

Effect of Butanol Challenge and Temperature on Lipid Composition and Membrane Fluidity of Butanol-Tolerant *Clostridium acetobutylicum*

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The effect of butanol challenge (0, 1.0, 1.5% [vol/vol]) and growth temperature (22, 37, 42°C) on the membrane composition and fluidity of *Clostridium acetobutylicum* ATCC 824 and a butanol-tolerant mutant, SA-2, was examined in chemically defined medium. Growth of strain ATCC 824 into the stationary phase coincided with a gradual increase in the percent saturated to percent unsaturated (SU) fatty acid ratio. When challenged with butanol at 22 and 37°C, ATCC 824 demonstrated an immediate (within 30 min) dose-response increase in the SU ratio. This strain showed little additional change over a 48-h fermentation. Compared with ATCC 824, growth of SA-2 into the late stationary phase at 22 or 37°C resulted in an overall greater increase in the SU ratio for both unchallenged and challenged cells. This effect was minimized when SA-2 was challenged at 42°C, probably due to the combination of the membrane fluidizing effect of butanol and the elevated temperature. Growth at 42°C resulted in an increase in longer acyl chain fatty acids at the expense of shorter acyl chains for both strains. The membrane fluidity exhibited by SA-2 remained essentially constant at various butanol challenge and temperature combinations, while that for the ATCC 824 strain increased with increasing butanol challenge. By synthesizing an increased amount of saturated fatty acids, the butanol-tolerant SA-2 strain has apparently developed a mechanism for maintaining a more stable membrane environment. Growth of the microorganism is necessary for butanol to fluidize the membrane. Incorporation of exogenous fatty acids (18:1) did not significantly improve the butanol tolerance of either strain. Since SA-2 was able to produce only trace amounts of either butanol or acetone, increased tolerance to butanol does not necessarily coincide with greater solvent yields in this strain.

Currently, the acetone-butanol-ethanol (ABE) fermentation method does not compete economically with the petrochemical route for butanol production. The energy requirements for distillate recovery of butanol (normally present at less than 2 to 3% [wt/vol] in the fermentation broth) exceed the net energy gain (30). It has been calculated that increasing the butanol concentration from 1.2 to 1.9% (wt/wt) would halve the energy consumption for distillation (30). The primary reason for the low butanol concentration is the toxicity of the butanol to the producing microorganism, since, in most cases, there is plenty of substrate remaining after the fermentation terminates.

Results of studies in other biological systems have shown that alcohols act to disrupt the cell membrane structure and thereby inhibit cellular processes (11, 18, 35).

The exact mechanism by which alcohols affect the membrane bilayer is not known. Ingram and Buttke (11) suggested that the short-chain alcohols (i.e., one to four carbons) solidify or freeze the membrane by intercalating between and restricting movement of the acyl chains. Longer-chain alcohols (i.e., four to nine carbons) fluidize the membrane by anchoring at the phospholipid head groups and increase the distance between the acyl chains. Also, the particular physical effect of the alcohol and the consequent biological response of the microorganism are dependent on other environmental factors such as temperature and pH.

It has been reported that butanol causes an increase in the fluidity of lipid dispersions derived from *Clostridium acetobutylicum* ATCC 824 (36). The biological response of the microorganism to offset the disruptive effect of butanol is

to increase the synthesis of saturated acyl chains at the expense of unsaturated chains. A similar response is observed when *Clostridium butyricum* is exposed to elevated temperatures (14). It has been further suggested (36) that if the cell could not alter its lipid composition in response to butanol, the cell would be even more sensitive to this alcohol than is observed. The objective of this investigation was to examine the effect of butanol challenge and temperature on the fatty acid composition and membrane fluidity of wild-type and butanol-tolerant *C. acetobutylicum* strains. A comparison of the membrane-related changes in the two strains should help to elucidate the basis for increased butanol tolerance.

MATERIALS AND METHODS

Strains and culture maintenance. *C. acetobutylicum* ATCC 824 was grown anaerobically at 37°C for 72 h in either brain heart infusion (BHI) broth or cooked meat medium (Difco Laboratories, Detroit, Mich.) and subsequently stored at room temperature under anaerobic conditions as spores. Before use in a particular experimental study, the ATCC 824 strain was subjected to a heat shock treatment (80°C for 10 min followed by cooling on ice for 2 min). The butanol-tolerant SA-2 mutant was derived from the ATCC 824 strain by using a serial enrichment procedure that was carried out essentially as described previously (21). This strain was maintained by weekly transfer through BHI and chemically defined P2 medium (R. Machanoff, personal communication) plus 1.5% butanol. SA-2 was inoculated at the 1% (vol/vol) level into BHI broth and incubated at 37°C for 15 h or until the culture exhibited active growth. The cells (5 ml) were then inoculated into 90 ml of P2 medium. P2 medium was

