic acid, palmitoleic acid, or palmitic acid.

Table 2 shows a complete list of the polar lipids of *Z. mobilis*. Glycolipids were not observed in these experiments. The phospholipids found were identified by one- and two-dimensional thin-layer chromatography, authentic standards, and specific detection methods. All

TABLE 2. Phospholipid identification of *Z. mobilis* ATCC 10988

Phospholipid	Result with the following identification method[a]					
	Iodine vapor	^{32}P	Phosphate spray	Ninhydrin spray	Dragendorff spray reagent	α-napthol spray reagent
Cardiolipin	+	+	+	−	−	−
Phosphatidic acid	+	+	−	−	−	−
Phosphatidylglycerol	+	+	+	−	−	+
Phosphatidylethanolamine	+	+	+	+	+	−
Unknown	+	+	+	−	+	−
Lysophosphatidylethanolamine	+	+	−	+	+	−
Phosphatidylcholine	+	+	+	−	+	−

[a] +, Positive reaction with reagent; −, negative reaction with reagent.

of the phospholipids were observed with iodine vapor. Radioactive phosphate was incorporated into all of these during growth, proving their biosynthesis by *Z. mobilis*. Figure 1 shows an autoradiogram of the phospholipids of a stationary-phase culture after separation by thin-layer chromatography. All of the major phospholipid components showed a positive reaction with phosphate stain. The two components which were not positive were present in very low concentrations, probably too low to be visualized. Only phosphatidylethanolamine and lysophosphatidylethanolamine were ninhydrin positive. The ethanolamines, choline, and an unknown were positive with Dragendorff reagent. The sugar reagent, α-naphthol, was positive for phosphatidylglycerol.

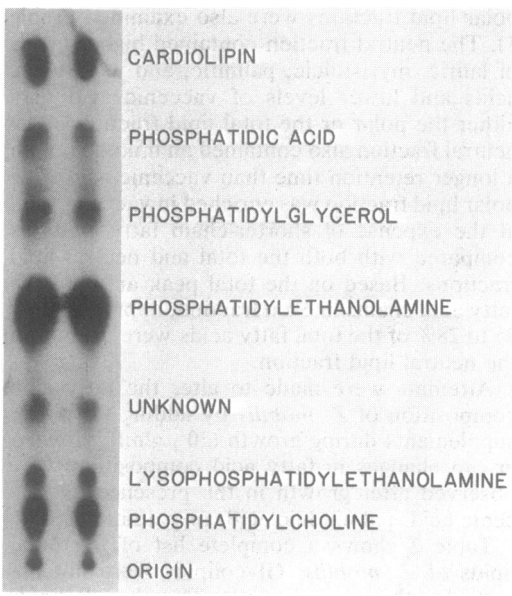

FIG. 1. Autoradiogram of phospholipids of stationary-stage cells of *Z. mobilis* ATCC 10988 uniformly labeled with radioactive phosphate.

Table 3 shows a comparison of the phospholipid composition of log- and stationary-phase cells. In both, the principal phospholipid was phosphatidylethanolamine. Stationary-phase cells contained higher levels of cardiolipin and lower levels of phosphatidylglycerol than log-phase cells.

Both the total fatty acid and phospholipid compositions of all of the six strains of *Z. mobilis* studied were remarkably similar. Phosphatidylethanolamine was the most abundant phospholipid in all cases and ranged from 49 to 60%. Vaccenic acid (18:1) was the most abundant fatty acid in all cases and ranged from 58 to 69% of the total fatty acids.

Effect of glucose concentration on growth and membrane composition. Figure 2 shows the effects of glucose on the growth of *Z. mobilis*. This organism is obligately fermentative, and glucose is required for growth even in rich media (13, 42). In medium containing 0.06 or 0.56 M glucose, the generation time of the organism was approximately 2 h. In medium containing 0.83 M glucose, the generation time of the organism was

TABLE 3. Phospholipid composition of *Z. mobilis* ATCC 10988[a]

Phospholipid	Composition (% total)	
	Log-phase cells	Stationary-phase cells
Cardiolipin + phosphatidic acid[b]	2	20
Phosphatidylglycerol	20	5
Phosphatidylethanolamine	61	55
Lysophosphatidylethanolamine + unknown	4	4
Phosphatidylcholine	13	16

[a] Cells were grown in 0.56 M glucose without added ethanol and harvested in log phase (optical density at 550 nm, 0.6) and in stationary phase at 48 h (optical density at 550 nm, >2.0). The results presented are the average of two experiments.

