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Development of Ethanol Tolerance in *Clostridium thermocellum*: Effect of Growth Temperature†

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The growth of *Clostridium thermocellum* ATCC 27405 and of C9, an ethanol-resistant mutant of this strain, at different ethanol concentrations and temperatures was characterized. After ethanol addition, cultures continued to grow for 1 to 2 h at rates similar to those observed before ethanol was added and then entered a period of growth arrest, the duration of which was a function of the age of inocula. After this period, cultures grew at an exponential rate that was a function of ethanol concentration. The wild-type strain showed a higher energy of activation for growth than the ethanol-tolerant derivative. The optimum growth temperature of the wild type decreased as the concentration of the ethanol challenge increased, whereas the optimum growth temperature for C9 remained constant. The results are discussed in terms of what is known about the effects of ethanol and temperature on membrane composition and fluidity.

Clostridium thermocellum, an anaerobic, thermophilic, cellulolytic bacterium (22), has a potential role in producing ethanol from biomass. When this organism grows on cellulose as the sole carbon source, ethanol, acetic acid, lactic acid, CO_2, and H_2 are produced (32). *C. thermocellum* could thus be used to convert cellulose to ethanol in a single-step process, eliminating the need for biomass pretreatment. However, unlike other alcohol-producing microbes, such as yeasts, *C. thermocellum* is strongly inhibited at relatively low ethanol concentrations (5 g/liter). Consequently, the final ethanol concentration in the fermentation broth is low. To develop strains of *C. thermocellum* that can tolerate high ethanol concentrations, we need to understand the biochemical basis of ethanol tolerance. As a starting point we examined the relationship between growth temperature, ethanol concentration in the medium, and growth rate of both *C. thermocellum* and an ethanol-tolerant derivative of this microorganism.

MATERIALS AND METHODS

Organisms and culture conditions. *C. thermocellum* ATCC 27405 and its ethanol-resistant derivative, C9, were grown routinely under anaerobic conditions at 60°C in Hungate tubes (9 by 180 mm) with 10 ml of a medium (CM4-Cb) containing (grams per liter): cellobiose, 7.0; yeast extract, 5.0 (Difco); $(NH_4)_2SO_4$, 1.3; $MgCl_2$, 0.75; $CaCl_2$, 0.1; K_2HPO_4, 2.9; KH_2PO_4, 1.5; cysteine, 0.5; $FeSO_4$, 1.25×10^{-6}; and resazurin, 2.0×10^{-6}. At times the cellobiose was replaced by either 5 g of Solka Flock cellulose (Brown Co., Berlin, N.H.) (CM4-cellulose) or 5 g of α-cellulose (Sigma Chemical Co., St. Louis, Mo.) (CM4-α-cellulose). After autoclaving (15 min at 121°C), the tubes were flushed with sterile nitrogen gas for 15 s each.

Strain maintenance. Stock cultures of *C. thermocellum* ATCC 27405, grown in CM4-cellulose for 48 h at 60°C, were refrigerated at 4 to 5°C. Stock cultures of the C9 ethanol-resistant mutant, grown for 48 h at 60°C on CM4-α-cellulose containing 20 g of absolute ethanol per liter, were also maintained at 4 to 5°C and were transferred into the same medium every 8 to 10 weeks.

Selection of ethanol-resistant strains. The ethanol-resistant strain C9 was derived from wild-type *C. thermocellum* ATCC 27405 by an enrichment procedure. Parent strain cultures were inoculated into CM4-cellulose broth with ethanol (5 g/liter) and incubated for 120 h at 60°C. The tubes were then agitated, and after 1 h (to allow for cellulose particle settling), optical densities at 660 nm (OD_{660}) were measured in a Turner 330 spectrophotometer. Cultures with the highest OD_{660} were transferred sequentially to fresh medium containing increasing amounts of ethanol. After the ninth transfer, a strain capable of growing in ethanol at 25 g/liter was obtained. Single-colony isolates were obtained on CM4-cellobiose agar, and their ethanol tolerance was confirmed by their ability to grow in both CM-4 cellobiose and CM4-cellulose containing ethanol at 25 g/liter.