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composed of the following separately prepared solutions (in grams per 100 ml of distilled water, unless indicated otherwise): 20 g of glucose–790 ml of distilled water (solution I), 0.5 g of K_2HPO_4 –0.5 g of KH_2PO_4 –2.2 g of CH_3COONH_4 (solution II), 2.0 g of $MgSO_4 \cdot 7H_2O$ –0.1 g of $MnSO_4 \cdot H_2O$ –0.1 g of $NaCl$ –0.1 g of $FeSO_4 \cdot 7H_2O$ (solution III), and 100 mg of *p*-aminobenzoic acid–100 mg of thiamine–1 mg of biotin (solution IV). Solutions I and II were autoclaved separately, cooled to room temperature, and subsequently mixed. Solutions III and IV were filter sterilized with a 0.2 μ m pore size filter (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.). Portions (10 and 1 ml) of solutions III and IV, respectively, were added aseptically to the glucose-buffer solution. The final pH of the P2 medium was 6.6. Following 1 h of incubation in P2, the culture was challenged with filter-sterilized *n*-butanol solution to give a final concentration of 1.5% (vol/vol). The culture was incubated for an additional 48 h to allow for cell growth and then stored anaerobically at room temperature.

Culture conditions, media, and chemical reagents. *C. acetobutylicum* cultures were routinely grown under an anaerobic atmosphere (85% N_2 , 10% CO_2 , 5% H_2) in an anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, Mich.). Media, distilled water, and *n*-butanol were placed in the anaerobic chamber at least 24 h before use to ensure the complete removal of oxygen. Glucose-soluble medium (21) was used in the development of butanol tolerance in the SA-2 strain. BHI and P2 media were used to grow cells for challenge studies, lipid determinations, fluorescence depolarization, and mutant stability studies. *trans*-Parinaric acid (*trans*-9,11,13,15-octadecatetraenoic acid) was obtained from Molecular Probes (Junction City, Ore.) and purified for use by dissolving in absolute ethanol and passage through a silicic acid column (Sep-Pak 51900; Waters Associates, Inc., Milford, Mass.). A stock solution (10 mg of ethanol per ml) prepared with 0.1% BHT as an antioxidant was stored at $-80^\circ C$ in the dark under N_2 . A working solution (0.5 mM) was prepared by diluting the stock solution 1:100 with ethanol. This solution was kept at $-20^\circ C$ in the dark under N_2 . Glassware used in the lipid determinations was cleaned with Nochromix solution (Godax Laboratories, Inc., New York, N.Y.) and rinsed repeatedly with distilled water before use.

Characterization of fermentation profiles. The fermentation profiles of *C. acetobutylicum* ATCC 824 and the butanol-tolerant SA-2 derivative were examined at different temperatures (22, 37, $42^\circ C$) and butanol challenges (0, 1.0, 1.5%). Inocula were prepared by transferring 40 ml of the SA-2 or heat-shocked ATCC 824 culture into 400 ml of BHI broth in a 1-liter screw-cap Erlenmeyer flask. The cultures were incubated at $37^\circ C$ for 4 h and then shifted to the experimental temperature for an additional 20 h. The BHI culture of each strain was divided into three 100-ml portions which were subsequently used to inoculate three 2-liter bottles (Schott-Duran, Mainz, Federal Republic of Germany through Bellco Glass, Inc., Vineland, N.J.) containing 1.8 liters of P2 medium. The cultures were incubated for 1 h and then challenged with 100 ml of sterile butanol solution to give final concentrations of 0, 1.0, and 1.5% (vol/vol). The time of butanol addition was designated as time zero. Samples (150 ml) were taken at 0.5, 4, 8, 12, 16, 24, 36, and 48 h. From each sample, a 15-ml fraction was removed to measure growth and pH changes. Growth was monitored by measuring the optical density at 600 nm with a spectrophotometer (Spectronic 20; Bausch & Lomb, Inc., Rochester, N.Y.). Specific growth rates for the wild-type and SA-2 strains at

different temperature and challenge combinations were determined as described previously (21). The remaining 135 ml of sample was centrifuged at $16,300 \times g$ for 15 min at $4^\circ C$. The supernatant was decanted and stored at $-20^\circ C$ until the fermentation end products were analyzed by gas-liquid chromatography. The cell pellets were deposited into screw-cap, 1.2-ml cryotubes (Nunc, Roskilde, Denmark, through Vanguard International, Neptune, N.J.) and stored at $-196^\circ C$ under liquid nitrogen until polarization and fatty acid compositional analyses were carried out.