[b] Primarily cardiolipin.

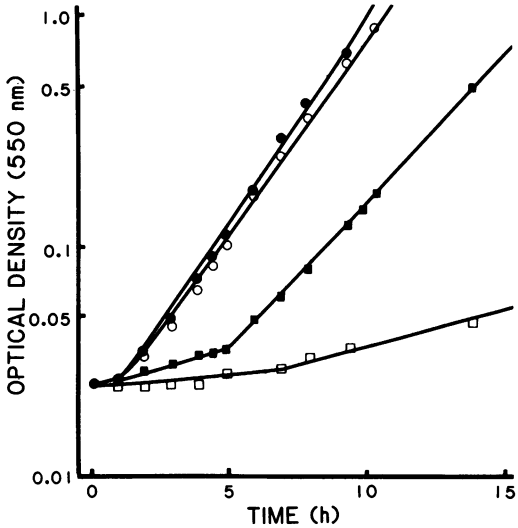

FIG. 2. Effects of glucose on growth of *Z. mobilis* ATCC 10988. An overnight culture was diluted 1:100 into fresh medium containing various concentrations of glucose. Symbols: ●, 0.06 M glucose; ○, 0.28 M glucose; ■, 0.56 M glucose; □, 1.11 M glucose.

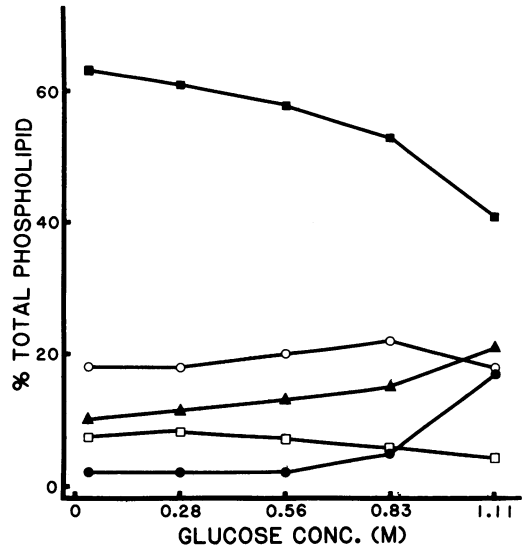

FIG. 3. Effect of glucose on phospholipid composition of *Z. mobilis* ATCC 10988. Symbols: ●, cardiolipin plus phosphatidic acid; ○, phosphatidylglycerol; ■, phosphatidylethanolamine; □, lysophosphatidylethanolamine plus unknown; ▲, phosphatidylcholine.

increased to 2.5 h. In medium containing 1.11 M glucose, growth of the organism was slow and somewhat erratic with a generation time greater than 13 h.

Table 4 shows the effects of glucose on the phospholipid-to-protein ratio in isolated membranes. These ratios were similar in membranes from cells grown in either 0.06 or 0.56 M glucose. However, a decrease in the lipid-to-protein ratio was observed in membranes from cells grown in the highest concentration of glucose (1.11 M).

Figure 3 shows the effects of glucose on phospholipid composition. Cells grown in various glucose concentrations were harvested in exponential phase. With increasing concentrations of glucose, an increase in cardiolipin plus phosphatidic acid and in phosphatidylcholine was observed at the expense of phosphatidylethanolamine and lysophosphatidylethanolamine.

The total fatty acid composition of exponential-phase cells grown in various concentrations of glucose is shown in Fig. 4. With increasing concentrations of glucose, vaccenic acid increased slightly at the expense of palmitic and palmitoleic acids. Since *Z. mobilis* contains appreciable quantities of neutral lipids, other glucose-induced changes in polar membrane lipids may have been masked. Thus, we examined the effects of glucose on the fatty acid composition of the polar lipid fraction (not shown). The polar lipid fraction was even more enriched in vaccenic acid than the total lipid fraction. Increasing concentrations of glucose caused only slight changes in the fatty acid composition of the polar lipid fraction. In the presence of 1.11 M glucose, small increases in myristic and palmitic acids were observed, with a decrease in vaccenic acid. These changes may be related to poor growth in the presence of 1.11 M glucose.

The reversibility of the glucose-induced changes was also investigated. Log-phase cultures grown in medium containing 1.11 M glucose were used to inoculate (1:100) fresh medium containing 0.56 M glucose, and the cells were harvested in exponential phase. Both the total fatty acid and phospholipid compositions of these cells were identical to control cultures grown only in the presence of 0.56 M glucose.

