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Clostridium acetobutylicum Mutants That Produce Butyraldehyde and Altered Quantities of Solvents

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Spontaneous mutants of *Clostridium acetobutylicum* NRRL B643 that were resistant to allyl alcohol (AA) were selected and characterized. These mutants contained 10- to 100-fold reduced activities of butanol and ethanol alcohol dehydrogenase. The AA mutants formed two groups and produced no ethanol. Type 1 AA mutants produced significant amounts of a new solvent, butyraldehyde, and contained normal levels of the coenzyme A-dependent butyraldehyde dehydrogenase (BAD). Type 2 AA mutants produced no significant butyraldehyde and lower levels of all solvents, and they contained 45- to 100-fold lower activity levels of BAD. Following ethyl methanesulfonate mutagenesis, low-acid-producing (Acid$^-$) mutants were selected and characterized as superinduced solvent producers, yielding more than 99% of theoretical glucose carbon as solvents and only small amounts of acetate and butyrate. Following ethyl methanesulfonate mutagenesis, 13 sporulation-negative (Spo$^-$) mutants were characterized; and 3 were found to produce only butyrate and acetate, a minor amount of acetone, and no alcohols. These Spo$^-$ mutants contained reduced butanol dehydrogenase activity and no BAD enzyme activity. The data support the view that the type 2 AA, the Acid$^-$, and the Spo$^-$ mutants somehow alter normal regulated expression of the solvent pathway in *C. acetobutylicum*.

Because of renewed interest in microbial conversion of biomass to commodity chemicals, there has been a flurry of physiologic and biochemical research centered on solvent-forming organisms such as *Clostridium acetobutylicum* (21). Davies and Stephenson (5) showed that these bacteria produce acetate and butyrate during exponential growth in batch culture and then switch and form butanol, acetone, and ethanol during the early stationary phase of growth. Using continuous culture, Gottschalk and co-workers (2, 3) showed that the physiologic triggers to shift the bacteria from an acidogenic to a solventogenic fermentation were a low pH; a low concentration of butyrate, acetate, or both; and growth-limiting phosphate or sulfate but plentiful nitrogen and carbon sources. The same set of signals has been shown by us and others (1, 21) to induce the biosynthesis of all of the terminal enzymes that catalyze solvent production.

To understand the molecular basis for the signals that regulate the pathway switching mechanism in this fermentation, it is essential to obtain mutants that show altered regulatory properties and to develop a genetic system for *C. acetobutylicum*. Three general approaches for obtaining fermentation mutants in clostridia have been employed. First, mutants in the structural genes for alcohol dehydrogenases as well as regulatory mutants, may be selected by virtue of their resistance to allyl alcohol (AA). AA is oxidized by alcohol dehydrogenases to a toxic aldehyde, acrolein. Thus, mutant organisms that synthesize altered forms of butanol dehydrogenase (BDH) or ethanol dehydrogenase (ADH) or that are deficient in these enzymes survive. Such mutants have been isolated from yeasts (15, 16, 27), *Escherichia coli* (14), and recently, *C. acetobutylicum* (7). Second, three strategies have been reported for producing low-acid-producing mutants in clostridia. Since *Clostridium saccharolyticum* can grow on pyruvate, following mutagenesis strains that were pyruvate negative were selected and found to produce mostly ethanol and little acetate from hexoses (18). A mutant of *Clostridium thermosaccharolyticum* that overproduced ethanol and that was blocked in acetate production was isolated following mutagenesis and selection for fluoroacetate resistance (22). Using dye selection (bromcresol purple), strains of *Clostridium thermocellum* were isolated that converted cellobiose to an 8:1 ratio of ethanol-acetate instead of the 1:1 ratio found in the parent strain (6). Finally, Jones et al. (11) demonstrated that a group of early-stage, sporulation-negative (Spo$^-$) *C. acetobutylicum* isolates produced little or no solvents. Further work by this group of investigators showed that the physiologic signals for switching to solvent production and initiation of endospore formation were the same, suggesting that the regulation of these two processes is somehow connected (13).

We report here the isolation of two types of AA-resistant mutants with reduced and altered alcohol dehydrogenase activity. One type produced significant amounts of a new solvent, butyraldehyde. The second type of AA mutants appeared to be a class of regulatory mutant. Also, low-acid-producing mutants (Acid$^-$) and Spo$^-$ mutants were isolated and characterized as likely regulatory mutants.

(Some of this work has been reported previously [P. Rogers, C. Quinn, and J. C. McDilda, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, O51, p. 270].)

