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Ethanol Production by Thermophilic Bacteria: Physiological Comparison of Solvent Effects on Parent and Alcohol-Tolerant Strains of *Clostridium thermohydrosulfuricum*

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The effects of temperature, solvents, and cultural conditions on the fermentative physiology of an ethanol-tolerant (56 g/liter at 60°C) and parent strain of *Clostridium thermohydrosulfuricum* were compared. An ethanol-tolerant mutant was selected by successive transfer of the parent strain into media with progressively higher ethanol concentrations. Physiological differences noted in the mutant included enhanced growth, tolerance to various solvents, and alterations in the substrate range and the fermentation end product ratio. Ethanol tolerance was temperature dependent in the mutant but not in the parent strain. The mutant grew with ethanol concentrations up to 8.0% (wt/vol) at 45°C, but only up to 3.3% (wt/vol) at 68°C. Low ethanol concentration (0.2 to 1.6% [wt/vol]) progressively inhibited the parent strain to where glucose was not fermented at 2.0% (wt/vol) ethanol. Both strains grew and produced alcohols on glucose complex medium at 60°C in the presence of either 5% methanol or acetone, and these solvents when added at low concentration stimulated fermentative metabolism. The mutant produced ethanol at high concentrations and displayed an ethanol/glucose ratio (mole/mole) of 1.0 in media where initial ethanol concentrations were ≤4.0% (wt/vol), whereas when ethanol concentration was changed from 0.1% to 1.6% (wt/vol), the ethanol/glucose ratio for the parent strain changed from 1.6 to 0.6. These data indicate that *C. thermohydrosulfuricum* strains are tolerant of solvents and that low ethanol tolerance is not a result of disruption of membrane fluidity or glycolytic enzyme activity.

Thermophilic saccharide fermentations have been suggested as potential novel systems for industrial ethanol production for three major reasons (14, 15, 18–20, 22, 23). Thermoanaerobes, unlike *Saccharomyces* or *Zymomonas* species, can ferment biomass polymers (i.e., cellulose, hemicellulose, or starch) or hexoses and pentoses directly to ethanol. Thermophiles also possess very high metabolic transformation rates. Last, thermophilic fermentations may allow less energy-intensive process design if a continuous ethanol recovery process by reduced pressure distillation at 60°C can be developed. Considerable emphasis has been placed on the utilization of cocultures (18–20) such as *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum* (16) to ferment delignified wood to ethanol. The major limitations of utilizing thermophilic fermentations include the formation of other carbon waste products and the low final ethanol concentration (<2.0%) achieved with the parental strains.

Recently, Herrero and Gomez (7–10) have studied the mechanism of ethanol tolerance in *C. thermocellum*. These authors selected an ethanol-resistant strain which unlike the parent strain grew at medium ethanol concentrations >20 but <35 g/liter (8). However, these low concentrations of ethanol inhibited the growth rate of *C. thermocellum*. The low ethanol tolerance of *C. thermocellum* was ascribed to the general effect of a solvent on increasing membrane fluidity (9, 10) and to specific inhibition of some glycolytic enzyme(s) involved in transformation of hexose into glyceraldehyde-3-phosphate (7).

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C. thermohydrosulfuricum 39E ferments starch and a wide variety of hexose- or pentose-derived saccharides including xylose and glucose into ethanol as the major reduced end product (16, 21). In the present paper, we compare the effects of solvent concentration on growth and end product formation of an ethanol-resistant mutant strain with those of the parent *C. thermohydrosulfuricum* strain. The data indicate that the parent and mutant strains of *C. thermohydrosulfuricum* are more solvent tolerant than *C. thermocellum* strains (7–10) and that the mechanisms by which ethanol inhibits growth are quite different.

MATERIALS AND METHODS

Organisms and culture conditions. *C. thermohydrosulfuricum* 39E was isolated from Octopus Spring at Yellowstone National Park (21) and is in the American Type Culture Collection (Rockville, Md.) as ATCC 33223. Stringent anaerobic culture techniques (21) were employed for all experimental studies.