Effects of ethanol on growth and membrane composition. Figure 5 shows the effects of ethanol on the growth of *Z. mobilis*. Ethanol was

TABLE 4. Effects of glucose and ethanol on phospholipid-to-protein ratio of *Z. mobilis* ATCC 10988

Glucose (M)	Ratio of lipid phosphate (μM) to membrane protein (mg) at the following ethanol concn (M):[a]		
	0	0.7	1.3
0.06	0.50 (0.03)	0.31 (0.01)	0.21 (0.02)
0.56	0.55 (0.07)	0.33 (0.01)	No growth
1.11	0.34 (0.06)	No growth	No growth

[a] Mean (standard deviation); $n = 3$.

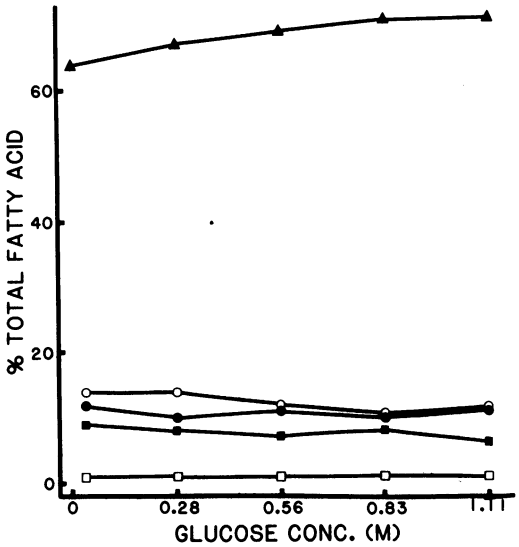

FIG. 4. Effect of glucose on total fatty acid composition of *Z. mobilis* ATCC 10988. Symbols: ●, myristic acid; ○, palmitic acid; ■, palmitoleic acid; □, stearic acid; ▲, vaccenic acid. Trace amounts of lauric acid, myristoleic acid, and an unknown were also found.

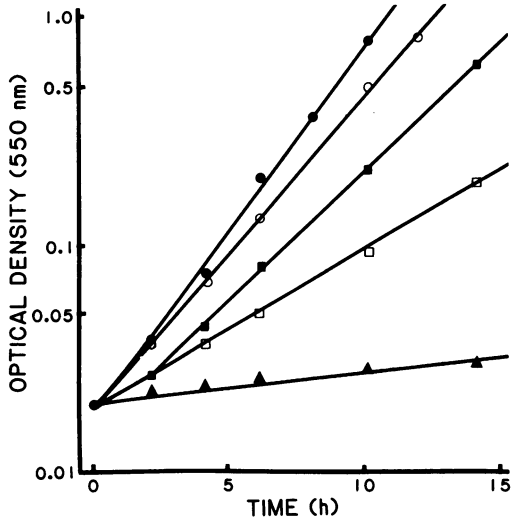

FIG. 5. Effect of ethanol on growth of *Z. mobilis* ATCC 10988. An overnight culture was diluted 1:100 into fresh medium containing 0.56 M glucose and various concentrations of ethanol. Symbols: ●, no ethanol; ○, 0.35 M ethanol; ■, 0.70 M ethanol; □, 1.04 M ethanol; ▲, 1.39 M ethanol.

added to the growth medium immediately before inoculation. Cells grown in 0.56 M glucose without added ethanol had a generation time of about 2 h, whereas cells grown in the presence of 1.04 M ethanol had a generation time of about 4 h. No growth was observed in concentrations of ethanol above 1.39 M. Alcohol is also produced by the organism during growth. However, less than 0.03 M ethanol was produced by cultures which had grown to an optical density of 1.0 (550 nm). This low level of ethanol did not have a major effect on alcohol concentration at the time of harvest. Table 5 shows a comparison of generation times in various glucose and ethanol concentrations. The inhibition of growth by ethanol was enhanced by higher concentrations of glucose.

Table 4 shows the effects of ethanol on the phospholipid-to-protein ratio of membranes from *Z. mobilis*. Increasing concentrations of ethanol caused a decrease in the lipid/protein ratio.

