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NOTES

Effect of Butanol on Lipid Composition and Fluidity of *Clostridium acetobutylicum* ATCC 824

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Butanol, at sub-growth-inhibitory levels, caused a ca. 20 to 30% increase in fluidity of lipid dispersions from *Clostridium acetobutylicum*. When grown in the presence of butanol or into stationary phase, *C. acetobutylicum* synthesized increased levels of saturated acyl chains at the expense of unsaturated chains.

Short-chain aliphatic alcohols are important industrial chemicals which are currently synthesized chemically from petroleum-derived precursors. Microbial pathways for production of some of these alcohols exist and may be exploited for future production. Several members of the genus *Clostridium* produce butanol during the late stages of culture growth and during sporulation, but the toxic effects of butanol severely limit the extent of production. The amphiphilic nature of butanol suggests that its toxic effects may be due to interactions with cell membranes and their components.

A recent study has shown that butanol inhibits several membrane transport systems and enzymes in *Clostridium acetobutylicum*. Specifically, alanine and 3-*O*-methyl-glucose uptake and membrane ATPase activity are severely reduced by near-toxic levels of butanol (5).

Although numerous studies have been carried out on the effects of ethanol on membranes of procaryotic and eucaryotic microorganisms (1, 2, 6), the effects of butanol on the physical and biochemical properties of membranes are largely unknown. We have studied, and report here, a direct effect of butanol on membrane fluidity and the effects of growth in the presence of butanol on the acyl chain lipid composition of *C. acetobutylicum* ATCC 824.

In our hands, *C. acetobutylicum* ATCC 824 grows at a normal rate in the presence of 0.5% added butanol, at a slightly reduced rate in 1% butanol, and not at all in 1.5% butanol. Thus, we decided to investigate the direct effect of butanol (up to 1.5%) on the physical properties of the cell membranes by use of electron spin resonance-spin label analysis. We were unable to obtain reproducible data for membranes of intact cells, so we extracted the lipids from mid-exponential-phase cell cultures and formed lipid dispersions. The hydrocarbon spin label 7N14 and butanol (0 to 1.5%) were added to the dispersions, and the electron spin resonance spectra were recorded as previously described (3). For comparison, we investigated the effect of butanol on the fluidity of dispersions consisting of either pure dimyristoylphosphatidylcholine or lipids extracted from *Escherichia coli* K-12. The results, tabulated as rotational correlation time, τ_c, for 7N14 in the lipid dispersion, showed that the presence of butanol caused a 20 to 30% decrease in τ_c in all three lipid preparations (Table 1). These results can be interpreted as a significant fluidizing effect of butanol on the bulk lipid regions of *E. coli* and *C. acetobutylicum* membranes. In all cases, τ_c values for *E. coli* and *C. acetobutylicum* lipid dispersions were similar. Repeated experiments recording τ_c over a temperature range from 15 to 50°C showed no evidence for a phase transition in either *E. coli* or *C. acetobutylicum* lipid dispersions, whereas, as expected, a sharp transition was seen in the dimyristoylphosphatidylcholine dispersion at 22 to 25°C (data not shown).

This observation that butanol caused a direct fluidization of the lipid regions of the membranes of *C. acetobutylicum* suggested that the bacteria might respond to this physical perturbation by altering their membrane lipid composition, perhaps similar to the homeoviscous adaptation of *E. coli* to maintain membrane fluidity at an acceptable level for cell function (7). Thus, we examined the effect of growth in the presence of butanol on the acyl chain composition of the lipids of *C. acetobutylicum* by adding butanol to early-exponential-phase cultures. Samples were harvested at mid-exponential phase and in stationary phase, at a time when approximately half of the cells had formed endospores and

TABLE 1. Effect of butanol on rotational mobility of spin-labeled hydrocarbon in lipid dispersions at 35°C

Lipids[a]	Butanol (% [vol/vol])	τ_c[b] ($\times 10^{-10}$ s)
Dimyristoylphosphatidylcholine	0	7.3 ± 0.5
	0.5	5.2
	1.5	5.4
E. coli	0	8.8
	0.5	7.0
	1.5	6.9
C. acetobutylicum	0	8.3
	0.5	5.5
	1.5	6.0

[a] Pure dimyristoylphosphatidylcholine or lipids extracted from bacteria were dispersed in water; butanol was then added to give the desired concentration.

[b] Spin label 7N14 was added to give 10^{-4} M to the lipid plus butanol preparations, and electron spin resonance spectra were recorded over a temperature range from 15 to 50°C. The 35°C data are representative. The estimated error is $\pm 0.5 \times 10^{-10}$ s.