An ethanol-resistant mutant, 39EA, was selected from the parental strain as described below. Strain 39E was routinely grown on complex medium in 30-ml anaerobic pressure tubes (Bellco Glass, Inc., Vineland, N.J.) that contained yeast extract, Trypticase, trace salts, and vitamins (TYE medium) (16) with either 0.5% xylose or glucose as the fermentable carbohydrate. Mutant strain 39EA was maintained on this medium but with 50 g of absolute ethanol per liter. Culture media were autoclaved for 30 to 45 min to ensure killing the extremely heat-resistant spores of thermoanaerobes (11).

All experiments were performed in anaerobic pressure tubes that contained 10 ml of TYE medium, 0.5% of the fermentable carbohydrate indicated, and the amount of

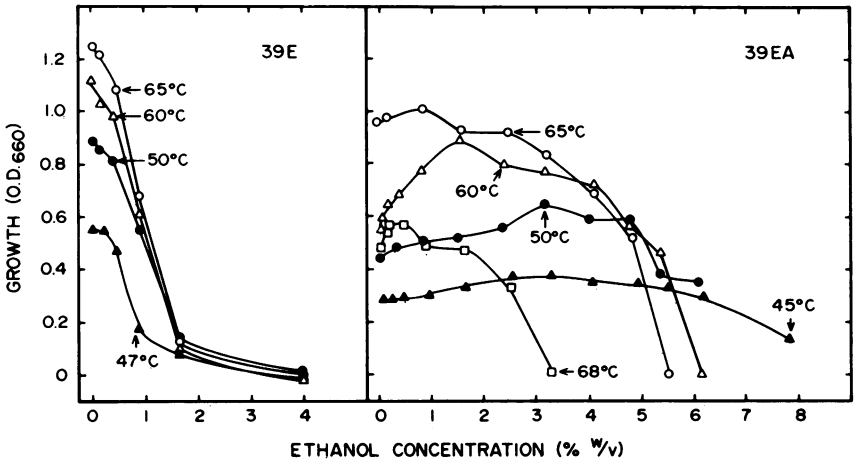

FIG. 1. Influence of temperature and ethanol concentration on final growth yield of *C. thermohydrosulfuricum* strains. The parent (39E) and ethanol-resistant mutant (39EA) were grown in anaerobic pressure tubes that contained 10 ml of TYE–0.5% glucose medium. The limits of growth are shown by the last point. At ethanol concentrations above these values, the growth yields were below 0.05.

solvent described. The tubes were preincubated in a water bath at the experimental temperature and inoculated with 1 ml of an exponential-phase culture.

The tubes were not shaken and were incubated at 60°C unless indicated. Cultures were routinely checked for purity by streaking onto plates of TYE medium that contained 0.5% glucose and 2.0% purified agar (Difco Laboratories, Detroit, Mich.), with or without 4.0% ethanol for strain 39EA or 39E, respectively. This was performed in an anaerobic glove bag (Coy Laboratory Products, Ann Arbor, Mich.). Only strain 39EA grew in the presence of 4.0% ethanol on agar plates.

Electron microscopy. The same procedures described by Hyun et. al. (11) were employed.

Measurement of growth, substrate consumption, and end product formation. Growth was measured by the increase in optical density at 660 nm (OD_{660}) by the insertion of the experimental tube into a Spectronic 20 Spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). All growth data reported represent the average of 5 to 10 replicate experimental determinations.

Fermentation products were routinely determined by gas chromatographic and enzymatic procedures described elsewhere (14, 16). These procedures were not suitable for end product measurement in the presence of high solvent concentrations because of interference. Therefore, fermentation balances were based on use of ^{14}C-labeled glucose and separation of the substrate and the ^{14}C-labeled end products by high-pressure liquid chromatography.

A Perkin-Elmer series 3 liquid chromatograph equipped with a Sigma 10 data station (Perkin Elmer Corp., Norwalk, Conn.) and refractive index detector (Laboratory Data Control, Riveria Beach, Fla.) was employed. Separation of substrate and end product was achieved on a Bio-Rad Aminex ion-exclusion HPX87H column (300 by 7.8 mm) fitted with a microguard precolumn (Bio-Rad Laboratories, Richmond, Calif.). H_2SO_4 (0.014 M) was used as the solvent at a flow rate of 0.8 ml/min and at ambient temperature. Culture broth samples (0.4 ml) were acidified with 25 μl of 10 M phosphoric acid and centrifuged at 5,000 × *g*. Samples (100 μl) were loaded onto the column, and fractions were collected with a Gilson FC100 Microfractionator (Gilson Medical Electronics, Middleton, Wis.). Fractions that corresponded to coincidence peaks for ^{14}C-labeled substrate and end product standards were placed into Instagel (Packard Instrument Co., Inc., Downers Grove, Ill.) and counted in a Packard Tricarb scintillation counter.