Inhibition studies. We assessed the extent to which ethanol inhibits growth of both *C. thermocellum* ATCC 27405 and C9. Hungate tubes, containing 8 ml of CM4-cellobiose broth at 1.25 times its normal concentration and preequilibrated at the experimental temperature (48 to 64°C), were inoculated (using a hypodermic syringe) with 1 ml of a culture growing exponentially in CM4-Cb at the same temperature. These inocula were prepared as follows: 1 ml of the stock culture was inoculated into 9 ml of CM4-Cb and

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incubated overnight at 60°C; 1 ml of this culture was then transferred into fresh CM4-Cb medium and incubated at the experimental temperature. After 10 to 12 h, 1 ml of the culture was again transferred to CM4-Cb, preequilibrated to the experimental temperature. The cultures were incubated at the experimental temperature for 0.5 to 1 h before being challenged with ethanol.

Portions of 1 ml of various aqueous oxygen-free ethanol solutions, equilibrated at the same temperature, were then injected into growing cultures. Oxygen-free distilled water (1-ml volumes) was injected into control cultures. Oxygen-free ethanol solutions were prepared by adding known quantities of absolute ethanol (prewarmed at 60°C; density, 0.76 g/liter) into the proper volumes of recently sterilized distilled water. Both ethanol and water were kept under N_2 atmosphere inside Hungate tubes, and mixing was done by injection of ethanol into the water tubes. The addition of these ethanol solutions to CM4-Cb did not cause changes in the color of resazurin. The OD_{660} was measured as a function of time.

Statistical methods. Specific rates (μ) were calculated using the slopes of the least-squares regression lines, which fitted the data of the natural logarithm of OD_{660} as a function of time. To calculate Arrhenius activation energies, linear regression analyses were performed on the natural logarithm of μ (growth rates, min^{-1}) as a function of the reciprocal value of the absolute temperature (°K).

RESULTS

Ethanol-resistant mutant selection. Ethanol-resistant mutants were obtained by the enrichment procedure described above. One of these mutants, strain C9, grew (with about 50 to 60% inhibition) in ethanol concentrations at which the parent strain could not grow. C9 and the parent strain had the same optimum temperature for growth and the same growth rate at 60°C, but the maximum OD_{660} for C9, reached at stationary phase (0.55 to 0.60), was lower than that attained by the parent (0.75 to 0.85). This mutant's specific properties, in relation to its ethanol tolerance, are described later.

Response of *C. thermocellum* to ethanol. We characterized the behavior of *C. thermocellum* in response to an ethanol challenge. Figure 1 presents results from a typical experiment. The culture continued to grow at a rate similar to the uninhibited one for about 45 to 90 min after ethanol addition (phase I). After a period of growth arrest (phase L), the culture resumed exponential growth at an inhibited rate (phase II). The growth rate during phase I and the duration of phase L were functions of the physiological state of the inoculum (Fig. 2). In the presence of ethanol, phase I was shortened and phase L was lengthened when stationary-phase cultures, instead of exponential-phase cultures, were used as inocula. No phase L was observed

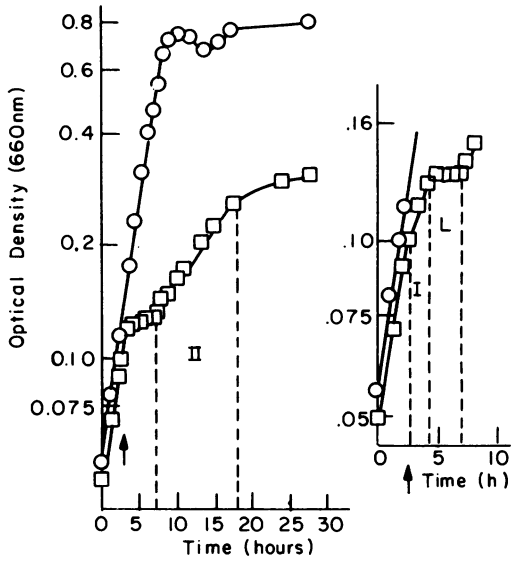

FIG. 1. *Growth response of C. thermocellum ATCC 27405 to an ethanol challenge at 60°C. ↑, Time of ethanol addition; ○, no ethanol added; □, 11 g of ethanol per liter.*

