APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1989, p. 2729–2731
0099-2240/89/102729-03$02.00/0

Vol. 55, No. 10

Electron Spin Resonance Analysis of the Effect of Butanol on the Membrane Fluidity of Intact Cells of *Clostridium acetobutylicum*

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Received 9 March 1989/Accepted 25 July 1989

Analysis of electron spin resonance spectra of 5-doxyl stearic acid in aqueous suspensions of *Clostridium acetobutylicum* ATCC 824 and the butanol-tolerant SA-2 derivative during a small-scale fermentation at three different butanol challenge levels indicated that the SA-2 strain is able to respond to the physical fluidizing effect of high (1.5%) butanol challenge by reducing its membrane fluidity at 12 and 30 h. The wild-type 824 strain was unable to so respond when challenged at the 1.5% level.

The acetone-butanol-ethanol fermentation continues to be of interest as a potentially viable approach for converting waste carbohydrates to valuable chemicals. The primary limitation of this fermentation relates to the toxicity of the butanol end product on the producing microorganism, *Clostridium acetobutylicum*, and the resulting low concentration of solvent in the fermentation broth. If the amount of butanol produced in the fermentor could be increased from 1.2 to 2% (wt/vol), then the distillative energy recovery costs would be cut in half (7). Such an increase in butanol concentration would make this fermentation an economically viable process.

Studies in other biological systems have shown that alcohols act to disrupt cell membrane structure and thereby inhibit cellular processes (4, 5, 8). Recent work in our laboratory (1) involved an examination into the effect of butanol challenge and temperature on the lipid composition and membrane fluidity of butanol-tolerant *C. acetobutylicum*. Cell membrane fluidity analysis by fluorescence depolarization using *trans*-parinaric acid indicated that the membrane fluidity exhibited by the butanol-tolerant SA-2 strain remained essentially constant at various butanol challenge and temperature combinations, whereas the membrane fluidity of the ATCC 824 wild-type strain increased with increasing butanol challenge. We suggested that by synthesizing an increased amount of saturated fatty acids, the butanol-tolerant strain has developed a mechanism for maintaining a homeoviscous membrane environment. Earlier work by Vollherbst et al. (9) using electron spin resonance (ESR) spin label analysis found that butanol added at subinhibitory levels caused a ca. 20 to 30% increase in fluidity of lipid dispersions from *C. acetobutylicum* ATCC 824. However, these workers were unable to obtain reproducible data using the hydrocarbon spin label 7N14 and the membranes of intact cells and therefore extracted the lipids from mid-exponential-phase cell cultures and caused lipid dispersions to form. The objective of our study was to examine the effect of butanol challenge on the membrane fluidity of intact cells of *C. acetobutylicum* ATCC 824 and the butanol-tolerant SA-2 strain over the course of a 30-h fermentation, using ESR analysis with the 5-doxyl stearic acid spin label probe (6).

The *C. acetobutylicum* ATCC 824 and SA-2 strains were grown and maintained as described previously (1). Brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) cultures (24 h) were inoculated (5% [vol/vol]) into 1.8 liter of chemically defined P2 medium (1) and incubated at 37°C for 1 h before butanol challenge (final butanol concentrations in the culture were 0, 1.0, and 1.5% [vol/vol]). The time of challenge was recorded as time zero. Cultures were incubated at 37°C, and 15-ml samples were collected at 4, 12, and 30 h and then centrifuged at 16,300 × *g* for 15 min at 4°C. The cell pellets were deposited into 1.2-ml cryotubes (Nunc, Roskilde, Denmark) and stored at −196°C under liquid nitrogen.