MATERIALS AND METHODS

Bacteria and growth conditions. *C. acetobutylicum* NRRL B643 was obtained from L. K. Nakamura, Northern Regional Research Center, Peoria, Ill. Cells were grown in a yeast extract medium (YEM) consisting of the glucose-minimal medium described by O'Brien and Morris (19), without biotin or *p*-aminobenzoic acid, and supplemented with the following, per liter: yeast extract, 8 g; casein hydrolysate, 2.2 g; asparagine, 1.0 g; and cysteine, 0.5 g. The pH was adjusted to 7.0. The plating medium was YEM with 2% agar. YEM agar plates containing AA (0.2 to 0.15 mM) or crotyl alcohol (0.22 mM) were prepared by adding the alcohol to the agar medium that was cooled to 45°C after it was autoclaved and just before it was poured into petri

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dishes. BCP agar was YEM agar containing bromcresol purple (0.4 g/liter). Growth of cells and manipulation of cells for mutagenesis and selection were carried out in an anaerobic chamber (model 1024; Forma Scientific, Marietta, Ohio) in an atmosphere of 5% H_2–10% CO_2–85% N_2. A spore stock of *C. acetobutylicum* was spread on yeast extract agar, and colonies were selected from plates incubated for 18 to 20 h at 37°C for inoculation into liquid YEM.

Mutagenesis and mutant selection. For mutagenesis, exponential-phase *C. acetobutylicum* (about 2×10^8 cells in 1 ml of YEM) were treated in plastic Eppendorf tubes with 0.05 ml of ethyl methanesulfonate (EMS) at 35°C for 30 or 60 min. Following centrifugation and two washings with fresh sterile YEM, cell samples were distributed into tubes with YEM for outgrowth for 18 h at 35°C, and cells were spread onto YEM agar (50 to 150 CFU per plate) and incubated for 24 h at 37°C.

For detection of low-acid-producing mutants, colonies grown for 24 h on YEM agar after EMS treatment were replicated onto BCP agar and incubated for 4 to 5 h at 35°C. Yellow halos formed around normal colonies, while low-acid-producing colonies remained purple or gray.

For detection of sporulation-negative strains, 3- to 4-day-old colonies from EMS-treated cultures were replicated onto fresh YEM agar, and the master plates were exposed to iodine vapor for 3 to 5 min at room temperature in air (17). Colonies that stained dark blue contained granulose, while yellow colonies contained granulose-free cells and were usually asporogenic. Replicas of yellow colonies (Spo^-) were picked and purified from the anaerobic replica plate. Microscopic examination confirmed the absence of spores in most all granulose-negative colonies.

For selection of AA-resistant mutants, exponential-phase cells of *C. acetobutylicum* spread onto YEM agar were exposed to 50 to 100 μl of AA or crotyl alcohol added to paper disks (diameter, 12 mm) on the agar surface. After 48 to 72 h of incubation at 35°C, small colonies which appeared in the clear zones were picked and purified on yeast extract agar containing AA or crotyl alcohol.

Determination of fermentation products. Strains of *C. acetobutylicum* were grown in 5 ml of YEM supplemented with 5 to 7.5% glucose at 35°C for 48 h, unless indicated otherwise. Acetate and butyrate were determined as follows. Acidified culture supernatants were extracted with ether, the ether extract was freed of water, and 2- to 10-μl samples were injected into a gas-liquid chromatograph (Packard Instrument Co., Inc., Rockville, Md.) with a glass column (6 ft [1.8 m] by 4 mm) packed with Supelco 1-1841 (10% SP-100–1% H_3PO_4 on 100/120 Chromosorb W) at 147°C, with He as the carrier gas injected at 70 ml/min with a thermal conductivity detector. Solvents were extracted into toluene from alkaline culture supernatants and freed of water, and 2- to 10-μl toluene samples were injected into the gas-liquid chromatograph with a glass column (6 ft [1.8 m] by 4 mm) with Supelco 1-1813 (80/120 Carbopak–3% SP-1500) at 100°C, with He used as the carrier gas injected at 75 ml/min with a thermal conductivity detector. The amount of solvents and acids was determined by comparison with standards that were extracted similarly to culture samples and is expressed as millimolar in the original culture medium.

Preparation of cell extracts and enzyme assays. Vegetative cells from YEM plates were inoculated into 100 ml of YEM with 5% glucose and grown at 37°C anaerobically for 20 to 24 h to the early stationary phase. Cells were harvested and washed by centrifugation at 5,000 rpm for 10 min at 4°C in a centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.), and washed cells were suspended in 1/20 volume of iced PM buffer (25 mM potassium phosphate [pH 7.4] with 10 mM mercaptoethanol) or iced TM buffer (25 mM Tris hydrochloride [pH 8.5] with 10 mM mercaptoethanol). The concentrated cell suspensions were broken by three 1-min pulses of ultrasonic treatment at 4°C, and the cell extracts were centrifuged at 15,000 rpm in a centrifuge (Sorvall) for 30 min at 4°C. A coenzyme A (CoA)-dependent butyraldehyde dehydrogenase (BAD) similar to acetaldehyde:NAD oxidoreductase (CoA acylating; EC 1.2.1.10) was assayed for enzyme activity in the reverse direction in a 1.0-ml reaction mixture containing the following: potassium 2-(*N*-cyclohexylamino)ethane sulfonate buffer (pH 9.0), 75 μmol; NAD^+ 1.0 μmol; CoA, reduced form, 0.2 μmol; dithiothreitol, 10 μmol; cell extract in PM buffer, 25 to 250 μg of protein; and butyraldehyde, 10 μmol. Reaction mixtures were preincubated for 10 min at room temperature before the reaction was initiated by adding butyraldehyde. The rate of NADH formation at 340 nm was determined on a spectrophotometer (Response II; Gilford Instrument Laboratories, Inc., Oberlin, Ohio) with a kinetic program.