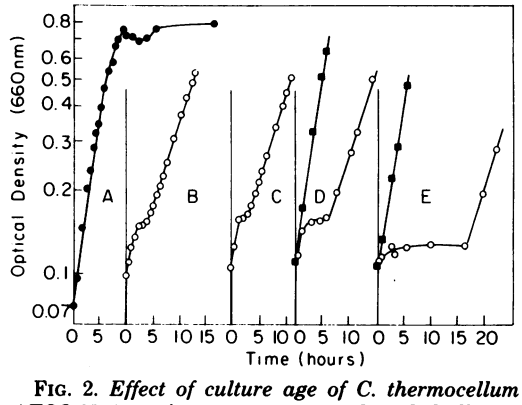

FIG. 2. *Effect of culture age of C. thermocellum ATCC 27405 on its response to an ethanol challenge. At different times during culture growth, portions were withdrawn and diluted, with fresh broth preequilibrated to 60°C, to an OD_{660} of approximately 0.1. The final ethanol concentration of the fresh broth was 7.5 g/liter. (A) ●, Growth curve without ethanol. (B) ○, Ethanol added at an OD of 0.1. (C) ○, Ethanol added at an OD of 0.2. (D) ○, Ethanol added to a 10-h culture; ■, control (no ethanol added) 10-h culture. (E) ○, Ethanol added to a 14-h culture; ■, control (no ethanol added) 14-h culture.*

when stationary-phase cultures were not challenged with ethanol (Fig. 2). At ethanol concentrations of 5 g/liter, the wild type showed a period of growth arrest (phase L) at 60°C, whereas the resistant strain required concentrations as high as 20 g/liter to show the period of

growth arrest (results not shown). However, the resistant strain showed the period of growth arrest at lower concentrations when growing temperature was either below or above 60°C (results not shown).

During phase II, the specific growth rate was a reproducible function of ethanol concentration and not a function of the physiological state of the inoculum. Since phase II took place when OD_{660} values were high enough to allow the control to grow exponentially, and was also the longest observed sustained exponential growth period, we used the specific growth rate during phase II to characterize the ethanol resistance of *C. thermocellum* strains.

The specific growth rate at a given ethanol concentration (μ_x^{II}) was obtained as the slope of the least-squares line that fit the natural logarithm of OD_{660} versus time data points. These regression analyses were performed on values that gave slopes with maximum correlation coefficients entering the maximum number of data points. Eight control replicates were done at each temperature (with independent inocula) to obtain the uninhibited growth rate. Inhibited growth rates were found to be reproducible in all experiments. The data are presented either as μ_x^{II}, as μ_x^{II}/μ_0, or as percentage of inhibition (%I) defined as: $\%I = (1 - \mu_x^{II}/\mu_0) \cdot 100$, where μ_0 is the uninhibited growth rate and μ_x^{II} is the growth rate in the presence of x grams of ethanol per liter, during phase II.

Effect of ethanol concentration on the growth of *C. thermocellum* at 60°C. Figure 3 is a semilog plot of μ_x^{II}/μ_0 versus ethanol concentration for both the wild type and C9. The inhibited growth rate was an exponential function of the ethanol concentration. Regression lines on these data provided, for the ATCC 27405 strain, a slope that was one order of magnitude higher than that of C9, showing that C9 had higher ethanol tolerance. At an ethanol concentration of 25 g/liter, the wild type had a negative growth rate, possibly caused by cell lysis, whereas the resistant strain was able to grow at a rate which was 40 to 50% of the uninhibited one. The growth rate of the parent strain was inhibited by 50% at 4 to 5 g/liter.

Growth of *C. thermocellum* at different temperatures. Figure 4, an Arrhenius plot of the uninhibited growth rates of both strains, shows the dependence of growth rates on temperature. Activation energies were obtained as the slopes of the regression lines of the natural logarithm of the specific growth rate as a function of the reciprocal value of the absolute temperature (°K). Growth rates of eight independent cultures were measured at each temperature shown (Table 1).