The fermentation balance studies were preformed in pressure tubes that containg TYE medium, 0.4% glucose and ca. 2 μCi of [*U*-^{14}C]glucose (348.2 mCi/mmol; New England Nuclear Corp., Boston, Mass.), plus the amount of solvent indicated. Immediately after inoculation, a sample was removed by syringe to determine the specific activity of glucose. The carbon fermentation balance was determined at the end of growth (24 h), and the data reported represent the mean of duplicate determinations.

All growth data were repeatable within 95% confidence limits. The standard deviation limit values did not vary more than 5% in the reported growth experiments.

RESULTS

Selection and characterization of the ethanol-resistant mutant. An ethanol-tolerant strain, 39EA, was derived from parental strain 39E by sequential transfer of the culture in TYE medium that contained 0.5% xylose and progressively higher amounts of added ethanol. After ca. 25 transfers, the culture was capable of growth at 60°C in 50 g of ethanol per liter. The selection of ethanol-resistant cultures by this procedure was repeated three times. A stock culture of strain 39EA was prepared by transferring a single colony isolated on a TYE-glucose agar medium plate in the anaerobic glovebag to a pressure tube that contained TYE medium, 0.5% glucose, and 5.0% ethanol.

The inhibitory effects of ethanol on microbial growth can be decreased by lowering the incubation temperature (2). Thus, experiments were designed to compare the effects of added ethanol concentration and temperature on growth of strains 39E and 39EA. The results of these studies are shown in Fig. 1 and 2. Increasing ethanol concentration drastically decreased the final cell density and the growth rate of the parental strain at incubation temperatures up to 65°C. Significant growth (i.e., final change in OD_{660}, >0.2; specific growth rate (μ), >0.002) of the parental strain 39E was not detectable above 20 g ethanol/l (i.e., it lacks ethanol tolerance). On the other hand, the growth response of strain 39EA was temperature dependent, and good growth at

greater than 20 g/liter confirmed that it was an ethanol-resistant mutant. Ethanol tolerance of the mutant was significantly enhanced at lower growth temperatures. Thus, the mutant strain 39EA grew at 45°C with ethanol concentrations above 70 g/liter but it did not grow above 30 g of ethanol per liter at 68°C. The growth rate and yield (i.e., OD_{660}) of the mutant were significantly higher at incubation temperatures above 50°C. An ethanol concentration of 56 g/liter and an incubation temperature of 60°C appeared maximal for combined growth and ethanol tolerance.

Other experiments were initiated to compare the physiological properties of the parent strain and the ethanol-resistant mutant. Both strains reduced thiosulfate to H_2S, but significant differences were observed in the carbon sources consumed and in the glucose fermentation end product yield (Table 1). The ethanol-resistant mutant did not ferment pyruvate or starch and produced more lactate and less ethanol than the parent strain.

Figure 3 illustrates a typical growth curve for both strains at 62°C on glucose in TYE medium. The parent strain displayed a biphasic growth curve (21) with a primary and secondary doubling time of 1.7 and 4.6 h, respectively. The mutant showed a monophasic growth curve with a 3.0-h doubling time. Cell density of the mutant was significantly lower than that of the parental strain at 10 h. The parental strain, however, lysed readily in the early-stationary growth phase. The mutant strain did not display significant cell lysis even after growth at high ethanol concentrations (i.e., 50 g/liter).

Figure 4 compares the ultrastructural appearance of late-exponential-phase cells of the parental strain grown without ethanol added to the medium with the mutant strain grown in 60 g of ethanol per liter. A significant portion of the parental strain was comprised of lysed cells. High ethanol concentrations appeared to inhibit membrane functions of the mutant strain because the following cell abnormalities were observed in thin sections: long filamentous cells which lacked division planes; cells with numerous distorted internal membranes; and large, swollen balloon-type cells with distorted walls. When the mutant was grown at 40 g of ethanol per liter, the cell distortions observed were less significant.