The NADP-dependent alcohol dehydrogenase (alcohol:NADP oxidoreductase; EC 1.1.1.2) was also assayed in the reverse direction in a 1.0-ml reaction mixture containing the following: Tris hydrochloride buffer, 100 μmol; dithiothreitol, 10 μmol; magnesium acetate, 5 μmol; $NADP^+$, 0.32 μmol; cell extract in TM buffer, 250 μg of protein; and butanol, 50 μmol, or ethanol, 20 μmol. The reaction mixtures were preincubated for 10 min at room temperature before the reaction was initiated by adding the alcohol. The rate of NADPH formation was measured as described above. One unit of enzyme activity for both dehydrogenases is defined as reduction of 1 nmol of $NAD(P)^+$ per min.

Phosphate butyryltransferase (butyryl-CoA:orthophosphate butyryltransferase; EC 2.3.1.19) and phosphate acetyltransferase (EC 2.3.1.8) were assayed in the reverse direction by using a modification of a standard method (4). A 1.0-ml reaction mixture contained the following: potassium morpholinopropane sulfonate buffer (pH 7.5), 75 μmol; CoA, reduced form, 0.2 μmol; butyryl phosphate or acetyl phosphate, 3.0 μmol; and cell extract in PM buffer, 0.12 μmol of inorganic phosphate and 10 to 20 μg of protein. The reaction was initiated by adding acyl phosphate, and the rate of acyl-CoA formation at 233 nm was determined spectrophotometrically as described above. One unit of enzyme activity for both transferases is defined as the formation of 1 μmol of acyl-CoA per min.

Butyrate kinase (ATP:butyrate phosphotransferase; EC 2.7.2.7) and acetate kinase (ATP:acetate phosphotransferase; EC 2.7.2.1) were assayed in the forward direction by measuring the formation of ATP from butyryl phosphate continuously by coupling through added yeast hexokinase and glucose-6-phosphate dehydrogenase to the reduction of $NADP^+$ (12). A 1.0-ml reaction mixture contained the following: potassium morpholinopropane sulfonate buffer (pH 7.5), 75 μmol; magnesium chloride, 5 μmol; ADP, 1.0 μmol; butyryl phosphate or acetyl phosphate, 1.5 μmol; yeast hexokinase, 5 units, yeast glucose-6-phosphate dehydrogenase, 2.5 units; and cell extract in PM buffer, 5 to 10 μg of protein. The reaction was initiated by adding butyryl phosphate or acetyl phosphate, and reduction of $NADP^+$ was measured spectrophotometrically at 340 nm as described above. One unit of kinase activity is defined as 1 μmol of ATP produced ($NADP^+$ reduced) per min.

Materials. Butyryl phosphate was synthesized and purified to 95 to 97% purity by the method (procedure B) for preparation of acetyl phosphate outlined by Stadtman (23)

by using butyric anhydride (Eastman Chemical Co, Rochester, N.Y.) and K_2HPO_4. EMS (99%) was from Aldrich Chemical Co. (Milwaukee, Wis.). Coenzymes, enzymes, ADP, dithiothreitol, and acetylphosphate were from Sigma Chemical Co. (St. Louis, Mo.). The other chemicals used were of the highest purity available.

RESULTS

Selection of AA-resistant mutants. *C. acetobutylicum* B643 cells spread onto YEM agar plates were exposed to paper pads with AA or crotyl alcohol as described above. Twenty-five spontaneous AA-resistant mutants were selected and purified on YEM agar containing AA (0.15 M). None of these mutants grew at AA concentrations of greater than 0.22 M. Ten AA-resistant strains were selected for further analysis.

Pretreatment of strain B643 cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, EMS, or UV light at a variety of times and concentrations or doses did not increase the yield of AA-resistant mutants observed after a period of outgrowth.

Fermentation products of AA-resistant mutants. The fermentation products of 10 AA-resistant mutants were determined following growth for 48 h in YEM containing 200 mM glucose (Table 1). The AA-resistant strains all showed a significantly different pattern of products compared with that of the parent strain and fell into two groups. Type 1 AA-resistant strains produced significant quantities of a new solvent identified as butyraldehyde, which was not produced by the parent strain or five other wild-type strains of *C. acetobutylicum* analyzed after fermentation of glucose (data not shown). Strains AA-3 and AA-C4 produced particularly high levels of butyraldehyde, but the optimal conditions for producing maximum amounts of this solvent by any of these strains was not studied. Type 1 strains produced no or trace amounts of ethanol, lower than normal levels of butanol, and about normal amounts of acetone (Table 1). Type 2 strains also produced no ethanol, but in contrast to type 1 strains, three type 2 strains produced either no butyraldehyde or a low level of this solvent. Type 2 strains also produced low levels of acetone, suggesting a general reduced capacity for solvent production. Except for strains AA-C4 and AA-C5, all AA-resistant strains produced significantly higher levels of acetic acid than did the parent strain.