Quantitation of fermentation end products. The quantitation of butyric acid, acetone, and butanol was accomplished by using a gas chromatograph (5700A; Hewlett-Packard Co., Avondale, Pa.) equipped with a flame ionization detector. An integrator (3390A; Hewlett-Packard) was used to analyze the data and plot chromatographs. For the determination of butyric acid, the supernatant was mixed (1:1) with formic acid (88%) prior to analysis. A 10- μ l syringe (Hamilton Co., Reno, Nev.) was used to inject a 2- μ l fraction of acidified sample into a glass column (6 ft [1.83 m] by 2 mm [inner diameter]; Supelco, Inc., Bellefonte, Pa.) packed with 60/80 Carbowax C–0.3% Carbowax 20M–0.1% H_3PO_4 . The column temperature was held at $160^\circ C$. Gas flow rates were as follow: N_2 , 60 ml/min; H_2 , 60 ml/min; and air, 240 ml/min. A 0.5% aqueous solution of butyric acid was used as the standard. Butanol and acetone were determined by injecting a 2- μ l fraction of supernatant into a glass column (6 ft [1.83 m] by 2 mm [inner diameter]) packed with 80/100 Carbowax C–0.1% SP-1000 (Supelco). The column temperature was held at $120^\circ C$. Gas flow rates were as follow: N_2 , 20 ml/min; H_2 , 20 ml/min; and air, 240 ml/min. A 1% aqueous solution of butanol was used as the standard.

Analysis of fatty acid composition. Fatty acid methyl esters (FAMES) were prepared by using whole cells and a methanolic-based reagent (29). The reagent was prepared by mixing 20 ml of a 25% (vol/vol) solution of sodium methoxide (Aldrich Chemical Co., Inc., Milwaukee, Wis.) with 40 ml of anhydrous methanol. Then, 1 ml of wet-packed cells, 1 ml of benzene, and 1 ml of methanolic base reagent were added into a Teflon (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.)-lined screw-cap tube (18 by 150 mm). The tube was sealed and heated in a water bath at $80^\circ C$ for 20 min. The sample was allowed to cool to room temperature before 3 ml of water and 3 ml of diethyl ether were added and vortexed. The lower aqueous phase was removed and discarded, while the upper benzene-ether layer was washed twice with 2 ml of water and dried over sodium sulfate crystals. The solvent layer, containing the FAMES, was transferred to glass scintillation vials (15 ml, 27.3 by 59.5 mm) with metal foil-lined screw caps and stored under N_2 at $-20^\circ C$. Immediately before analysis, the remaining solvents were removed by evaporation to dryness under a gentle stream of N_2 . A 2- μ l fraction of FAMES dissolved in carbon disulfide (Aldrich) was injected into a glass column (6 ft [1.83 m] by 2 mm [inner diameter]) packed with GP 3% SP-2100 DOH on 100/120 Supelcoport (Supelco). The column temperature was programmed for 150 to $250^\circ C$ at $4^\circ C$ /min. The FAMES were identified by comparing the retention times with bacterial FAME standards (Supelco).

Analysis of cell membrane fluidity. Changes in the cell membrane state (lateral phase separation) were followed by using fluorescent depolarization (18, 19, 23, 25, 27, 32, 33). Cells were suspended in 6 ml of 0.1 M potassium citrate buffer to give a scattering optical density at 300 nm of 0.2. *trans*-Parinaric acid (0.5 mM; 9 μ l) was added to 3 ml of sample, after which it was incubated for 30 min at the

particular temperature of cell growth. Fluorescence polarization studies were done with a spectrofluorimeter (650-10s; The Perkin-Elmer Corp., Norwalk, Conn.) fitted with a thermostatted turret cell holder (650-0139; Hitachi), cell stirrer, and polaroid filter rotator (C. N. Wood Manufacturing, Newtown, Pa.). Excitation occurred at 325 nm, while emission was read at 420 nm. Fluorescence intensity was measured for parallel (I_{\parallel}) and perpendicular (I_{\perp}) orientations of the emission filter. The polarization ratio was calculated as $[(I_{\parallel} - I_{\perp}) / (I_{\perp} + I_{\perp s})] \times G$, where $I_{\parallel s}$ and $I_{\perp s}$ are corrections for scattering, and G is the grating factor equal to the orientations of the filters (4).

Immediate effect of butanol on cell membrane fluidity. The immediate effect of butanol challenge on the membrane fluidity of *C. acetobutylicum* ATCC 824 and the butanol-tolerant SA-2 mutant was also determined. Cells were grown as described above, except that 500 ml of P2 culture was challenged with 0 and 1.0% (vol/vol) butanol. Cells were initially sampled at 0.5- and 1.0-h intervals and thereafter for up to 9 h. Both strains were also challenged under nongrowth (P2 medium without ammonium acetate) conditions.

Incorporation of exogenous fatty acids. Elaidic and oleic acids (Sigma Chemical Co., St. Louis, Mo.) were incorporated into the plasma membranes of *C. acetobutylicum* ATCC 824 and the SA-2 mutant by the method described by Khuller and Goldfine (15). Cells were grown anaerobically in a Casamino Acids (Difco)-based medium (3) with and without added biotin. The fatty acids were dissolved in 95% ethanol to a final concentration of 25 mg/liter and filter sterilized. A 0.2-ml portion of this mixture was added as a supplement to the biotin-free medium. To minimize turbidity resulting from the fatty acids, 0.002% Brij 35 (Sigma) was added to the fatty acid supplement. Late-exponential-phase cells of the control and fatty acid-supplemented *C. acetobutylicum* ATCC 824 and SA-2 mutant were transferred to P2, and the growth responses of these strains to butanol challenges (0, 1.0, 1.5% [vol/vol]) were monitored.