TABLE 1. General metabolic comparison of the parental and ethanol-resistant mutant strains of *C. thermohydrosulfuricum*

Metabolic feature	Parent (39E)	Mutant (39EA)
Growth substrates[a]		
Xylose	+	+
Glucose	+	+
Cellobiose	+	+
Starch	+	−
Lactose	+	+
Fructose	+	+
Pyruvate	+	−
Fermentation products (μmol/10 ml of glucose medium)[b]		
Ethanol	350	150
Acetate	18	16
Lactate	51	130
CO_2	360	154
H_2	30	23
Carbon recovery (%)	97	96

[a] Both strains were tested at 60°C in TYE medium with 0.4% substrate, and OD_{660} was determined after 10- and 24-h incubations for 39E and 39EA, respectively. +, $OD_{660} > 0.3$; −, $OD_{660} < 0.1$.

[b] Both strains were grown at 60°C on TYE medium with 0.4% glucose in anaerobic pressure tubes. Fermentation balances were determined after 28 h.

Influence of solvents on growth and fermentation product formation. Experiments were initiated to assess whether the basis for ethanol resistance of the mutant was related to enhanced solvent tolerance. Table 2 compares the effects of

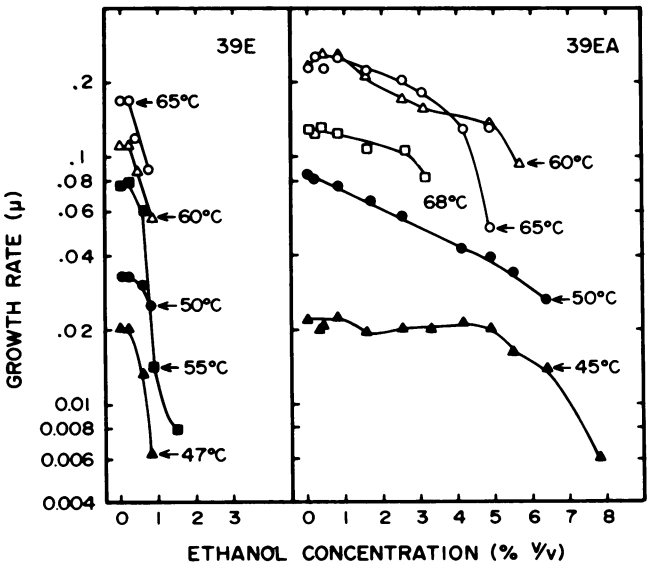

FIG. 2. Influence of temperature and ethanol concentration on growth rate of *C. thermohydrosulfuricum* strains. The parent (39E) and ethanol-resistant mutant (39EA) were grown in anaerobic pressure tubes that contained 10 ml of TYE–0.5% glucose medium. The limits of growth are shown by the last point. At ethanol concentrations above these values, the specific growth rates were below 0.002.

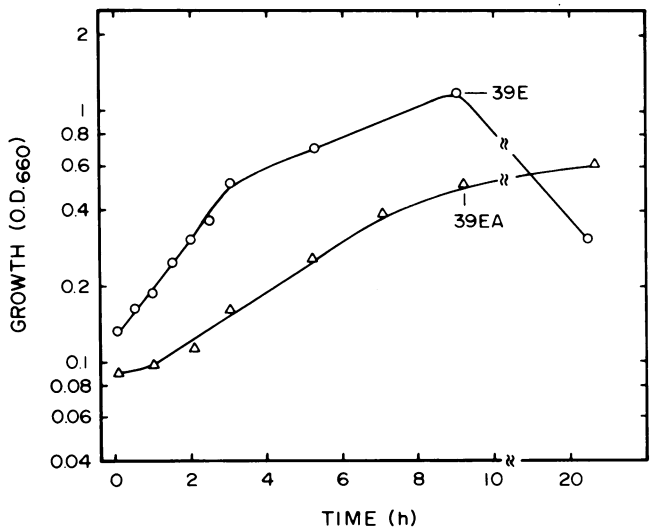

FIG. 3. Comparison of the growth response of the parent (39E) and ethanol-resistant mutant (39EA) of *C. thermohydrosulfuricum*. Both strains were grown at 65°C in anaerobic pressure tubes that contained 10 ml of TYE–0.5% glucose medium without added ethanol. It should be noted that all growth rates reported for strain 39E in this paper were those calculated for the secondary growth phase.