TABLE 1. Fermentation products of AA-resistant mutants of *C. acetobutylicum*

Strain		Concn (mmol/liter) of the following fermentation product[a]:					
Type	No.	Butanol	Acetone	Ethanol	Butyraldehyde	Butyric	Acetic
Parent	B643	92	47	10	0	16	6
1	AA-3	32	30	tr[b]	14	21	11
1	AA-11	7.9	18	0	5.4	21	18
1	AA-13	18	41	0	5.1	22	17
1	AA-C1	10	27	0	6.0	15	14
1	AA-C3	14	46	0	9.4	16	12
1	AA-C4	38	55	0	23	16	7.2
1	AA-C5	19	53	tr	6.4	15	7.9
2	AA-2	7.2	17	0	0	18	13
2	AA-9	19	12	0	0	16	22
2	AA-37	19	10	0	2.2	18	15

[a] The listed bacterial strains were grown for 48 h in YEM containing 200 mM glucose. Solvents and acids were extracted from the growth media, and the amounts were determined by gas-liquid chromatography as outlined in the text. Butyraldehyde was identified by comparison with the retention times of standard butyraldehyde on two different columns.

[b] tr, Trace amount was detected.

Enzymatic analysis of AA-resistant mutants. From the results shown in Table 1 it can be predicted that AA-resistant mutants contain altered alcohol dehydrogenase activities that allow them to survive because they do not convert significant amounts of AA to the toxic aldehyde acroline. Enzyme activities required for the production of butanol and ethanol from butyryl-CoA and acetyl-CoA in *C. acetobutylicum* are a CoA-acylating aldehyde dehydrogenase and an NADP-dependent alcohol dehydrogenase (21). These enzymes are induced 50- to 100-fold in early-stationary-phase cells just prior to the appearance of solvents and remain at high specific activities throughout solvent production (N. Palosaari and P. Rogers, Fed. Proc. **46**:2293, 1987). The AA-resistant strains were grown for 24 h (solvent production phase), cell extracts were prepared, and the enzyme activities producing alcohols were measured (alcohol reactions; Table 2). Both BDH- and ADH-specific activities were from 10- to 100-fold lower in the AA-resistant mutant strains than in the parent strain. Since in all 10 mutants both BDH and ADH were reduced coordinately, it appears that *C. acetobutylicum* possesses a single alcohol dehydrogenase enzyme. To understand more clearly the nature of the change in the mutant enzymes, we compared the kinetics of the reaction catalyzed by the mutant AA-3 enzyme and parent strain enzymes (Fig. 1). The mutant AA-3 enzyme in a crude extract had a negligible affinity for either ethanol or AA as substrates, while it still acted on butanol. The apparent K_m for butanol was about 8.5 mM for the wild-type enzyme and about 80 mM for the AA-3 enzyme. These data provide an explanation for both the AA resistance of the mutant strains and the observed absence of ethanol during fermentation (Table 1).

We found that the enzyme catalyzing acetaldehyde and butyraldehyde formation from the respective acyl-CoAs is a single BAD (Palosaari and Rogers, Fed. Proc. **46**:2293, 1987). The AA-resistant strains classified as type 1 contained normal to high levels of BAD activity (Table 2), which is consistent with the observed production of significant butyraldehyde by these mutants (Table 1). In contrast, the AA-resistant strains classified as type 2 (Table 1) possessed 45- to 100-fold lower levels of BAD, which was coordinate with the lower BDH and ADH activities. These strains did not produce significant butyraldehyde or acetone, suggesting that they may be a type of regulatory mutant (see below).

The specific activities of the four enzymes required for the production of acetic and butyric acids from acetyl-CoA and butyryl-CoA were determined (acid reactions; Table 2). Both phosphobutyryl transferase and phosphoacetyl transferase activities of the AA-resistant strains were 5 to 20 times higher than those of the parent strain in cells grown for 24 h. In contrast, the specific activities of butyrate kinase and acetate kinase in extracts of the AA-resistant mutants was either the same or slightly lower than that in extracts of the parent strain.

Morphology of the AA-resistant mutants. All of the AA-resistant strains were grown on solid medium for 72 h, and colonies were examined for the presence of granulose by treatment with iodine vapors. All but one of the mutants (AA-C3) made various amounts of granulose. From microscopic examinations, strain AA-C3 was deficient in spore formation, while all other AA-resistant strains formed spores. Although some of the other strains showed lower numbers of spores than the parent strain after 72 h on plates, this variability was not regarded as significant.