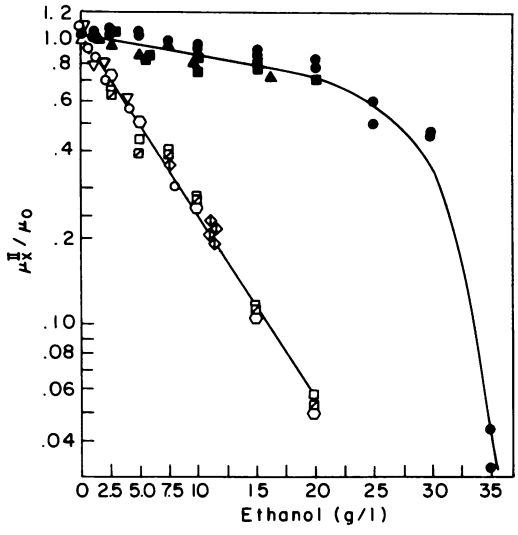

FIG. 3. *Effect of different ethanol concentrations on growth of C. thermocellum ATCC 27405 (open symbols) and C9 (closed symbols). The different symbols correspond to independent experiments. Incubation temperature was 60°C.*

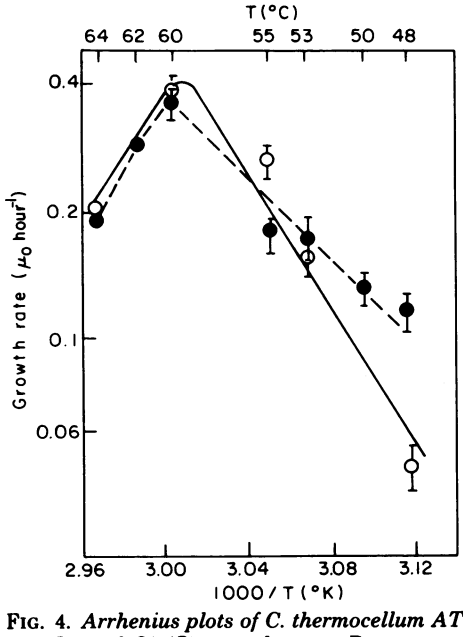

FIG. 4. *Arrhenius plots of C. thermocellum ATCC 27405 (○) and C9 (●) growth rates. Bars represent sample standard deviations (eight replicates).*

Effect of ethanol concentration on *C. thermocellum* growth at temperatures above and below the optimum. Figure 5 presents the same type of plot as Fig. 3, but at temperatures in both the suboptimal and supraoptimal ranges. In the suboptimal range (48 to 55°C), differences between the wild type and

TABLE 1. *Activation energies for growth of C. thermocellum ATCC 27405 and C9*

Strain	Ea ± SD[a]		99% confidence interval	
	kcal/mol	kJ/mol	kcal/mol	kJ/mol
ATCC 27405	35.9 ± 2.0	150.3 ± 8.4	30.9, 40.9	129.3, 171.2
C9	19.0 ± 1.0	79.5 ± 4.2	16.5, 21.5	69.1, 90.0

[a] When a Student's t test is carried out using a pooled standard deviation (SD) of measurements performed in both strains, the two Ea values are significantly different at $P \leq 0.0005$.