The effects of ethanol on phospholipid composition are shown in Fig. 6. This experiment was done in two ways. In one (A), cells were harvested as they produced ethanol during growth in medium containing 0.56 M glucose, irrespective of cell density. In the other (B), cells were grown in the presence of added ethanol and harvested in exponential phase (0.6 optical density). In both types of experiments, the level of cardiolipin increased at the expense of phosphatidylglycerol and phosphatidylethanolamine.

The effects of ethanol on the total fatty acid composition of *Z. mobilis* were also examined by both approaches (Fig. 7). Cells were allowed to accumulate alcohol during fermentation (Fig. 7A). In addition, cells were harvested during exponential growth in the presence of added ethanol (Fig. 7B). In both types of experiments, a slight decrease in vaccenic acid and a slight increase in palmitic acid were observed. Since *Z. mobilis* contains appreciable quantities of neutral lipids, these neutral lipids may have masked other alcohol-induced changes in the fatty acid composition of the polar membrane lipids. To investigate this possibility, we separated polar fractions by column chromatography and analyzed them. Growth in the presence of added ethanol caused no change in the fatty acid composition of the polar lipid fraction.

The reversibility of the effects seen in the presence of high concentrations of ethanol was

TABLE 5. Effect of growth conditions on generation time of *Z. mobilis* ATCC 10988

Glucose (M)	Generation time (h) in presence of the following ethanol concn (M):			
	0	0.35	0.70	1.04
0.06	1.7	2.0	2.2	2.7
0.56	1.8	2.2	2.8	4.2
0.83	2.4	3.5	4.3	8.0
1.11	>13.0[a]	>13.0[a]	No growth	No growth

[a] Growth slow and erratic.

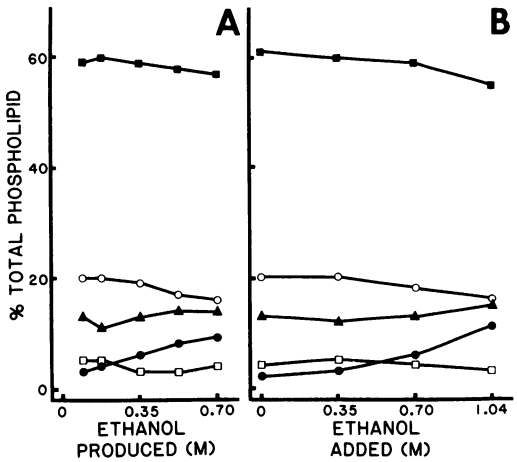

FIG. 6. Effect of ethanol on phospholipid composition of *Z. mobilis* ATCC 10988. Symbols: ●, cardiolipin plus phosphatidic acid; ○, phosphatidylglycerol; ■, phosphatidylethanolamine; □, lysophosphatidylethanolamine plus unknown; ▲, phosphatidylcholine. (A) Cells allowed to accumulate ethanol during fermentation; (B) cells harvested in exponential phase in the presence of added ethanol.

examined by diluting a culture (1:100) grown in 1.04 M ethanol into fresh medium containing 0.56 M glucose with no added ethanol. Cells were then harvested in exponential phase, and the lipid composition was examined. Both the fatty acid and phospholipid compositions of these cells were identical to those of cells grown with inocula from overnight cultures in 0.56 M glucose without added ethanol.

DISCUSSION

Many microorganisms have been shown to alter their lipid composition during growth in the presence of ethanol (3, 15, 16, 20, 23, 28, 32, 35, 45). For *E. coli* (15, 18, 20) and *S. cerevisiae* (3), growth in the presence of ethanol results in an increase in 18:1 fatty acid (up to 57%) at the expense of 16:0 and, to a lesser extent, 16:1. These changes in *E. coli* have been proposed as being adaptive, facilitating enhanced growth and survival in the presence of ethanol (20). *Lactobacillus heterohiochii*, an organism capable of tolerating over 20% ethanol (46, 48), contains phospholipids rich in 20- to 24-carbon monounsaturated and cyclopropane fatty acids (48). Other organisms, such as *Mycobacterium smegmatis* (45) and the protozoan, *Tetrahymena pyriformis* (28), also increase the unsaturation and chain length of their fatty acyl groups during growth in the presence of ethanol. In this study, over 60% of the total fatty acids in *Z. mobilis* were found to be vaccenic acid (18:1), consistent with a previous report (29). Our results indicate that the membranes of *Z. mobilis* are even further enriched in vaccenic acid, with this fatty acid representing approximately 78% of the acyl chains in the polar lipid fraction. Although this composition did not change to a major extent as a result of ethanol addition or production, the fatty acid composition of the polar lipids of *Z. mobilis* represents an extreme of the changes which are induced by ethanol in *E. coli* (18) and in *S. cerevisiae* (3). Thus, the fatty acid composition of *Z. mobilis* may represent an evolutionary adaptation for survival in the presence of ethanol.