TABLE 2. Enzyme activities of AA-resistant mutants of *C. acetobutylicum*

Strain		Enzyme activity (units/mg of protein) for[a]:						
		Alcohol reactions			Acid reactions			
Type	No.	BAD	BDH	ADH	PBT	PAT	BK	AK
Parent	B643	9.1	4.4	1.3	0.14	0.26	5.8	7.5
1	AA-3	6.1	0.08	0.05	1.9	2.2	3.6	7.5
1	AA-11	5.0	0.08	<0.03	1.2	4.2	5.2	7.2
1	AA-13	3.1	0.3	0.14	1.4	2.7	6.3	7.8
1	AA-C1	2.2	0.1	0.03	ND[b]	ND	ND	ND
1	AA-C3	17.6	0.2	0.12	0.7	0.62	2.0	2.6
1	AA-C4	79.8	0.18	0.06	2.0	4.1	2.7	2.4
1	AA-C5	42.0	1.3	0.07	0.46	0.62	2.2	2.1
2	AA-2	0.06	0.18	0.1	ND	ND	1.8	3.4
2	AA-9	0.11	0.38	0.3	3.5	0.14	3.5	2.3
2	AA-37	0.21	0.2	0.1	3.9	0.4	1.5	1.0

[a] Crude extracts of cells of the indicated strains grown for 24 h were assayed for enzyme activities as described in the text and are indicated as follows: BDH, $NADP^+$-dependent alcohol dehydrogenase with butanol as substrate; ADH, $NADP^+$-dependent alcohol dehydrogenase with ethanol as substrate; BAD, NAD^+-dependent butyrylaldehyde dehydrogenase; PBT, phosphobutyryl transferase; PAT, phosphoacetyl transferase; BK, butyrate kinase; and AK, acetate kinase.
[b] ND, Not determined.

Low-acid-producing mutants of *C. acetobutylicum*. Following treatment of cells with EMS, separated colonies on YEM agar were replicated onto BCP agar; and six mutant colonies were selected that appeared to produce little or no acid by the procedure given above. Four of these mutants were found to produce a great excess of solvents, only a trace of butyrate, and a small amount of acetic acid after 48 h of growth. The data for strain B18 shown in Table 3 are typical of these $Acid^-$ strains. Analysis of the alcohol-producing enzymes, BDH and BAD, in crude extracts of cells grown for 24 h showed approximately twofold higher specific activities than the parent strain (Table 4). In contrast, there was no significant change in the acid-producing enzymes, even though little butyrate was present. The time course of acid and solvent production of $Acid^-$ strain B18 and the parent strain revealed that small amounts of butyrate and acetate were produced for up to 12 h and they were then rapidly consumed by 24 h (Fig. 2). These data indicate that the $Acid^-$ mutants are probably superinduced for solventogenesis but are still regulated to produce solvents after an initial phase of acid production.

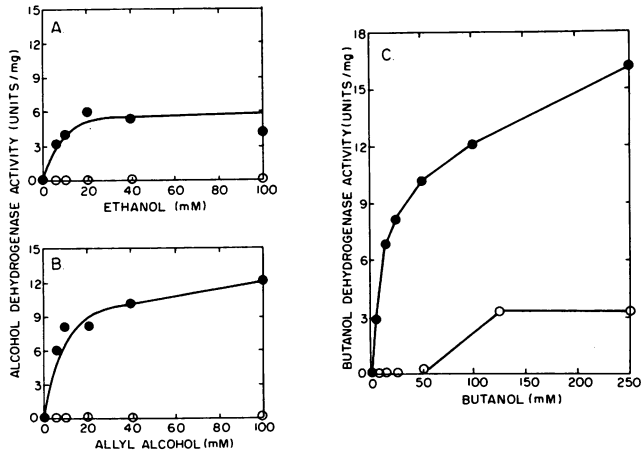

FIG. 1. Comparison of alcohol dehydrogenase activities from an AA-resistant mutant and the *C. acetobutylicum* parent strain. Crude extracts in TM buffer prepared from early-stationary-phase cells (24 h of growth) of strain B643 (●) and mutant strain AA-3 (○) were assayed for NADPH-dependent alcohol dehydrogenase activity at various concentrations of ethanol (A), AA (B), and butanol (C). Reaction mixtures were assayed as described in the text. The dehydrogenase activities shown are all adjusted to units per milligram of extract protein for comparison.

TABLE 3. Products of $Acid^-$ and Spo^- mutants with altered solvent production

Strain	Concn (mmol/liter) of the following product[a]:					
	Butanol	Acetone	Ethanol	Butyrate	Acetate	CO_2 (calculated)[b]
B18 ($Acid^-$)	220	98	100	0.2	1.7	835
6A (Spo^-)	0	7	0	87	22	221
B643 (parent)	168	129	40	29	5	826

[a] Products were measured by gas-liquid chromatography on extracts from media of cultures grown for 48 h. The medium contained 350 mM glucose. Strain 6A grew poorly and utilized only 30% of the added glucose.
[b] CO_2 was calculated from the quantities of the other products.