Preliminary ^1H nuclear magnetic resonance (NMR) analysis was carried out to determine whether the probe, 5-doxyl stearic acid (formula weight = 384; Molecular Probes, Junction City, Oreg.) would interact with the membranes of intact *C. acetobutylicum* cells in aqueous suspension. The NMR spin lattice relaxation times (T_1) of aqueous suspensions of intact cells, protoplasts, and extracted lipids from *C. acetobutylicum* with and without added spin label or probe were determined. A working stock solution of the probe was prepared by dissolving 6.92 mg in 0.3 ml of 100% ethanol to give a final concentration of 60 mM. The solution was stored at −20°C under N_2 in the dark until use. Ten microliters of the working solution was transferred into tubes (6 mm [outer diameter] by 50 mm) which had been previously shortened to ca. 12 mm. The probe was dried onto the bottom of the tube by using a gentle stream of N_2. The cells were thawed at room temperature and washed three times with 5 mM EDTA plus 0.15 M KCl in P2 buffer (5.0 g of K_2HPO_4 per liter, 5.0 g of KH_2PO_4 per liter, 22 g of CH_3COONH_4 per liter; pH 7.0) to chelate iron and manganese ions. The cells were diluted 1:10 in the wash solution. From this, 0.2-ml aliquots were transferred into the tubes containing the probe and gently stirred with a glass rod. The tubes were inserted into the bottom of high-resolution glass NMR receiving tubes (7 mm [outer diameter]; Wilmad Glass Co., Inc., Buena, N.J.). Samples were analyzed on a Bruker ^1H NMR process analyzer (model PC-10; IBM, White Plains, N.Y.). T_1 was determined at 10 MHz by the inversion recovery sequence (3) which can be written in the following notation: (180° − t −90° − AT − RD), where AT represents the time for which the free-induction decay is acquired and RD is the recycling time. In this study, RD is 5 or 10 s. Values of t varied from 0.01 to 2 s for $T_1 < \sim 0.6$ s and from 0.05 to 4 s for $T_1 > \sim 0.6$ s.

The ^1H NMR spin lattice relaxation times (T_1) of aqueous

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TABLE 1. NMR spin-lattice relaxation times of aqueous suspensions of 5-doxyl stearic acid in three different preparations from *C. acetobutylicum* ATCC 824 with and without added 5-doxyl stearic acid

Sample description	[Probe] (μmol/ml)	Dilution ratio (sample/buffer)	T_1[a] of sample (in seconds)		ΔT_1[b] (%)
			Without added probe	With added probe	
Intact cells	7.5	1:5[c]	0.246 ± 0.007 (29.6°C)	0.251 ± 0.007 (23.5°C)	+2
	7.5	1:10[c]	0.458 ± 0.009	0.449 ± 0.010	−2
	3	1:10[d]	0.846 ± 0.031	0.718 ± 0.015	−15
Protoplasts[e]	7.5	1:5[c]	0.101 ± 0.002	0.097 ± 0.002	−4
	3	1:10[d]	0.565 ± 0.024	0.566 ± 0.024	+0.2
	3	1:20[d]	1.204 ± 0.51	1.025 ± 0.033	−15
Lipids[f]	7.5		1.647 ± 0.095	0.443 ± 0.028 (28.2°C)	−73
Probe	7.5		0.347 ± 0.061		
Buffer[c]			3.12 ± 0.200		

[a] The temperature for sample analysis was 30°C, unless otherwise indicated.
[b] ΔT_1 (%) = $[T_1(\text{sample}) - T_1(\text{sample + probe})]/[T_1(\text{sample})] \times 100$ (the more negative the ΔT, the more interaction of the probe with the sample).
[c] P2 buffer (5.0 g of K_2HPO_4 per liter, 5.0 g of KH_2PO_4 per liter, 22 g of CH_3COONH_4 per liter; pH 7.0).
[d] 5 mM EDTA plus 0.15 M KCl in P2 buffer.
[e] Protoplasts prepared from exponential cells grown in 2 liters of P2 plus 0.2% glycine were collected by centrifugation (12,000 × *g* for 10 min at room temperature) and stored under liquid nitrogen until use. The pellet was allowed to thaw before suspension into 180 ml of P2 buffer (pH 7.0) containing 0.3 M sucrose, 25 mM $CaCl_2$, and 25 mM $MgCl_2$. Lysozyme (0.2 g) was dissolved in 20 ml of the same P2 buffer and allowed to stand for 1 h at room temperature before mixing with cell suspension. The mixture was allowed to incubate for 45 min at room temperature before centrifugation (2,600 × *g* for 10 min at room temperature). The protoplasts were washed twice with P2 buffer containing 0.15 M KCl before NMR analysis.
[f] Lipids were prepared as described previously (1).