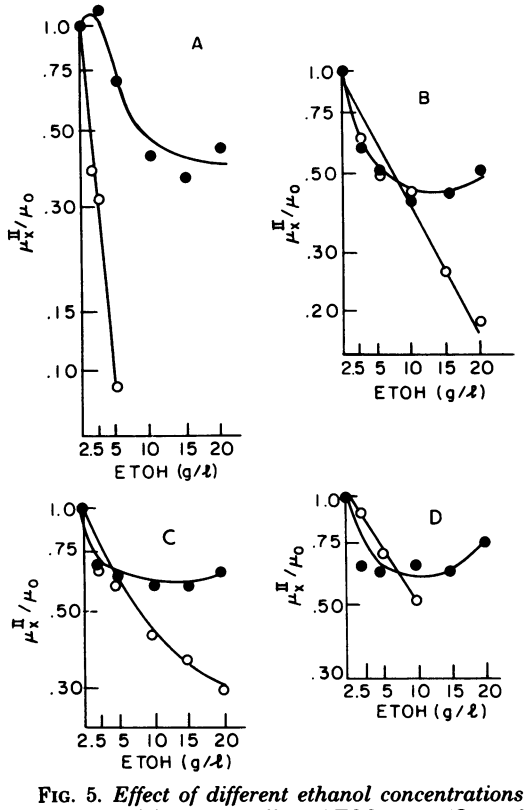

FIG. 5. *Effect of different ethanol concentrations on growth of C. thermocellum ATCC 27405 (○) and C9 (●) at 64°C (A), 55°C (B), 53°C (C), and 48°C (D).*

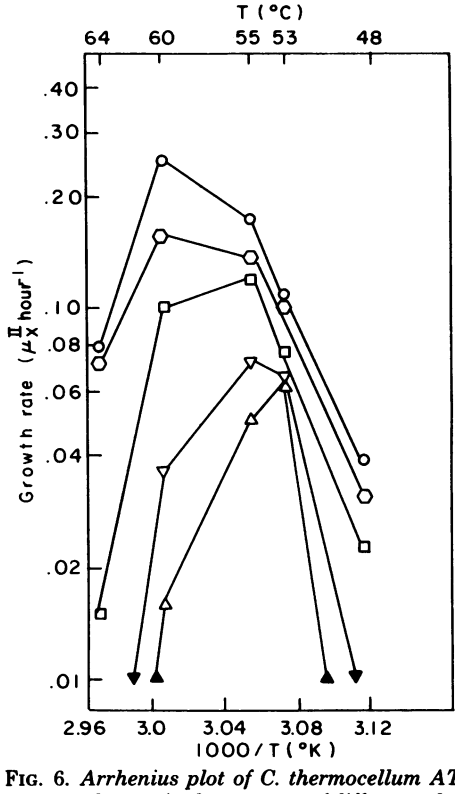

FIG. 6. *Arrhenius plot of C. thermocellum ATCC 27405 growth rates in the presence of different ethanol concentrations: ○, 2.5 g/liter; ○, 5 g/liter; □, 10 g/liter; ▽, 15 g/liter; △, 20 g/liter. Closed symbols indicate values obtained by interpolation on curves of μ_x^{II} versus temperature when lysis, rather than growth, was observed.*

the resistant strain were found only at ethanol concentrations higher than 10 g/liter, at which the growth of wild type was either strongly inhibited or stopped, whereas the resistant strain still grew at measurable rates. Above the optimum (60°C), a small increase in ethanol concentration strongly inhibited the growth of the wild type but not that of the ethanol-tolerant strain. At 64°C the growth of the wild-type strain was completely inhibited at an ethanol concentration of 10 g/liter. At 48°C the growth of the wild-type strain was completely inhibited at an ethanol concentration of 15 g/liter.

Figure 6 is an Arrhenius plot of inhibited growth rates of ATCC 27405, obtained at different ethanol concentrations. Figure 7 is the same plot for C9. The ethanol-resistant strain showed the same optimum growth temperature at ethanol concentrations of 2.5 to 20 g/liter. In the same ethanol concentration range, the wild type's optimum growth temperature was lowered as the ethanol concentration increased.

DISCUSSION

We investigated ethanol tolerance, as related to growth temperature, in *C. thermocellum* ATCC 27405 and in its ethanol-resistant derivative C9.