Extraction of total lipids. Total lipids of *C. acetobutylicum* ATCC 824 and the SA-2 mutant were extracted by a modification of the method described by Bligh and Dyer (2) and Mahfouz et al. (22). The cellular material (whole cells or membrane fragments) was transferred to a 125-ml Erlenmeyer flask, and 30 ml of a 1:2 ratio of chloroform-methanol was added. The slurry was agitated at 150 rpm on a laboratory rotary shaker (model G2; New Brunswick Scientific Co., Inc., Edison, N.J.) for 3 h. The suspension was transferred to a 50-ml polypropylene centrifuge tube with a plug seal cap (Corning Glass Works, Corning, N.Y.) and centrifuged at $900 \times g$ for 5 min. To the supernatant was added 10 ml of chloroform and 10 ml of water, which was vortexed and then centrifuged at $900 \times g$ for 5 min to facilitate phase separation. The upper aqueous layer and the interface were discarded. The lower chloroform layer was washed with 10 ml of 0.1 M KCl. After additional centrifugation ($900 \times g$ for 5 min), the upper layer and interface were again discarded. The lower chloroform layer was washed two times with deionized distilled water. The lipid extract was dried under N_2 and suspended in 0.1 ml of a 19:1 ratio of chloroform-methanol.

Analysis of phospholipids. Quantitation of total phospholipids was carried out colorimetrically with ammonium ferrothiocyanate (34). Individual phospholipids were separated by thin-layer chromatography (10, 13). Lipids (1 to 2 mg) were applied onto silica gel 60 thin-layer chromatographic plates without a fluorescence indicator (EM Science,

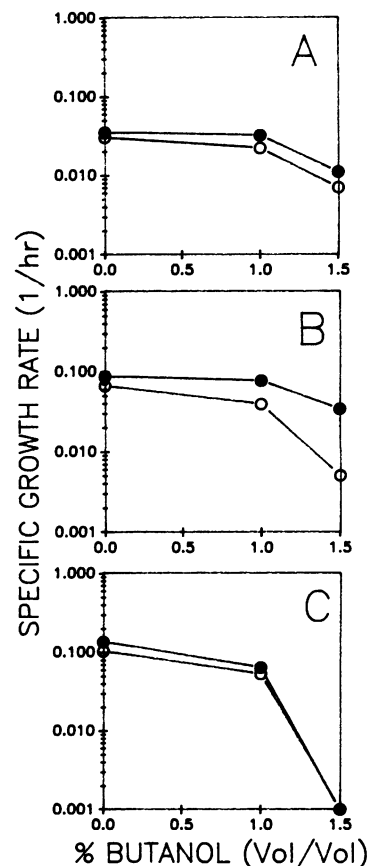


FIG. 1. Effect of butanol challenge at three different temperatures (22°C [A], 37°C [B], 42°C [C]) on the specific growth rate of *C. acetobutylicum* ATCC 824 (○) and SA-2 (●) in chemically defined P2 medium.

Darmstadt, Federal Republic of Germany). Thin-layer chromatographic plates were run at room temperature in two dimensions with the following solvent systems: (i) chloroform-methanol-7 M ammonium hydroxide (12:7:1) and (ii) chloroform-methanol-acetic acid (65:25:8). Phospholipids were visualized and identified by spraying with phospray (Supelco). Plasmalogen content was determined colorimetrically with *p*-nitrophenylhydrazine (26). A standard curve was prepared by using *cis*-11-hexadecanal (Sigma).

RESULTS

Butanol challenge of *C. acetobutylicum* at various temperatures. The effect of butanol challenge at three different temperatures (22, 37, 42°C) on the specific growth rate of *C. acetobutylicum* ATCC 824 and SA-2 in P2 medium is shown in Fig. 1. The growth response for SA-2 indicates that it has a higher tolerance to butanol than does the ATCC 824 strain at all butanol challenge and temperature combinations except 1.5% butanol and 42°C. When challenged in P2 medium at 37°C, the specific growth rate of the parent strain was inhibited 50% by 1.1% (vol/vol) butanol, whereas SA-2 was inhibited 50% by 1.4% butanol. This is equivalent to a 27% increase in butanol tolerance by the SA-2 strain. In the presence of 1.5% (vol/vol) butanol, the parent strain was unable to grow at 42°C and demonstrated only slight growth at 37 and 22°C. On the other hand, the SA-2 strain was able