Sporulation-negative mutants of *C. acetobutylicum*. After mutagenesis with EMS, 40 Spo^- mutants were selected for their inability to form granulose and were observed to form no spores. Thirteen Spo^- strains were examined for fermentation products following 48 h of growth in YEM. Five strains produced a normal wild-type pattern of solvents and acids, and five strains showed a 50 to 75% reduction in solvent formation (data not shown). Three strains produced no alcohols and small amounts of acetone (for example, strain 6A; Table 3).

As exemplified by the data for strain 6A, these solvent-negative strains produced no measurable BAD activity and a very low level of BDH activity that was found consistently (Table 4). The acid-producing enzyme activities were significantly higher than those of the parent strain. Thus, this class of Spo^- mutants may indicate the close connection between induction of sporulation and solventogenesis, as suggested previously (11, 13).

DISCUSSION

As far as we know, this is the first report of significant natural production of the solvent butyraldehyde by a mutant strain of *C. acetobutylicum* or any other bacteria. Because the boiling point of this solvent is about 75°C, compared with 118°C for butanol, the lower energy required for recovery of this solvent would make its production more attractive than production of butanol (20). Recently, there was a report (26)

TABLE 4. Enzyme activities of $Acid^-$ and Spo^- mutants with altered solvent production

Strain	Enzyme activity (units/mg of protein) of[a]:					
	BDH	BAD	PBT	PAT	BK	AK
B18 ($Acid^-$)	8.9	57.2	0.2	1.9	2.2	2.2
6A (Spo^-)	0.6	0.0	2.4	5.0	7.4	8.3
B643 (parent)	4.9	20.7	0.2	2.8	2.1	3.1

[a] Enzyme activities were assayed on crude extracts of cells grown for 24 h on YEM with 5% glucose. Abbreviations: BDH, butanol dehydrogenase; BAD, butyraldehyde dehydrogenase; PBT, phosphobutyryl transferase; PAT, phosphoacetyl transferase; BK, butyrate kinase; AK, acetate kinase.

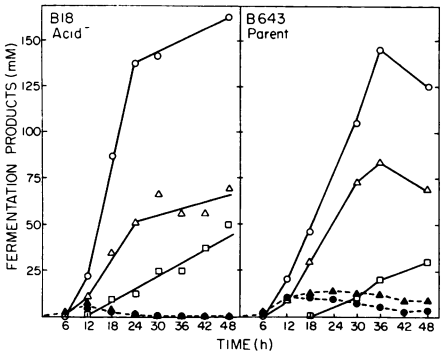

FIG. 2. Accumulation of fermentation products by an Acid⁻ mutant of *C. acetobutylicum*. Strains B18 (Acid⁻) and B643 (parent) were grown in 50 ml of YEM with 5.5% glucose. Samples of the culture medium taken at the indicated times were treated, extracted, and analyzed by gas-liquid chromatography for products, as described in the text. Symbols for fermentation products are as follows: ○, butanol; △, acetone, □, ethanol; ●, butyric acid; and ▲, acetic acid.

of the production of acetaldehyde by using mutants of *Zymomonas mobilis* with altered alcohol dehydrogenase.

The type 1 AA-resistant mutants analyzed in this study are most likely altered alcohol dehydrogenase mutants similar to those isolated from yeasts (14, 27) and *E. coli* (15) following selection in the presence of AA. Compared with the parent cells, all seven of these mutants produced normal amounts of acetone, low levels of alcohols, significant butyraldehyde, and abnormally low levels of BDH and ADH activities. It is most likely that *C. acetobutylicum* contains only one NADPH-dependent alcohol dehydrogenase, which is induced just prior to solvent production for conversion of aldehydes to alcohols. This is similar to the case with *Clostridium beijerinckii* (*Clostridium butylicum*), which was recently shown to have only one alcohol dehydrogenase ($NADP^+$ dependent) for producing ethanol, isopropanol, and butanol (10). Kinetic analysis of the AA-3 mutant for BDH and ADH production revealed a low affinity for both AA and ethanol, in contrast to the case for the parent cell enzyme. Thus, resistance of this type of mutant to AA is most likely due to a structural change in this enzyme.

We and others have reported previously (1, 21) that all of the enzymes required for the production of acetone, butanol, and ethanol are induced in *C. acetobutylicum* in batch cultures just after exponential growth is completed and at the onset of solvent production. Thus, we predict that selection of AA-resistant mutants might yield a type of regulatory mutant. The type 2 AA-resistant strains produced low amounts of all solvents and contained very low specific enzyme activities of BAD, as well as low activities of BDH and ADH. In *E. coli* the data predict that these two activities are carried out by one protein (14); however, our data show that for *C. acetobutylicum* the BAD and BDH activities are completely separated on TEAE cellulose columns (N. Palosaari and P. Rogers, manuscript in preparation). The simultaneous reduction of both activities in type 2 AA-resistant mutant strains suggests that there is either a common regulatory signal controlling BAD and BDH biosynthesis from two operons or that expression of both enzyme proteins from a single operon is blocked by a single mutation. Genetic analysis is needed to clarify this question. Recently, Durre et al. (7) reported that they isolated 10 AA-resistant mutants of *C. acetobutylicum* which, in contrast to our mutants, produced normal amounts of ethanol and no butanol, and had normal levels of BDH activity. We are at a loss for an explanation as to how these mutants survived in AA.