High levels of 18:1 are not limited to alcohol-producing bacteria such as *Z. mobilis*. Other organisms such as the photosynthetic bacterium, *Rhodopseudomonas sphaeroides* (5), and some of the pseudomonads also contain high levels of 18:1 but do not produce ethanol. These high levels of 18:1 may have been of evolutionary advantage for survival and competition in the presence of environmental stress.

One may speculate as to why many microorganisms (especially *E. coli* and *S. cerevisiae*) exhibit alcohol-induced changes in fatty acid composition whereas the fatty acid composition of *Z. mobilis* remains relatively unaffected by ethanol. Both *E. coli* and *S. cerevisiae* possess diverse metabolic capabilities, including both oxidative and fermentative metabolisms. The expression of these is regulated and dependent upon the availability of suitable acceptors for respiration. By contrast, *Z. mobilis* is very limit-

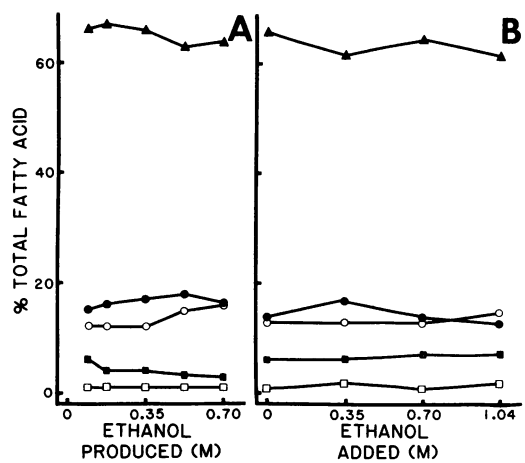

FIG. 7. Effect of ethanol on total fatty acid composition of *Z. mobilis* ATCC 10988. Symbols: ●, myristic acid; ○, palmitic acid; ■, palmitoleic acid; □, stearic acid; ▲, vaccenic acid. Trace amounts of lauric acid, myristoleic acid, and an unknown were also found. (A) Cells allowed to accumulate ethanol during fermentation; (B) cells harvested in exponential phase in the paresence of added ethanol.

ed in metabolic capability. *Z. mobilis* is obligately fermentative and produces CO_2 and ethanol as fermentation end products from glucose. In the absence of metabolic diversity, this organism may have evolved to synthesize only the fatty acid composition most advantageous for growth and survival in the presence of ethanol.

On the basis of our studies with *Z. mobilis* and those of other workers with different organisms (3, 15, 18, 20, 23, 28, 32, 45), a clear trend in the relationship between fatty acid structure and ethanol tolerance is emerging. In all cases, longer-chain fatty acids appear beneficial. These longer acyl chains are either monounsaturated or branched chain, modifications which may be essential for their mixing with other lipids or for their utilization by the acyl transferase enzymes during phospholipid assembly. Long-chain saturated fatty acids are not produced and may be harmful. Phospholipid molecules containing long saturated acyl chains have high melting temperatures and promote phase separation and other adverse effects within the membrane (19).

Increases in the chain lengths of monounsaturated or branched-chain fatty acids would tend to compensate for some of the physical changes in membrane structure caused by the presence of ethanol. Ethanol would tend to weaken the water lattice structure, decreasing the strength of hydrophobic interactions (51) which maintain membrane integrity. In addition, the presence of ethanol within the hydrophobic core of the membrane would tend to decrease the extent of van der Waals interactions, increasing the freedom of motion, and to increase the polarity of this region. All of these effects would decrease the integrity of the primary permeability barrier of the cell. Indeed, it is well established that ethanol promotes membrane leakage in both bacteria (11) and eucaryotic cells (37). Longer-chain fatty acids would increase the surface area for hydrophobic and van der Waals interactions and decrease the polarity within the hydrophobic core of the membrane, restoring the primary permeability barrier of the cell during growth in the presence of ethanol.