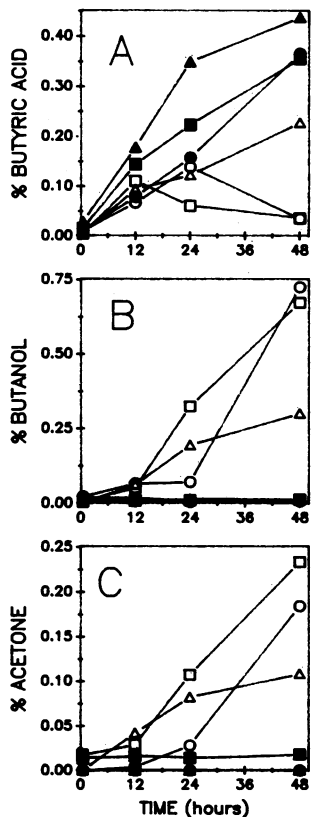


FIG. 2. End products (butyric acid [A], butanol [B], acetone [C]) produced by *C. acetobutylicum* ATCC 824 (open symbols) and SA-2 (closed symbols) at 22, 37, and 42°C. Symbols: ○ and ●, 22°C; □ and ■, 37°C; △ and ▲, 42°C.

to grow at the 1.5% butanol challenge level at either 37 or 22°C; however, this strain was unable to grow when so challenged at 42°C. The maximum specific growth rates for unchallenged *C. acetobutylicum* ATCC 824 and SA-2 strains were 0.066 and 0.087, respectively. Although the specific growth rates (calculated between 0 and 8 h) were consistently higher for the mutant versus the wild-type strain, the unchallenged SA-2 strain demonstrated a lower maximum cell density over the course of the 48-h fermentation (data not shown). This coincided with an altered pH profile that was characterized by the lack of a definite pH break point during the fermentation by SA-2. The pH of the fermentation broth continued to drop to as low as 4.3. Conversely, the unchallenged parent strain demonstrated pH break points at 5.2, 5.1, and 5.0 at 22, 37, and 42°C, respectively. The production of butyric acid, butanol, and acetone by both strains over the course of a 48-h fermentation at 22, 37, or 42°C in P2 medium was examined (Fig. 2). The SA-2 strain produced increasing amounts of butyric acid at each temperature, with the most being produced at 42°C. Although the butyric acid concentration also increased over the course of the fermentation when the ATCC 824 strain was grown at 42°C, there was an initial increase followed by a decrease to a low butyric acid concentration when this strain was grown at either 22 or 37°C. These results correspond with the production of butanol when the ATCC 824 strain was grown at either 22 or 37°C, while butanol production was lower for this strain when grown at 42°C. On the other hand, the SA-2

strain produced only butyric acid and little or no butanol or acetone.

Changes in fatty acid composition. The fatty acids routinely detected in *C. acetobutylicum* ATCC 824 and the SA-2 mutant were 12:0, 14:0, 16:1, 16:0, 17:0-cyclic, 18:1, 18:0, and 19:0-cyclic. Although eight different fatty acids were identified, three of them (i.e., 16:0, 16:1, 18:1) accounted for greater than 90% of the total. Since 17:0-cyclic and 19:0-cyclic are relatively bulky fatty acyl residues, they were included as part of the unsaturated fatty acids. The changes in the percent saturated to percent unsaturated fatty acid ratios (SU) in the parent and SA-2 mutant over the course of a small-scale batch fermentation in P2 medium at three different temperature and butanol challenge levels are shown in Fig. 3. At 22, 37, and 42°C, unchallenged growth of the ATCC 824 strain into the stationary phase coincided with a gradual increase in the SU ratio. The addition of 1.0 or 1.5% (vol/vol) butanol to cells grown at 22 and 37°C caused an immediate (within 30 min) dose response increase in the SU ratio. There was little further change in the SU ratio for the challenged cells over the course of the fermentation following this initial response. On the other hand, the SA-2 mutant was characterized by having fewer 16:0 and more 16:1 and 18:1 acyl chains than the ATCC 824 strain during the mid-exponential and early stationary phases at these temperatures. This was reflected by a lower initial SU ratio for