The Acid⁻ mutants described here were interesting because they converted more than 99% of C_2 units derived from glucose to solvents, in contrast to about 80 to 90% observed for wild-type *C. acetobutylicum*. However, these mutants accumulated a small but significant amount of acids during the first 12 h of the fermentation, and the levels of the four acid-producing enzymes were similar to the levels in the wild-type cells (Table 4). The recycling of butyrate and acetate is believed to occur through the first enzyme in the pathway for acetone formation, acetoacetyl-CoA CoA transferase, by which butyryl-CoA and acetyl-CoA are produced and then funneled into alcohol production (9). Decarboxylation of acetoacetate then yields the solvent acetone. The Acid⁻ mutants not only produced more solvents more rapidly than did the parent strain (Fig. 2) but, in contrast to the parent strain, recycled most all of the acids formed earlier. These data, together with the high levels of dehydrogenases at 24 h (Table 4), indicate that our Acid⁻ mutants were most likely superinduced for all of the solvent enzymes following a short period of acid production.

It was reported that early-stage sporulation mutants of *C. acetobutylicum* unable to form a clostridial stage did not produce solvents, although sporulation mutants blocked after the clostridial stage produced normal levels of solvents (11). Long et al. (13) showed that the requirements for the shift from the acidogenic to a solventogenic fermentation paralleled the requirements for triggering endospore formation. Conversely, Meinecke, et al. (17) isolated stable asporogenous mutants from a continuous culture under phosphate-limited conditions that produced solvents normally. Thus, although the two processes are intimately connected, induction of endosporulation is not a prerequisite for solvent production. We have reported here that those Spo⁻ mutants that did not make significant solvents were also severely deficient in the normally induced alcohol-producing enzymes BAD and BDH. The excess acids accumulated by these solvent-negative mutants are apparently toxic, slowing growth early and preventing complete utilization of the glucose for fermentation (Table 3). Thus, the specific induction of solventogenic enzymes is most likely linked to some early regulatory step in the program leading to endospore formation. Since the 2 AA-resistant mutants were all capable of sporulation, the low levels of BDH and BAD observed probably reflect a distinctly different regulatory signal than is apparent in the Spo⁻ strains.

It has been known for some time that the four acid-forming enzymes phosphobutyryl transferase, phosphoacetyl transferase, butyrate kinase, and acetate kinase are four separate proteins in clostridia (24, 25). During growth of *C. acetobutylicum* over a 48-h period in batch culture, the specific activities of these four enzymes were only two to three times higher during acid production than during solvent production (1; our observations). However, in one report (8) a 60-fold drop of phosphobutyryl transferase activity was observed from 15 to 24 h, and it remained low during solvent formation. It may be that the high levels of phosphobutyryl transferase reported here for both Spo⁻ mutants and some of the AA-resistant mutants reflect a further regulatory process in cells restricted for solvent production.

How these suspected regulatory mutants are related and the identification of the molecular signals required for solvent enzyme induction must await genetic analysis.