suspensions of intact cells, protoplasts, and extracted lipids from *C. acetobutylicum* ATCC 824 with and without added 5-doxyl stearic acid can be seen in Table 1. Equivalent percent ΔT_1 values (−15) obtained for both intact cells and protoplasts when 3.0 μmol of probe per ml was used indicated that the extent of interaction or binding of the probe in such samples was similar. On the basis of these preliminary results, it was decided that it would be possible to detect membrane fluidity changes of intact *C. acetobutylicum* cells by using ESR with the 5-doxyl stearic acid probe. This observation is further supported by Finne and Matches (2), who found that the spin label 12-*N*-oxyl-oxazolidin-stearic acid showed the same rotational correlation time in intact clostridial cells as in the lipid preparations extracted from the same cells.

In preparation for ESR measurements, the cells were prepared as described above, with the following exceptions: (i) a 20 mM 5-doxyl stearic acid solution was utilized, (ii) the cell washing steps were omitted, and (iii) cell material (0.1 ml) was transferred to the tubes containing the probe. After the samples were stirred, they were drawn by capillary action into Pyrex capillary tubes (1 mm [inner diameter] by 100 mm). The ends were quickly flame-sealed. ESR spectra were determined using a Varian E9EPR spectrometer (Varian, Inc., Sunnyvale, Calif.) equipped with a Varian E102 microwave bridge. The cavity (Varian E-238) was mounted so that the sample was horizontal, to prevent the cells from settling out of the sensitive volume of the cavity. A standard Varian Flow Dewar was used for temperature control. All experiments were performed at room temperature. The typical spectrometer settings were: field center, 3228 G; operating frequency, 9.03 or 9.065 GHz; modulating amplitude, 2 G; sweep field, 200 G; microwave power 12 or 12.5 mW; sweep time, 0.128 or 0.064 s. The fluidity of the membrane was quantitated by measuring the broadening of the central peak of the 5-doxyl stearic acid which is incorporated to levels in excess of 90% (data not shown) into the membrane lipid bilayer. The peak-to-peak line width was measured in the first derivative ESR spectrum and is designated *W*. Additional details on the analysis and broadening mechanisms using 5-doxyl stearic acid were recently reported (6).

The calculated peak width values from ESR spectra of 5-doxyl stearic acid in aqueous suspensions of *C. acetobutylicum* ATCC 824 and the butanol-tolerant SA-2 derivative during a small-scale fermentation in P2 medium at three different butanol challenge levels can be seen in Fig. 1. The initial *W* (4 h) values for both strains were higher for the challenged (1.0 and 1.5% butanol) than for the unchallenged cell samples. At 12 and 30 h, there was a dramatic decrease in peak width (fluidity) for the SA-2 strain at all challenge concentrations. This result is consistent with the idea of a biological compensatory response (1) by this strain to either accumulated butyrate (0% challenge) or accumulated butyrate plus added butanol (1.0 and 1.5% challenges). Although there was a similar, albeit later, compensatory response manifested as a decrease in fluidity at 30 h for the 0 and 1% butanol-challenged 824 strain, at the higher 1.5% challenge level, this strain was unable to respond to the physical fluidizing effect of butanol. The ability of the SA-2 strain to compensate for the 1.5% added butanol challenge over the course of this 30-h fermentation is a reflection of the fact that growth of SA-2 into the late stationary phase at 37°C resulted in an overall greater increase in the saturated to unsaturated fatty acid ratios for both unchallenged and challenged cells when compared with the response by the ATCC 824 strain (1). *C. acetobutylicum* SA-2 is more butanol tolerant than the 824 strain because the former is able to alter its membrane more effectively at high concentrations of butanol and thereby maintain a more suitable membrane environment. It is the greater ability of the SA-2 strain to alter its lipid composition during the acetone-butanol-ethanol fermentation which appears to increase the tolerance of this microorganism to butanol.