The inhibited growth of ethanol-challenged

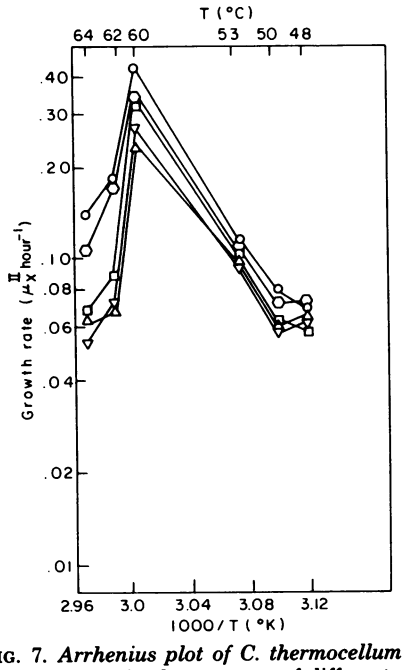

FIG. 7. *Arrhenius plot of C. thermocellum strain C9 growth rates in the presence of different ethanol concentrations.* ○, *2.5 g/liter;* ○, *5 g/liter;* □, *10 g/liter;* ▽, *15 g/liter;* △, *20 g/liter.*

cultures is characterized by three phases: (i) a short phase of little or no inhibition (phase I); (ii) a period of growth arrest, immediately after phase I (phase L); and (iii) a phase of inhibited but sustained exponential growth (phase II).

These results (Fig. 1) have important implications. It has been proposed (7) that an inhibition index equal to 1 − (ΔOD, inhibited culture)/(ΔOD, control culture) be used to evaluate the extent to which additives inhibit growth in bacterial cultures, and that the ΔOD values be measured over a constant period of time.

Since our results indicated that the duration of both phase I and phase L are dependent on inhibitor concentration and other culture conditions, inhibition data should be analyzed carefully and controls should be run to assess the presence or absence of phases I, II, and L.

That ethanol interferes with cell division has been shown in other organisms (8). Our results (Fig. 1) indicated that, during phase I, a proportion of the exponentially growing population can divide at the same rate in the presence of ethanol as in its absence. It appears that actively dividing cells can complete their division cycle in the presence of ethanol. The extended periods of growth arrest observed when stationary cultures are challenged with ethanol (Fig. 2) are probably caused by the inability of these cells to adjust quickly to the new conditions. Exponentially growing cultures involved actively in biosynthesis are more likely to adjust to environmental changes that require biosynthetic pathway redirection for membrane structure adjustment. *Escherichia coli* cultures also show a period of growth arrest as well as altered membrane fatty acid composition when ethanol is added (12); the duration of the period of growth arrest may be the time required for cells to undergo homeoviscous adaptation in response to membrane fluidity changes caused by ethanol addition.

Alternatively, it has been shown by Abbott (1) that *Acinetobacter calcoaceticus*, growing on low ethanol concentrations, exhibits phases I, L, and II when challenged with high ethanol concentrations. He proposed that, when confronted with high ethanol concentrations, *A. calcoaceticus* rapidly oxidizes ethanol to acetate. As the ethanol is converted to acetate, the microorganism continues to grow at its initial rate until the ethanol is depleted or the acetate reaches inhibitory concentrations. A period of growth arrest then ensues during which the microorganism begins to slowly utilize the acetate. As the acetate is lowered the growth rate increases and reaches its previously high levels. However, unlike *A. calcoaceticus*, *C. thermocellum* is not able to utilize acetate or ethanol as carbon sources. In addition, there is no evidence that *C. thermocellum* can oxidize ethanol to acetate.

Maintenance of optimum membrane fluidity appears to be crucial to healthy cell physiology (5, 23, 26); at high temperatures, high fluidity (i.e., high degree of molecular motion in the lipid bilayer) may produce "leaky," unstable membranes. Low fluidity (i.e., a "frozen" bilayer) may deprive inactive integral proteins of either their required lipid cofactors or the lipophilic milieu needed for optimal activity. It would appear that membrane fluidity changes can affect growth rate by causing permeability or other membrane function alterations.

Numerous reports provide evidence that membrane fluidity is a regulated function (2, 3, 5, 6, 11, 14, 16, 17, 19–31). Biological systems respond to environmental factors that change membrane fluidity by synthesizing membrane constituents with properties that compensate for the external change. Thus, lowering growth temperature increases unsaturated fatty acid synthesis; unsaturated fatty acids are difficult to array in an orderly manner and therefore provide a less viscous milieu. Raising growth temperatures decreases unsaturated fatty acid synthesis.