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TABLE 2. Effect of stage of growth and added butanol on acyl chain composition of *C. acetobutylicum*

Added butanol concn (%)	Stage of growth[a]	% Total composition (acyl chain)[b]							% Total saturates	% Total unsaturates
		14:0	16:0	16:1	18:0	18:1	19 cyc	Unknowns		
0	Mid-log	6.6	43.6	15.4	2.8	22.1	5.1	4.7	58.1	37.5
	Stationary	7.8	57.4	12.8	4.4	4.9	7.2	4.1	76.8	17.7
0.5	Mid-log	1.3	53.2	9.1	3.0	22.4	7.1	4.9	64.6	31.5
	Stationary	5.5	59.5	12.1	3.4	4.6	10.9	3.9	79.3	16.7
1.0	Mid-log	1.2	58.3	7.3	4.6	15.5	8.7	4.8	72.8	22.8
	Stationary	3.5	62.6	7.6	3.2	7.9	10.1	4.9	79.4	15.5

[a] Cells from overnight stationary-phase cultures were diluted 1:100 into (0.01 M) PO_4-buffered growth medium (2% glucose, 0.5% yeast extract [pH 6.3]) containing 0, 0.5, or 1.0% butanol. Cultures were incubated at 37°C from an initial optical density of <0.1 and harvested at various optical density values. Mid-log phase is defined here to be an optical density of 0.5, and stationary phase is a 40-h culture having an optical density of >1.5.

[b] Cells were heated for 1 h at 100°C in 5 ml of 5% KOH–50% aqueous methanol to saponify the lipids. The samples were acidified by the dropwise addition of 6 N HCl. Methyl ester derivatives of the various fatty acids were prepared by the addition of 5 ml of 10 to 12% BCl_3 in methanol, followed by boiling for 5 min. After cooling, the mixture was extracted twice with $CHCl_3$-hexane (1:4). The combined solvent layers were evaporated to near dryness with nitrogen gas; 0.2 ml of hexane was then added, and the samples were stored at −20°C. Separation of the fatty acid methyl esters was accomplished by the use of a glass cyanosilicone column (5 ft. by 0.25 in. [152.4 by 0.64 cm]; Supelco, Inc., Bellefonte, Pa.) on a Varian 2400 gas chromatograph, using a temperature program of 150 to 225°C at a rate of increase of 4°C/min. The nitrogen carrier gas flow rate was 30 ml/min. The injection port and flame ionization detector were both run at 250°C, and peak quantitation was carried out with a Hewlett-Packard 3390A integrator. Peaks were identified by comparison with fatty acid methyl ester standards purchased from Supelco and by comparison with the data of Johnston and Goldfine (4). Repeated analyses of samples from the same culture gave results reproducible to within 1%, whereas variation of results from separate experiments averaged ca. 4%.

significant (0.1 to 0.2% extracellular concentration) levels of butanol had been produced by the cells. Lipid acyl chain compositions were determined by gas-liquid chromatography of fatty acyl methyl esters. The results (Table 2) showed that in the absence of added butanol the relative abundance of saturated chains was greater in stationary-phase cells (butanol production) than in exponential-phase cells (butyric acid production). The presence of added butanol affected the cellular acyl chain composition in much the same way as did growth into stationary phase. In the presence of 1% butanol during mid-exponential phase, levels of saturated acyl chains were increased, whereas levels of unsaturated chains were decreased.

The fluidity measurements and acyl chain composition determinations reported here are consistent with the suggestion that *C. acetobutylicum* has a homeoviscous response to the presence of butanol; i.e., in response to the membrane-perturbing physical effect of butanol, cellular fatty acid synthesis is shifted toward a higher saturated chain to unsaturated chain ratio. Regardless of the actual mechanism responsible for the changes in lipid composition resulting from either (i) growth in the presence of added butanol during exponential phase or (ii) growth into stationary phase (sporulation and intracellular conversion of butyric acid to butanol), it is possible that these changes play a role in determining the toxic effects of the presence of butanol. We suggest that, if the cell could not alter its lipid composition in response to the perturbing effect of butanol, the cell would be even more sensitive to this alcohol than is observed. Conversely, perhaps even greater changes in lipid composition might increase the tolerance level of the cells to butanol. To achieve such changes, it may be necessary to isolate a collection of specific lipid synthesis mutants of *C. acetobutylicum*.

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