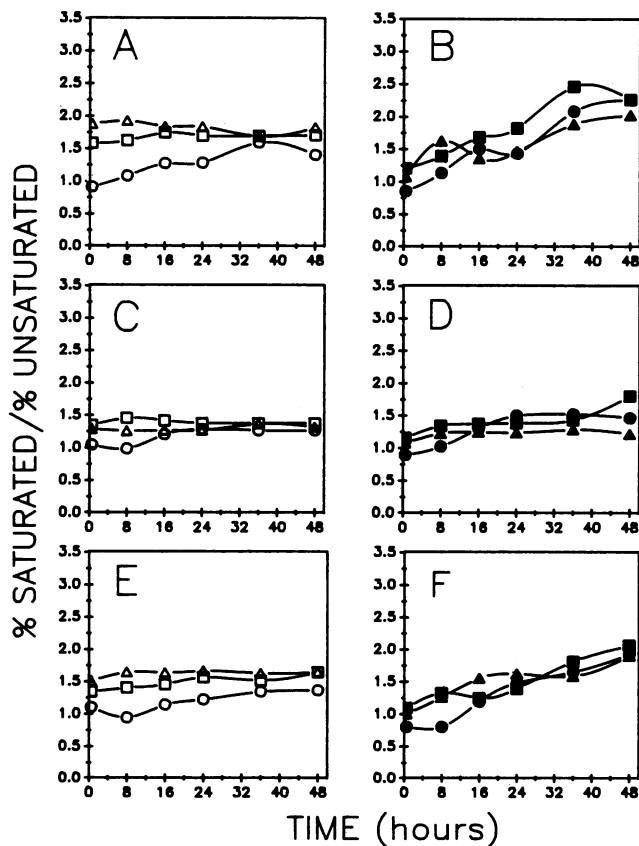


FIG. 3. Changes in the fatty acid composition (SU) for *C. acetobutylicum* ATCC 824 (open symbols) and SA-2 (closed symbols) during small-scale batch fermentation at 37°C (A and B), 42°C (C and D), and 22°C (E and F) in P2 medium with butanol challenges (0, 1, 1.5% [vol/vol]). Symbols: ○ and ●, control; □ and ■, 1% butanol; △ and ▲, 1.5% butanol.

challenged SA-2 cells. However, growth of SA-2 into the late stationary phase at 22 or 37°C resulted in an overall greater increase in the SU ratio for both unchallenged and challenged cells when compared to the response by the ATCC 824 strain. This effect was minimized when SA-2 was grown at 42°C.

Growth at 42°C caused an increase in the longer acyl chain fatty acids (i.e., 17:0-cyclic, 18:1, 18:0, 19:0-cyclic) with a decrease in the shorter acyl chains (i.e., 14:0, 16:1, 16:0) for both strains. The SA-2 mutant exhibited a smaller percent change in its acyl chain composition than did the parent strain when grown at 37 and 42°C (data not shown).

Because of the presence of neutral lipids (diglycerides and free fatty acids) in *Clostridium* spp. (13), it was necessary to determine if these lipids interfere with an examination of membrane lipid compositional changes. There was no statistical difference ($P = 0.1$) between the FAMES prepared from intact cells versus that prepared from cell membranes. Lipids extracted from intact cells and cell membranes also exhibited similar thin-layer chromatograms. Although neutral lipids are not normally associated with plasma membranes, the chromatograms indicated the presence of mono- and diglycerides, along with phospholipids, in both lipid preparations. It was, therefore, concluded that separation of the membranes from intact cells was not necessary in further analyses.

Analysis of cell membrane fluidity. The effect of growth at different temperatures and butanol challenge levels on the fluorescence polarization ratios for *C. acetobutylicum* ATCC 824 and SA-2 cell membranes during small-scale batch fermentation in P2 medium is shown in Fig. 4. When unchallenged at 22, 37, or 42°C, both ATCC 824 and SA-2 exhibited relatively constant polarization ratio (P) readings over the course of the fermentation. However, in the presence of 1.0 or 1.5% (vol/vol) butanol, the ATCC 824 strain was characterized by dramatic changes in P values, while SA-2 maintained essentially constant P values at these challenge levels. Furthermore, it appears that the effect of butanol is dominant over the solidifying effect of low temperature, since challenge with 1.5% butanol at 22°C resulted in a dramatic decrease in P , indicating a fluidizing effect. Cellular growth appears to be necessary for butanol to fluidize the cell membrane (see also below). This is manifested by less of a change in P in those cases in which there is no growth (e.g., 1.5% butanol challenge at 37 and 42°C).

The immediate effect of 1.0% butanol challenge at 37°C on the cell membrane fluidity of *C. acetobutylicum* ATCC 824 and SA-2 was examined by observing changes in polarization ratios starting at 0.5 h postchallenge. These effects were observed under growth as well as nongrowth conditions (Fig. 5). When unchallenged, the ATCC 824 strain demonstrated a 15.4% decrease in P within the first 2 h. The SA-2 strain showed a corresponding 6.4% decrease in P at 3 h. In both cases, there was a recovery back to starting values after ca. 6 to 8 h. When ATCC 824 cells were exposed to a 1.0% (vol/vol) butanol challenge, there was a dramatic decrease in P (43.6%), followed by recovery to starting values within 9 h. Under the same conditions, SA-2 displayed only a 12.3% reduction in P , which rapidly recovered to starting values within 4 h. Under nongrowth conditions, ATCC 824 and SA-2 demonstrated no apparent change in P in the presence or absence of butanol challenge.

Alterations in total lipid composition. There were no obvious differences between ATCC 824 and SA-2 in the relative abundance of phosphatidyl ethanolamine, phosphatidyl glycerol, and cardiolipin, as examined by thin-layer chromatog-

raphy. Consequently, no attempts were made to quantitate the results.

The presence of plasmalogens in exponential- and stationary-phase ATCC 824 and SA-2 cells was also examined. The 1.0% butanol-challenged ATCC 824 strain demonstrated a 102 and 95% increase in plasmalogen content in exponential- and stationary-phase cells, respectively. On the other hand, 1.0% butanol-challenged SA-2 demonstrated a 10 and 3% decrease in plasmalogen content for exponential- and stationary-phase cells, respectively.