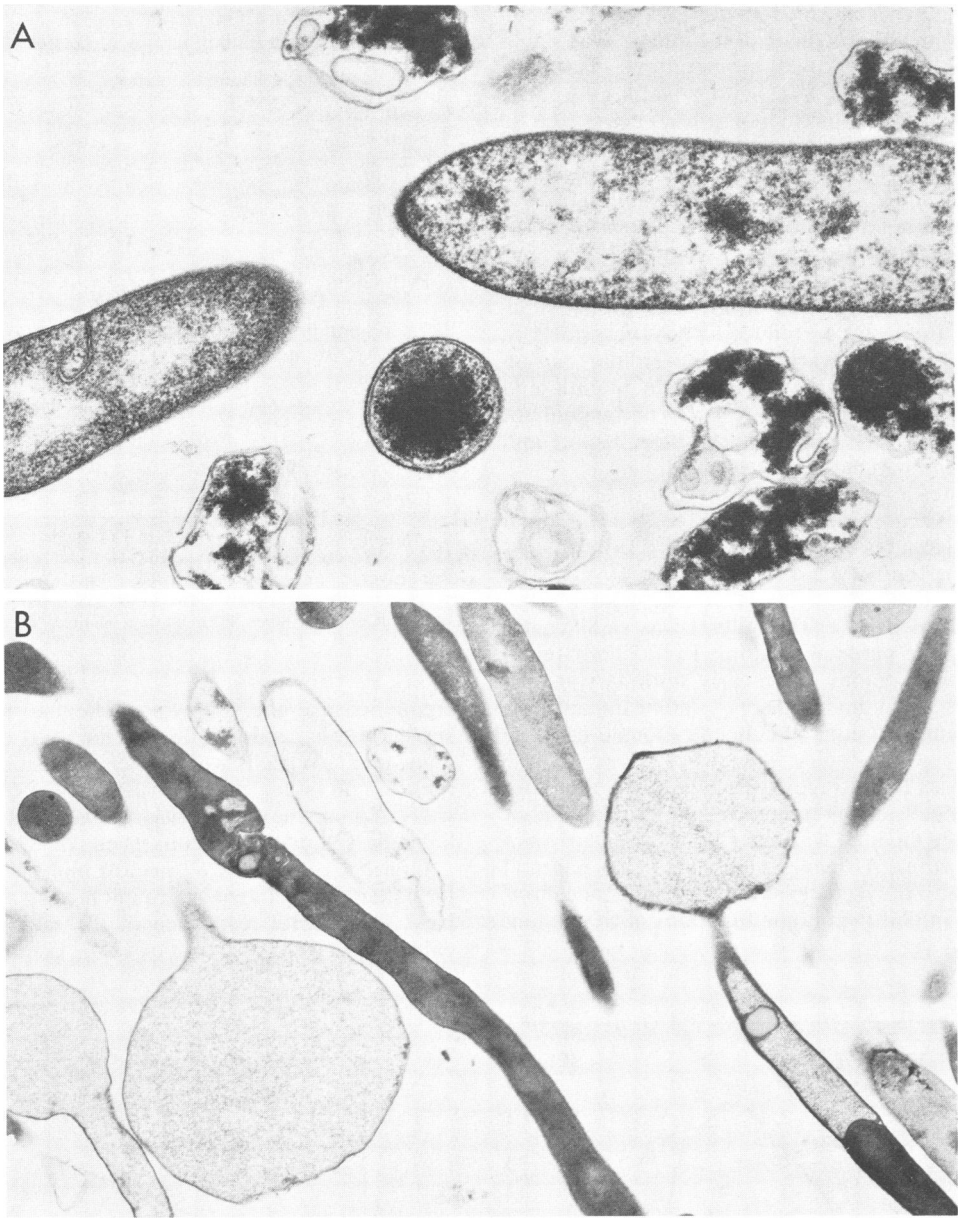

FIG. 4. Ultrastructural appearance of *C. thermohydrosulfuricum* strains. (A) Grazing section of the parent strain grown to late-exponential phase on TYE–0.5% glucose medium at 60°C. (B) Grazing sections of the ethanol-resistant mutant strain grown to late-exponential phase at 60°C on TYE-0.5% glucose medium with 60 g of ethanol per liter.