Ethanol induced other changes in the membrane composition of *Z. mobilis*. These included small shifts in phospholipid composition and a decrease in the lipid-to-protein ratio. Similar ethanol-induced decreases in the lipid-to-protein ratios of membranes and cells have been reported with *E. coli* (16), *M. smegmatis* (45), and *Bacillus subtilis* (32). These may also decrease cell permeability by decreasing the available area on the membrane surface for the partitioning of diffusible molecules. Alternatively, this may represent part of a general stress response resulting from slower growth.

Glucose also induced changes in the membrane composition of *Z. mobilis*. High concentrations of glucose reduced the rate of growth of *Z. mobilis* and increased the sensitivity of cultures to growth inhibition by ethanol. The extent of growth inhibition by ethanol and by high concentrations of glucose were roughly additive, suggesting some similarity in their mechanisms of action. Although these molecules are very dissimilar, chemically, high concentrations of both of these would have similar effects on the colligative properties of water. Both would tend to decrease the extent of water lattice structure and the strength of hydrophobic interactions.

Z. mobilis has extremely high levels of vaccenic acid (18:1) in the membrane lipids. The implication is that the increase in chain length of these phospholipids is beneficial for survival in the presence of ethanol. To firmly establish this, future studies will need to be done to isolate mutants in which the fatty acid composition can be controlled by the addition of exogenous lipids. Such mutants would allow direct tests of the beneficial role of lipids containing high levels of vaccenic acid in *Z. mobilis*.

During the course of these studies (V. C. Carey, and L. O. Ingram, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, K165, P.248), Tornabene et al. (47) and Ohta et al. (29) described the lipid composition of *Z. mobilis*. In most cases, these results are in agreement with 18:1 as the principal fatty acid component and phosphatidylethanolamine as the dominant phospholipid. However, unlike the results of Tornabene et al., our studies and those of Ohta et al. did not find significant levels of a 17:0 fatty acid. It is possible that this unusual component may represent either an error in identification or a contaminant of the cells introduced during analysis and processing. This latter possibility would be consistent with the reported absence of this fatty acid as a component of phospholipid molecules and the apparent abundance in a neutral lipid fraction. Since neutral lipids (di- and triglycerides) and polar lipids are synthesized via phosphatidic acid, using the same acyl transferase enzyme system, the total absence of 17:0 in the polar fraction suggests that this fatty acid may not be an esterified component.

ACKNOWLEDGMENTS

We thank A. Sekthira for his assistance in performing lipid-to-protein ratios and K. M. Dombek for his critical reading of this manuscript.

This investigation was supported by grant PCM-8204928 from the National Science Foundation, by grant AA 03816 from the National Institute of Alcohol Abuse and Alcoholism, and by the Florida Alcohol Research Center (NIAAA AA05793). L.O.I. is the recipient of a Career Development award from the National Institute of Alcohol Abuse and Alcoholism (KO2 00036).