DISCUSSION

The few studies that have been carried out on the lipid composition of the clostridia (14, 36) have been limited to the use of wild-type strains. By comparison with the wild-type strain, the use of a butanol-tolerant mutant of *C. acetobutylicum* in this study allows for an examination into the role of lipid composition and membrane fluidity in enhanced butanol tolerance.

E. H. Carnarius (U.S. patent 2,198,104, 1940) found that the butanol concentration could be increased by 3 to 5% by cooling a molasses mash from 30 to 24°C. This suggests that growth temperature, which affects membrane fluidity, may be used to offset the disruptive effect of butanol. Since both butanol and elevated temperature act to fluidize the cell membrane, these factors in combination would be expected

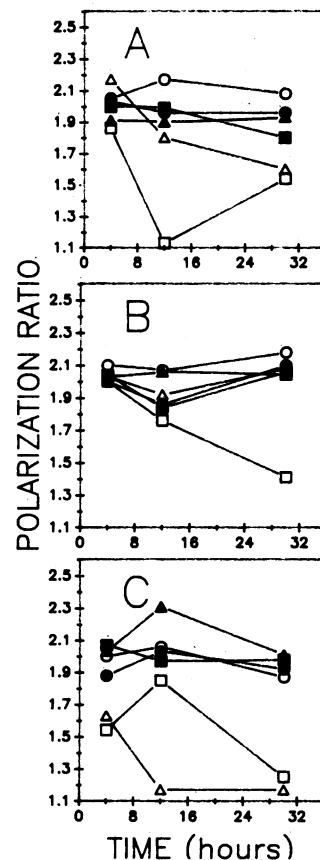


FIG. 4. Changes in the polarization ratio of *C. acetobutylicum* ATCC 824 (open symbols) and SA-2 (closed symbols) during small-scale batch fermentations in P2 medium at different temperatures (37°C [A], 42°C [B], 22°C [C]) and butanol challenges. Symbols: ○ and ●, control; □ and ■, 1% butanol; △ and ▲, 1.5% butanol.

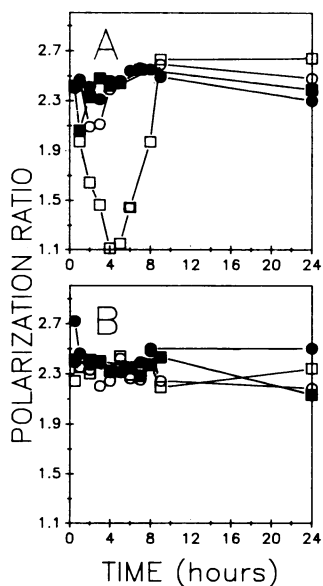


FIG. 5. Immediate changes in the polarization ratios of *C. acetobutylicum* ATCC 824 (open symbols) and SA-2 (closed symbols) when exposed to butanol challenges (0 and 1.0% [vol/vol]) under growth (A) and nongrowth (B) conditions. Symbols: ○ and ●, control; □ and ■, 1% butanol.

to act on the cell in a synergistically negative way. Conversely, lower growth temperatures would be expected to offset the fluidizing effect of butanol on the membrane, since lower growth temperatures tend to solidify the membrane bilayer. As expected, an elevated growth temperature (42°C) in combination with a 1.5% butanol challenge had the most deleterious effect on cell growth of both *C. acetobutylicum* strains. On the other hand, growth at lower temperatures (22 and 37°C) improved the ability of both strains to tolerate butanol, despite the fact that the unchallenged growth rate at 22°C was lower than that at the other two temperatures tested.

Although SA-2 demonstrated a 27% increase in butanol tolerance over that of the wild-type strain, the pH profile for this strain was not characteristic of ABE fermentation, since it did not exhibit a typical pH break point. Instead of switching from acidogenesis to solventogenesis, SA-2 continued to produce up to 0.44% (vol/vol) butyric acid and little or no butanol and acetone. The concentration of butyric acid produced is greater than the threshold level below which no growth inhibition occurs in *C. acetobutylicum* (5), and as such, it would be expected to have a detrimental effect on the cells. These results demonstrate that increased tolerance to butanol does not necessarily coincide with improved butanol production. Asther and Khan (1) reported that there is no direct correlation between ethanol tolerance and production capability in *Clostridium saccharolyticum*. The lack of a solventogenic phase transition in SA-2 may be attributed to repeated serial transfer (6, 9, 17) in P2 synthetic medium during the development and maintenance of butanol tolerance.

There have been some recent reports in which the effect of butanol on the membrane lipid composition of *C. acetobutylicum* has been described (20, 36). The results of these studies coincide with our findings, which demonstrate that there is a significant increase in the SU ratio of the fatty acid chains when these cells are exposed to butanol. However,

the quantitative values for individual fatty acids were different. This may be due to the culture conditions, as well as to the method used to synthesize the FAMES. In this study, we used a chemically defined medium to eliminate potential interference from the culture medium. The elevated levels of 18:1 fatty acids and the presence of 18:2 fatty acids reported by Vollherbst-Schneck et al. (36) and Lepage et al. (20), respectively, may be attributed to the yeast extract present in their culture media. In the preparation of FAMES, the sodium methoxide method required less cellular material and fewer processing steps than did the BCl_3 -methanol method used by the other investigators. In addition, the BCl_3 -methanol method has been reported to form artifacts and cause a reduction in unsaturated fatty acids (12).