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LITERATURE CITED

1. **Andersch, W., B. Hubert, and G. Gottschalk.** 1983. Level of enzymes involved in acetate, butyrate, acetone and butanol formation by *Clostridium acetobutylicum*. Eur. J. Appl. Microbiol. Biotechnol. **18**:327–332.
2. **Bahl, H., W. Andersch, and G. Gottschalk.** 1982. Continuous production of acetone and butanol by *Clostridium acetobutylicum* in a two-stage phosphate limited chemostat. Eur. J. Appl. Microbiol. Biotechnol. **15**:201–205.
3. **Bahl, H., and G. Gottschalk.** 1984. Parameters affecting solvent production by *Clostridium acetobutylicum* in continuous culture, p. 215–223. *In* D. I. C. Wang and C. D. Scott (ed.), Biotechnology and bioengineering Symposium no. 14, Sixth Symposium on Biotechnology for Fuels and Chemicals. John Wiley & Sons, Inc., New York.
4. **Bergmeyer, H. U., K. Gawehn, and M. Grassl.** 1974. Enzymes as biochemical reagents, p. 507–508. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis, vol. 1. Verlag Chemie International, Deerfield Beach, Fla.
5. **Davies, R., and M. Stephenson.** 1941. Studies on the acetone-butyl alcohol fermentation. I. Nutritional and other factors involved in the preparation of active suspensions of *Clostridium acetobutylicum*. Biochem. J. **35**:1320–1331.
6. **Duong, T.-V. C., E. A. Johnson, and A. L. Demain.** 1983. Thermophilic, anaerobic and cellulolytic bacteria, p. 156–195. *In* A. Weisman (ed.), Topics in enzyme and fermentation biotechnology 7. John Wiley & Sons, Inc., New York.
7. **Durre, P., A. Kuhn, and G. Gottschalk.** 1986. Treatment with allyl alcohol selects specifically for mutants of *Clostridium acetobutylicum* defective in butanol synthesis. FEMS Microbiol. Lett. **36**:77–81.
8. **Hartmanis, M. G. N., and S. Gatenbeck.** 1984. Intermediary metabolism in *Clostridium acetobutylicum*. Levels of enzymes involved in the formation of acetate and butyrate. Appl. Environ. Microbiol. **47**:1277–1283.
9. **Hartmanis, M. G. N., T. Klason, and S. Gatenbeck.** 1984. Uptake and activation of acetate and butyrate in *Clostridium acetobutylicum*. Appl. Microbiol. Biotechnol. **20**:66–71.
10. **Hiu, S. F., C.-X., Zhu, R.-T. Yan, and J.-S. Chen.** 1987. Different alcohol dehydrogenases in two strains of *Clostridium beijerinckii* (syn. *Clostridium butylicum*): a butanol-ethanol dehydrogenase and a butanol-ethanol-isopropanol dehydrogenase. Appl. Environ. Microbiol. **53**:697–703.
11. **Jones, D. T., A. van der Westhuizen, S. Long, E. R. Allcock, S. R. Reid, and D. R. Woods.** 1982. Solvent production and morphological changes in *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **43**:1434–1439.
12. **Lamprecht, W., and I. Trautschold.** 1974. Determination with hexokinase and glucose-6-phosphate dehydrogenase, p. 2101–2110. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis, vol. 4. Verlag Chemie International, Deerfield Beach, Fla.
13. **Long, S., D. T. Jones, and D. R. Woods.** 1984. Initiation of solvent production, clostridial stage and endospore formation in *Clostridium acetobutylicum* P262. Appl. Microbiol. Biotechnol. **20**:256–261.
14. **Lorowitz, W., and D. Clark.** 1982. *Escherichia coli* mutants with a temperature-sensitive alcohol dehydrogenase. J. Bacteriol. **152**:935–938.
15. **Lutsdorf, U., and R. Megnet.** 1968. Multiple forms of alcohol dehydrogenase in *Saccharomyces cerevisiae*. I. Physiological control of ADH-2 and properties of ADH-2 and ADH-4. Arch. Biochem. Biophys. **126**:933–944.
16. **Megnet, R.** 1967. Mutants partially deficient in alcohol dehydrogenase in *Schizosaccharomyces pombe*. Arch. Biochem. Biophys. **121**:194–207.
17. **Meinecke, B., H. Bahl, and G. Gottschalk.** 1984. Selection of an asporogenous strain of *Clostridium acetobutylicum* in continuous culture under phosphate limitation. Appl. Environ. Microbiol. **48**:1064–1065.
18. **Murray, W. D., K. B. Wemyss, and A. W. Khan.** 1983. Increased ethanol production and tolerance by a pyruvate-negative mutant of *Clostridium saccharolyticum*. Eur. J. Appl. Microbiol. Biotechnol. **18**:71–74.
19. **O'Brien, R. W., and J. G. Morris.** 1971. Oxygen and the growth and metabolism of *Clostridium acetobutylicum*. J. Gen. Microbiol. **68**:307–318.
20. **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. 249–304. *In* D. L. Wise (ed.), Organic chemicals from biomass. Benjamin/Cummings Publishing Co., Menlo Park, Calif.
21. **Rogers, P.** 1986. Genetics and biochemistry of *Clostridium* relevant to development of fermentation processes. Adv. Appl. Microbiol. **31**:1–60.
22. **Rothstein, D. M.** 1986. *Clostridium thermosaccharolyticum* strain deficient in acetate production. J. Bacteriol. **165**:319–320.
23. **Stadtman, E. R.** 1957. Preparation and assay of acetyl phosphate. Methods Enzymol. **3**:228–231.
24. **Twarog, R., and R. S. Wolfe.** 1962. Enzymatic phosphorylation of butyrate. J. Biol. Chem. **237**:2474–2477.
25. **Valentine, R. C., and R. S. Wolfe.** 1960. Purification and role of phospho-transbutyrylase. J. Biol. Chem. **235**:1948–1952.
26. **Wecker, M. S. A., and R. R. Zall.** 1987. Production of acetaldehyde by *Zymomonas mobilis*. Appl. Environ. Microbiol. **53**:2815–2820.
27. **Wills, C., and J. Phelps.** 1975. A technique for the isolation of yeast alcohol dehydrogenase mutants with altered substrate specificity. Arch. Biochem. Biophys. **167**:627–637.