various solvents on growth of the two strains. In general, the ethanol-resistant mutant was more tolerant of methanol, ethanol, and isopropanol than the parent strain, and low concentrations of these solvents stimulated growth of the mutant, whereas they inhibited the parental strain. As expected, low concentrations of acetone stimulated growth of both strains because *C. thermohydrosulfuricum* contains an alcohol dehydrogenase which reduces acetone to isopropanol (13), and this affords certain thermophilic species with an enhanced growth rate and yield (1). The parental strain displayed a much higher tolerance for methanol, acetone, and isopropanol than ethanol, and it grew in the presence of 2 to 5% of these solvents. Long-chain alcohols such as butanol were very toxic for growth of both strains. The addition of 2% butanol (270 mM) readily caused cells of both strains to lyse at 60°C. Neither strain displayed growth in the presence of 0.5% octanol, dodecanol, or ally alcohol.

More detailed studies were initiated to examine the influence of acetone, ethanol, and methanol concentration on the growth response of the parent strain (see Fig. 5 and 6). At an equal solvent concentration of 270 mM (0.86% [wt/vol] methanol, 1.2% [wt/vol] acetone or ethanol), the following was observed: acetone stimulated both the growth rate ratio and growth yield from 1 to 3.5 and from 1.1 to 1.4, respectively, ethanol reduced the growth rate ratio and yield from 1.1 to 0.5 and from 1.1 to 0.8, respectively, whereas methanol had little effect.

Figure 5 illustrates that the parental strain, although not tolerant of 3.0% ethanol, displays the same cell yield as the mutant in 3.0% acetone or methanol. Both strains displayed

higher tolerances for methanol than for acetone. The parent strain grew in methanol up to 69 g, whereas the mutant did so up to 100 g/liter (data not shown). Growth of the parental strain was higher on 2.0% acetone than in the absence of the solvent, yet it would not grow in 2.0% ethanol.

Figure 6 shows the effect of increasing concentrations of acetone, ethanol, and methanol on the growth rate ratio (i.e., without solvent/with solvent) of the two strains. Low concentrations of all three solvents enhanced the growth rate of the mutant strain, whereas only acetone and methanol enhanced the growth rate of the wild-type strain. Thus, low ethanol concentration specifically and not solvent concentration per se inhibits growth of the parental strain.

The effect of ethanol concentration on glucose fermentation of the two strains is shown in Table 3. Increasing ethanol concentration during growth of the parental strain progressively decreased glucose consumption and decreased the ethanol yield as a result of shifting the fermentation product ratios. At 1.6% ethanol, glucose consumption by strain 39E decreased ninefold, whereas lactate and acetate production increased at the expense of a 60% lower ethanol yield. On the other hand, a slight stimulation of glucose transformation to alcohol was observed at 2.0% ethanol for mutant strain 39EA. Ethanol was also a major end product of the mutant at 4.0% alcohol. Nonetheless, the best ethanol yield ratio of the mutant strain (1.0) was significantly lower than that of the parental strain (1.5) grown in the absence of

TABLE 2. Influence of various solvents on the final growth yield of the parent and ethanol-resistant mutant strains of *C. thermohydrosulfuricum*[a]

Solvent concn (% [wt/vol])	Growth (ΔOD_{660})	
	Parent (39E)	Mutant (39EA)
None	1.12	0.46
Methanol		
0.5	1.05	0.45
1.0	1.03	0.52
2.0	0.95	0.65
5.0	0.60	0.70
Acetone		
0.5	1.40	1.05
1.0	1.40	1.03
2.0	1.18	1.01
5.0	0.50	0.81
Ethanol		
0.5	1.0	0.52
1.0	0.85	0.80
2.0	0	0.76
5.0	0	0.40
Isopropanol		
0.5	0.95	0.55
1.0	0.80	0.60
2.0	0.40	0.65
5.0	0.09	0.30
Butanol		
0.5	0.42	0.50
1.0	0.14	0.35
2.0	0	0

[a] Both strains were grown in pressure tubes at 60°C on TYE medium that contained 0.5% glucose and the amount of solvent indicated. Growth was measured as final OD_{660} minus initial OD_{660}.