LITERATURE CITED

1. **Ames, G. F.** 1968. Lipids of *Salmonella typhimurium* and *Escherichia coli*: structure and metabolism. J. Bacteriol. **95**:833–843.
2. **Bartlett, G. R.** 1959. Phosphorus assay in column chromatography. J. Biol. Chem. **234**:466–468.
3. **Beavan, M. J., C. Charpentier, and A. H. Rose.** 1982. Production and tolerance of ethanol in relation to phospholipid fatty-acyl composition in *Saccharomyces cerevisiae* NCYC 431. J. Gen. Microbiol. **128**:1447–1455.
4. **Beiss, U.** 1964. Zur Papierchromatographischen auftrennung von Pflanzenlipiden. J. Chromatogr. **13**:104–110.
5. **Campbell, T. B., and D.R. Lueking.** 1983. Long-chain fatty acid assimilation by *Rhodopseudomonas sphaeroides*. J. Bacteriol. **153**:782–790.
6. **Cubero, J. M., and H. K. Mangold.** 1965. Chromatography on absorbent layers impregnated with $AgNO_3$. Microchem. J. **9**:227–236.
7. **Dadds, M. J. S., and P. A. Martin.** 1973. The genus *Zymomonas*—a review. J. Inst. Brew. London **79**:386–391.
8. **Dawes, E. A., D. W. Ribbons, and P. J. Large.** 1966. The route of ethanol formation in *Zymomonas mobilis*. Biochem. J. **98**:795–803.
9. **Dawson, R. M. C.** 1960. A hydrolytic procedure for the identification and estimation of individual phospholipids in biological samples. Biochem. J. **75**:45–53.
10. **De Ley, J., and J. Swings.** 1976. Phenotypic description, numerical analysis, and proposal for an improved taxonomy and nomenclature of the genus *Zymomonas* Kluyver and van Niel 1936. Int. J. Syst. Bacteriol. **26**:146–157.
11. **Enequist, H. G., T. R. Hirst, S. Harayama, S. J. S. Hardy, and L. L. Randall.** 1981. Energy is required for maturation of exported proteins in *Escherichia coli*. Eur. J. Biochem. **116**:227–233.
12. **Gibbs, M., and R. D. DeMoss.** 1951. Ethanol formation in *Pseudomonas lindneri*. Arch. Biochem. Biophys. **34**:478–479.
13. **Gibbs, M., and R. D. DeMoss.** 1954. Anaerobic dissimilation of ^{14}C-labeled glucose and fructose by *Pseudomonas lindneri*. J. Biol. Chem. **207**:689–694.
14. **Gilmore, R., N. Cohn, and M. Glaser.** 1979. Rotational relaxation times of 1,6-diphenyl-1,3,5-hexatriene in phospholipids isolated from LM cell membranes. Effects of phospholipid polar head-group and fatty acid composition. Biochemistry **18**:1050–1056.
15. **Ingram, L. O.** 1976. Adaptation of membrane lipids to alcohols. J. Bacteriol. **125**:670–678.
16. **Ingram, L. O.** 1977. Preferential inhibition of phosphatidyl ethanolamine synthesis in *E. coli* by alcohols. Can. J. Microbiol. **23**:779–789.
17. **Ingram, L. O.** 1981. Mechanism of lysis of *Escherichia coli* by ethanol and other chaotropic agents. J. Bacteriol. **146**:331–336.
18. **Ingram, L. O., B. F. Dickens, and T. M. Buttke.** 1980. Reversible effects of ethanol on *E. coli*. Adv. Exp. Med. Biol. **126**:299–337.
19. **Ingram, L. O., L. C. Eaton, G. W. Erdos, T. F. Tedder, and N. L. Vreeland.** 1982. Unsaturated fatty acid requirement in *Escherichia coli*: mechanism of palmitate-induced inhibition of growth of strain WNI. J. Membr. Biol. **65**:31–40.
20. **Ingram, L. O., N. S. Vreeland, and L. C. Eaton.** 1980. Alcohol tolerance in *Escherichia coli*. Pharmacol. Biochem. Behav. **13**:191–195.
21. **Issenberg, P., and I. Hornstein.** 1970. Analysis of volatile flavor components of foods, p. 295–328. *In* J. C. Giddings and R. A. Keller (ed.), Advances in chromatography. Marcel Dekker, Inc., New York.
22. **Kanfer, J., and E. P. Kennedy.** 1963. Metabolism and function of bacterial lipids. J. Biol. Chem. **238**:2919–2922.
23. **Kates, M., D. J. Kushner, and A. T. James.** 1962. The lipid composition of *Bacillius cereus* as influenced by the presence of alcohols in the culture medium. Can. J. Biochem. Physiol. **40**:83–93.
24. **Kluyver, A. J., and W. J. Hoppenbrouwers.** 1931. Ein merkwurdiges Garungsbakterium: Lindner's *Termobacterium mobile*. Arch. Mikrobiol. **2**:245–260.
25. **Lee, K. J., M. L. Skotnicki, D. E. Tribe, and P. L. Rogers.** 1980. Kinetic studies on a highly productive strain of *Zymomonas mobilis*. Biotechnol. Lett. **2**:339–344.
26. **Lee, K. J., D. E. Tribe, and P. L. Rogers.** 1979. Ethanol production by *Zymomonas mobilis* in continuous culture at high glucose concentrations. Biotechnol. Lett. **1**:421–426.
27. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
28. **Nandii-Kishore, S. G., S. M. Mattox, C. E. Martin, and G. A. Thompson, Jr.** 1979. Membrane changes during the growth of *Tetrahymena* in the presence of ethanol. Biochim. Biophys. Acta **551**:315–327.
29. **Ohta, K., K. Supanwong, and S. Hayashida.** 1981. Environmental effects on ethanol tolerance of *Zymomonas mobilis*. J. Ferment. Technol. **59**:435–439.
30. **Okafor, N.** 1975. Microbiology of Nigerian palm wine with particular reference to bacteria. J. Appl. Bacteriol. **38**:81–88.
31. **Ostovar, K., and P. G. Keeney.** 1973. Isolation and characterization of microorganisms involved in the fermentation of Trinidad's cacao beans. J. Food Sci. **38**:611–617.
32. **Rigomier, D., J. Bohin, and B. Lubochinsky.** 1980. Effects of ethanol and methanol on lipid metabolism in *Bacillus subtilis*. J. Gen. Microbiol. **121**:139–149.
33. **Rogers, P. L., K. J. Lee, and D. E. Tribe.** 1979. Kinetics of alcohol production by *Zymomonas mobilis* at high sugar concentrations. Biotechnol. Lett. **1**:165–170.
34. **Rogers, P. L., K. J. Lee, and D. E. Tribe.** 1980. High productivity ethanol fermentations with *Zymomonas mobilis*. Process Biochem. **15**:7–11.
35. **Rose, A. H., and M. J. Beavan.** 1981. End-product tolerance and ethanol, p. 513–531. *In* A. Hollaender, R. Rabson, P. Rogers, A. San Pietro, R. Valentine, and R. Wolfe (ed.), Trends in the biology of fermentations for fuel and chemicals. Plenum Publishing Corp., New York.
36. **Ruiz-Argueso, T., and A. Rodriguez-Navarro.** 1975. Microbiology of ripening honey. Appl. Microbiol. **30**:893–896.
37. **Seeman, P.** 1972. The membrane actions of anaesthetics and tranquilizers. Pharmacol. Rev. **24**:583–655.
38. **Siakotos, A. N., and G. Rouser.** 1965. Analytical separation of nonlipid water-soluble substances and gangliosides from other lipids by dextran gel column chromatography. J. Am. Oil Chem. Soc. **42**:913–919.
39. **Silbert, D. F., R. C. Ladenson, and J. L. Honegger.** 1973. The unsaturated fatty acid requirement in *Escherichia coli*. Biochim. Biophys. Acta **311**:349–361.
40. **Skotnicki, M. L., K. J. Lee, D. E. Tribe, and P. L. Rogers.** 1981. Comparison of ethanol production by different *Zymomonas* strains. Appl. Environ. Microbiol. **41**:889–893.
41. **Swings, J., and J. De Ley.** 1975. Genome deoxyribonucleic acid of the genus *Zymomonas* Kluyver and van Niel 1936: base composition, size, and similarities. Int. J. Syst. Bacteriol. **25**:324–328.
42. **Swings, J., and J. De Ley.** 1977. The biology of *Zymomonas*. Bacteriol. Rev. **41**:1–46.
43. **Swings, J., K. Kersters, and J. De Ley.** 1977. Taxonomic position of additional *Zymomonas mobilis* strains. Int. J. Syst. Bacteriol. **27**:271–273.
44. **Swings, J., and W. Van Pee.** 1977. Infra-red spectroscopy of *Zymomonas* cells. J. Gen. Appl. Microbiol. **23**:297–301.
45. **Taneja, R., and G. K. Khuller.** 1980. Ethanol-induced alterations in phospholipids and fatty acids of *Mycobacterium smegmatis* ATCC 607. FEMS Microbiol. Lett. **8**:83–85.
46. **Tanigawa, T., and M. Umezu.** 1979. Studies on the cell wall of Hiochi bacteria with special reference to the change of its thickness due to growth conditions. J. Gen.

Appl. Microbiol. 25:31–40.
47. **Tornabene, T. G., G. Holzer, A. S. Bittner, and K. Grohmann.** 1982. Characterization of the total extractable lipids of *Zymomonas mobilis* var. *mobilis*. Can. J. Microbiol. **28**:1107–1118.
48. **Uchida, K.** 1974. Occurrence of saturated and monounsaturated fatty acids with unusually-long-chains (C20-C30) in *Lactobacillus heterohiochii*, an alcoholophilic bacterium. Biochim. Biophys. Acta **348**:86–93.
49. **Vaskovsky, V. E., E. Y. Kostetsky, and I. M. Vasendin.** 1975. A universal reagent for phospholipid analysis. J. Chromatogr. **114**:129–141.
50. **Work, T. S., and E. Work (ed.)** 1972. Laboratory techniques in biochemistry and molecular biology, vol. 3, p. 420–423. American Elsevier Publishing Co., Inc., New York.
51. **Yaacobi, M., and A. Ben-Naim.** 1974. Solvophobic interaction. J. Phys. Chem. **78**:175–178.