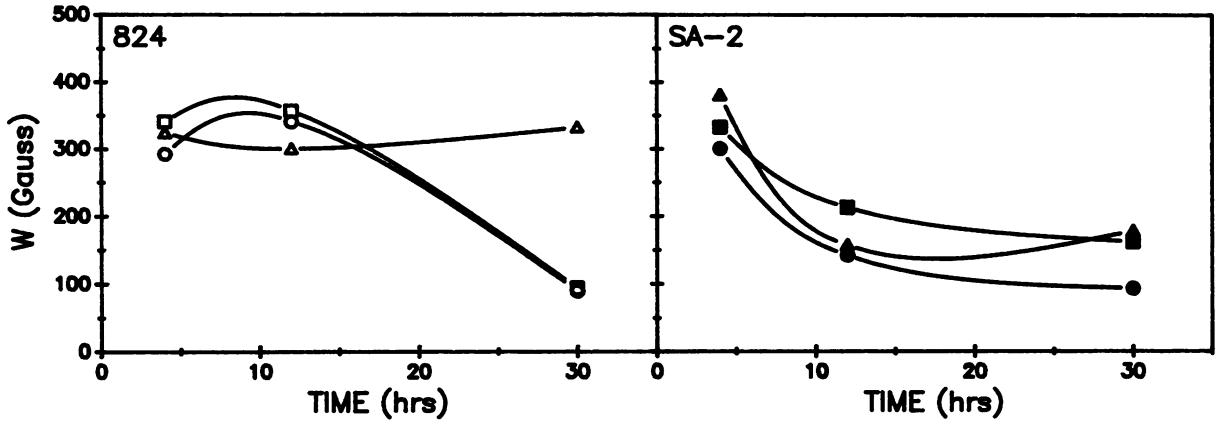

FIG. 1. Peak-to-peak line width values calculated from ESR spectra of 5-doxyl stearic acid in *C. acetobutylicum* ATCC 824 and SA-2 during small-scale batch fermentations in P2 medium at different butanol challenge levels. Symbols: ○ and ●, control; □ and ■, 1% butanol; △ and ▲, 1.5% butanol.

This study was supported in part by grant AG-83-CRSR-2-2249 from the U.S. Department of Agriculture Alcohol Research program, grant ICMB 86-0015-01 from the Illinois Corn Marketing Board, and Hatch grant 50-0314 from the University of Illinois Agricultural Experiment Station.

We thank Harold Swartz and Ion Baianu for their excellent suggestions and comments.

LITERATURE CITED

1. **Baer, S. H., H. P. Blaschek, and T. L. Smith.** 1987. Effect of butanol challenge and temperature on lipid composition and membrane fluidity of butanol-tolerant *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **53**:2854–2861.
2. **Finne, G., and J. R. Matches.** 1976. Spin-labeling studies on the lipids of psychrophilic, psychrotrophic, and mesophilic clostridia. J. Bacteriol. **125**:211–219.
3. **Gadlan, D. G.** 1982. Nuclear magnetic resonance and its applications to living systems, p. 117–122. Oxford University Press, New York.
4. **Ingram, L. O.** 1984. Effects of alcohols on micro-organisms. Adv. Microb. Physiol. **25**:253–300.
5. **Lenaz, G., E. Bertoli, G. Curatola, L. Mazzanti, and A. Bigi.** 1976. Lipid-protein interactions in mitochondria. Spin and fluorescence probe studies on the effect of n-alkanols on phospholipid vesicles and mitochondria membranes. Arch. Biochem. Biophys. **172**:278–288.
6. **Nettleton, D. O., P. D. Morse II, J. W. Dobrucki, H. M. Swartz, and N. J. F. Dodd.** 1988. Distribution of 5-doxyl stearic acid in the membranes of mammalian cells. Biochim. Biophys. Acta **944**: 315–320.
7. **Phillips, J. A., and A. E. Humphrey.** 1983. An overview of process technology for the production of liquid fuels and chemical feedstocks via fermentation, p. 294–304. *In* D. L. Wise (ed.), Organic chemicals from biomass. Benjamin-Cummings, Menlo Park, Calif.
8. **Thompson, G. A., Jr.** 1980. The regulation of membrane lipid metabolism. CRC Press, Inc., Boca Raton, Fla.
9. **Vollherbst, K., J. A. Sands, and B. A. Montenecourt.** 1984. Effect of butanol on lipid composition and fluidity of *Clostridium acetobutylicum* ATCC 824. Appl. Environ. Microbiol. **47**:193–194.