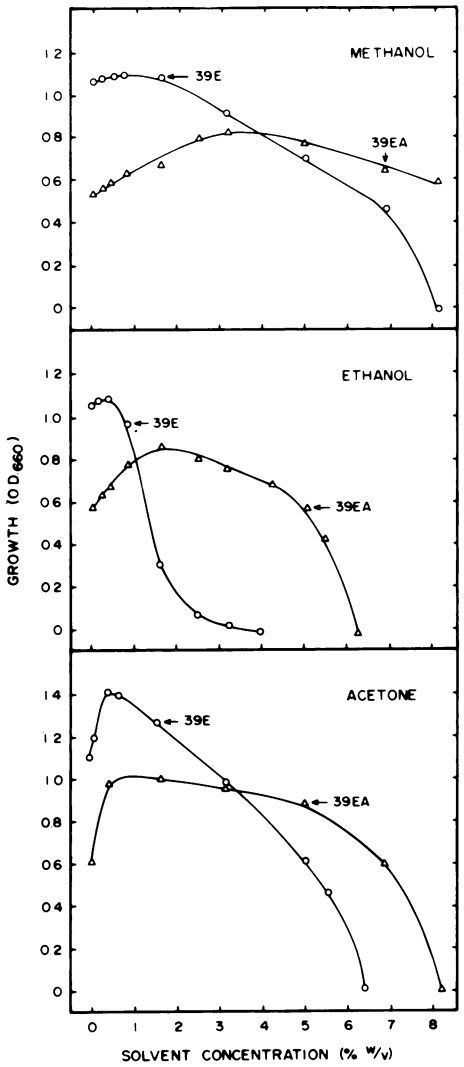

FIG. 5. Influence of different solvent concentrations on the final growth yield of *C. thermohydrosulfuricum*. The parent (39E) and the ethanol-resistant mutant (39EA) were grown at 60°C in anaerobic pressure tubes that contained TYE–0.5% glucose medium with the concentration of solvent indicated.

added ethanol. Ethanol production by the mutant strain appeared to be limited by low pH (5.0) caused by production of high levels of lactic and acetic acids.

Table 4 compares the influence of acetone and methanol on glucose fermentation by the two strains. The stimulatory effect of acetone on growth of both strains was a consequence of enhanced acetate production. Lactate and ethanol production decreased in the presence of acetone, and isopropanol was the major reduced carbon end product formed. Aside from doubling the acetate yield, end product formation by either strain was not significantly altered by 4.0% methanol. The ethanol yield ratio in the presence of methanol was 1.6 and 0.9 for the parental and mutant strains, respectively.

DISCUSSION

These data provide the first clear evidence that certain thermophilic *Clostridium* strains both produce alcohol and

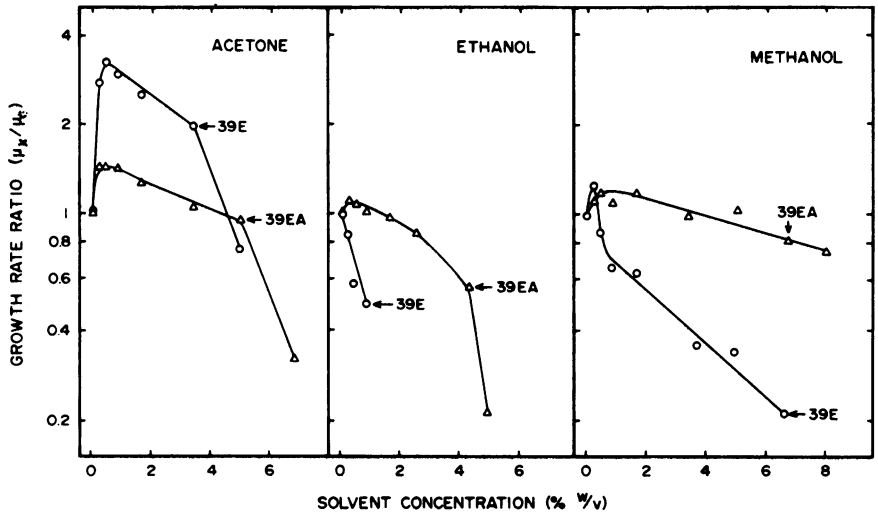

FIG. 6. Influence of different solvent concentrations on the growth rate ratio (μ_x with solvent/μ_0 without solvent) of *C. thermohydrosulfuricum* strains. The parent (39E) and the ethanol-resistant mutant (39EA) were grown at 60°C in anaerobic pressure tubes that contained TYE–0.5% glucose medium with the concentration of solvent indicated.