Amphiphilic molecules, such as *n*-alkanols, either fluidize or increase the bilayer's internal viscosity, depending on the site at which these

molecules partition and interact. In model bilayers, this site is a function of chain length and alkanol concentration. It has been proposed (15) that, at relatively low concentrations, ethanol fluidizes artificial (dipalmitoyl lecithin liposomes) bilayers by partitioning near their cores, i.e., at the methyl end of each acyl chain. At higher concentrations, ethanol may partition closer to the exterior of the bilayer, allowing rows of several ethanol molecules to substitute for the acyl chain and contribute to membrane stability (15).

In our inhibition experiments, the period of growth arrest was followed by a state of balanced, inhibited growth. Evidence accumulated in a variety of biological systems suggests that the membrane composition and properties of an ethanol-challenged culture change, and that this change is related to growth inhibition. Our measurements of growth rate, in the presence of ethanol (μ_x^{II}), should reflect the culture's adaptive response to ethanol.

Figure 4 shows the difference between the two strains in activation growth energies. Differences in activation energies have been related to changes in membrane composition (23). Figure 4 also shows that C9 can grow faster than the wild type at low temperatures (0.13 h^{-1} versus 0.06 h^{-1} at 48°C), suggesting that C9 can adapt its membrane viscosity upon "freezing" of its lipids, and thus maintain a higher growth rate than the parent strain. Our data suggest that, in the ethanol-resistant strain, ethanol resistance is associated with tolerance of low temperatures.

At 64°C, at increasing ethanol concentrations, the wild type showed a marked decrease in μ_x^{II}/μ_0 (Fig. 5) compared to that observed at suboptimal growth temperatures. Our interpretation of this result is that *C. thermocellum* responds to ethanol by producing a more fluid membrane, and that this response takes precedence over the adaptive response due to the increased growth temperature, meaning that even if an adaptive response had taken place in the opposite direction (e.g., an increase in membrane viscosity to sustain growth at high temperature), the presence of ethanol will trigger the response to fluidize the membrane. This fluidization would be less tolerated at higher than at lower temperatures. This conclusion is also substantiated by the decrease in the optimum growth temperature of the wild type (Fig. 6) observed as the ethanol concentration in the broth was raised.

It is difficult to extrapolate the meaning of low and high ethanol concentrations from artificial bilayers to functional bacterial biomembranes. In all cases, the ethanol amount present in the lipid environment will depend on the partition coefficient of ethanol into the membrane, which is a function of the chemical nature of the membrane's components. Assuming a lipid content of 5% (dry cell weight) and an average molecular weight of lipids such as that of dipalmitoyl lecithin (i.e., ~900 daltons), the lipid/ethanol molar ratios used in our in vivo experiments range from 2×10^{-4} (ethanol concentration of 1.25 g/liter) to 10^{-5} (ethanol concentration of 20 g/liter). These values are much lower than those required to promote fluidization in vitro (15). This indicates that under these conditions, ethanol would probably freeze the bilayer, a conclusion that agrees with Ingram's reports (3, 12, 14) on the physiological response of *E. coli* to ethanol. Ingram proposed that ethanol freezes the membrane, since the physiological response observed is an increase in the fluidity of lipid components.

The optimum growth temperature of the resistant strain remained at 60°C at ethanol concentrations up to 20 g/liter (Fig. 7), whereas that of the wild type decreased. Since the physical effect of ethanol is to "tighten" the membrane and the biological response is to produce a more fluid membrane, it follows that membrane fluidization would decrease the optimum growth temperature. Therefore, we suggest that the ethanol-tolerant strain has a diminished capacity to respond to an ethanol challenge as far as membrane fluidization is concerned. Although membrane fluidization might be the logical response of a microorganism to ethanol, this membrane change may interfere with normal cell physiology. In fact, Gill and Suisted (9) observed that several bacteria tend to maintain a constant membrane fatty acid composition, rather than constant fluidity at different growth temperatures. Other mechanisms to adjust membrane fluidity, such as changes in fatty acid positional distribution in the phospholipid molecules (16) and changes in the molecular species of phospholipids (4, 10, 13, 18), may also have unfavorable consequences for cell physiology.

ACKNOWLEDGMENTS

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