The influence of alcohols and growth temperatures on the membrane lipid bilayer has been studied extensively. Results of studies with model membranes (28), animal cell membranes (25), and bacterial membranes (11) provide overwhelming evidence that the addition of alcohols or changes in the growth temperature alter the membrane fluidity. This disrupts a variety of cellular functions by changing the physical properties of the membrane. To survive adverse environmental conditions, the microorganism must have the ability to adjust its lipid composition. This response, known as homeoviscous adaptation, is believed to offset the physical changes caused by the environment and permit the cell to maintain its membrane at the proper viscosity and surface ionic milieu for optimal cellular function (16). In *Escherichia coli*, fatty acid compositional changes are observed immediately in response to a shift in growth temperature (7). *C. acetobutylicum* responds in less than 5 h to the presence of solvents, mainly by increasing the percentage of 16:0 and 18:0 fatty acids and decreasing the percentage of 16:1 and 18:1 fatty acids (20). Our results agree with these findings. An increase in the SU ratios for *C. acetobutylicum* ATCC 824 over the course of the ABE fermentation was only observed in the absence of butanol challenge. On the other hand, the SA-2 mutant demonstrated a greater overall increase in the SU ratio than did the parent strain under both unchallenged and challenged conditions. It appears that the response to increase the SU ratio may be independent of a butanol trigger. Instead, the response may be due to an accumulation of acids such as butyric acid which may interact with the membrane in a way that elicits a biological response similar to that observed when *C. acetobutylicum* (20) and *E. coli* (31) are exposed to butanol. In any event, the greater lipid compositional changes by the SA-2 mutant during ABE fermentation appears to increase the tolerance of the microorganism to butanol.

With respect to growth temperatures, *C. acetobutylicum* ATCC 824 and SA-2 responded to an increase in temperature by increasing the fatty acid chain length. This is in agreement with the results reported for *C. acetobutylicum* ATCC 39057 (20) and *C. butyricum* (14).

J. C. Linden (Abstr. Int. Union Microbiol. Soc., P54:7, p. 142, 1982) suggested that the addition of oleic and elaidic acids improved butanol tolerance in *C. acetobutylicum*. Although the SA-2 butanol-tolerant mutant exhibited higher levels of 18:1 acyl chains than did the parent strain in the early stages of growth, the incorporation of additional 18:1 fatty acids into the cell membrane depressed the butanol tolerance of both strains. This suggests either that this specific alteration in fatty acid composition is not responsible for butanol tolerance in the SA-2 mutant or that the method used to incorporate the exogenous fatty acids into the membranes results in less tolerant cell lines.

Membrane fluidity is a term used to describe the structural disorder of hydrocarbon chains as well as their dynamic movement. Vollherbst-Schneck et al. (36) demonstrated, by the use of electron spin resonance-spin label analysis, that butanol causes a ca. 20 to 30% increase in the fluidity of lipid dispersions from mid-exponential-phase cell cultures of *C. acetobutylicum* ATCC 824. Results of our study, in which fluorescence depolarization on intact cells was used, show a dramatic fluidization of ATCC 824 cells when they were exposed to butanol, while SA-2 cells remained relatively stable. This supports the data on fatty acid compositional changes; the SA-2 strain is more butanol tolerant than ATCC 824, because the former is able to alter its membrane more effectively and thereby maintain a more suitable membrane environment. It also appears that growth is necessary to destabilize the membrane with butanol.

It has been suggested that plasmalogens represent important membrane-modulating components because of their limited existence in certain mammalian tissues (e.g., central nervous system, heart, and skeletal muscle) that are under extremely varied physiological conditions (8). Among microorganisms, plasmalogens have only been found in obligate anaerobic bacteria, in which they can constitute more than half of the total phospholipids (13). Our results indicate that a dramatic increase in the plasmalogen content did not improve the ability of the parent strain to grow in the presence of butanol challenge. In the case of the SA-2 mutant, there was very little change in its plasmalogen composition when SA-2 was exposed to butanol.

The SA-2 mutant is more butanol tolerant than the parent strain, because it is better able to adjust its membrane lipid composition in such a way so as to maintain a stable environment for cellular functions. The inability of the SA-2 strain to produce butanol may be a consequence of this adjustment. Since butyric acid is thought to reenter the cell to act as a carbon skeleton for butanol formation (24), it is an interesting possibility that alterations in membrane fatty acid composition are responsible for the lack of butanol formation by the SA-2 strain. Studies are under way to determine if other cellular processes associated with butanol production (i.e., granule accumulation, sporulation) are also affected in this strain.

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