grow at ethanol concentrations greater than 4% (wt/vol). Thus, the physiological basis for ethanol tolerance in *C. thermohydrosulfuricum* strains differs significantly from that reported for *C. thermocellum* (7–10), in which ethanol concentrations of less than 3.5% significantly decreased growth as a consequence of increased membrane fluidity and inhibition of glycolytic enzyme activity. The explanation for high general solvent tolerance of *C. thermohydrosulfuricum* may be a result of a unique membrane lipid composition. In this regard, *C. thermohydrosulfuricum* but not *C. thermocellum* contains a C_{30} dicarboxylic acid thought to exist as a tetraester of glycerol and which forms a unit membrane (T. Langworthy, personal communication). Membranes having this component as their major lipid may display more alcohol

TABLE 3. Influence of ethanol concentration on glucose fermentation by the parent and mutant strains of *C. thermohydrosulfuricum*[a]

Strain	Ethanol (% [wt/vol])	Growth (OD_{660})	pH	Glucose consumed (μmol)	End product ratio (mM/100 mM glucose)				Carbon recovery (%)	Ethanol yield (mol ethanol/mol glucose)
					Lactate	Acetate	Ethanol	CO_2		
Parent strain 39E	0	1.1	6.3	220	23	8	155	163	93	1.5
	0.4	1.0	6.4	212	19	8	147	153	86	1.5
	0.8	0.75	6.5	63	14	22	112	133	91	1.1
	1.6	0.3	6.6	27	30	37	63	100	93	0.6
Mutant strain 39EA	0	0.8	5.0	164	80	10	92	102	92	0.9
	0.8	0.7	5.1	155	85	9	87	96	94	0.9
	2.0	0.8	5.0	211	66	7	103	110	90	1.0
	4.0	0.6	5.0	157	82	8	74	83	87	0.7

[a] Anaerobic pressure tubes contained 10 ml of TYE medium with 0.4% glucose, 2 μCi of [^{14}C]glucose, and the amount of solvent indicated. Tubes were assayed after growth was completed at 60°C.

TABLE 4. Influence of acetone and methanol on glucose fermentation by the parent and mutant strains of *C. thermohydrosulfuricum*[a]

Solvent	Strain	Growth	pH	Glucose consumed (μmol)	Lactate	End product ratio (mol/100 mol of glucose)			Alcohol-ketone (μmol/tube)		Carbon recovery (%)
						Acetate	Ethanol	CO_2	Isopropanol produced	Acetone used	
None	39E	1.1	6.3	220	23	8	155	163			93
	39EA	0.8	5.0	164	80	10	92	102			92
0.8% Acetone	39E	1.4	4.9	215	15	140	24	164	470	470	89
	39EA	1.0	4.7	170	38	97	36	133	275	250	85
4.0% Methanol	39E	0.78	4.3	217	21	17	123	140			90
	39EA	0.80	5.1	118	86	21	81	103			97

[a] Anaerobic pressure tubes contained 10 ml of TYE medium with 0.4% glucose, 2 μCi of [^{14}C]glucose, and the amount of solvent indicated. Tubes were assayed after growth was completed (24 h) at 60°C.

tolerance because they would not be subject to solvent disruption of the hydrophobic interactions between free fatty acid side chains present in normal lipids.

The basis for low ethanol tolerance in the parent strain of *C. thermohydrosulfuricum* was not due to solvent effects on membrane integrity or activity, although this has been suggested as the general mechanism for solvent tolerance in other microorganisms (3–7, 12, 17). The parent strain readily grew at greater than 4% [wt/vol] acetone or methanol, but less than 2% ethanol inhibited growth and altered the end product yield. In a separate study (manuscript in preparation), we have demonstrated that regulation of the carbon and electron flow pathways in the parent and mutant strains differ as a consequence of specific enzymatic activity alterations, including the absence of an NAD-linked alcohol dehydrogenase activity in the ethanol-tolerant strain. Thus, the mechanism for low ethanol tolerance in the parent strain is ascribed here to the ethanol-dependent inhibition of end product formation. Finally, solvent-tolerant strains of *C. thermohydrosulfuricum* that can ferment xylose or glucose to greater than 4% ethanol may be of practical value in industrial alcohol production, provided that they can be further mutated or controlled so that the ethanol yield (i.e., moles of ethanol produced per moles of substrate consumed) is higher than the value of 1.0 reported here.

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