Properties of Allyl Alcohol-Resistant Mutants of *Clostridium butyricum* Grown on Glycerol

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Mutants of *Clostridium butyricum* E5 exhibiting resistance to allyl alcohol which produced the same quantities of 1,3-propanediol as the wild-type strain but more acetate than butyrate were isolated. The acetate-butyrate formation plays a major function in the regulation of the internal redox balance. Allyl alcohol resistance can be attributed not to the loss of 1,3-propanediol dehydrogenase but to a shift in the reductive properties of the enzyme. The data support the view that cellular regulation is modified to avoid intracellular accumulation of 3-hydroxypropionaldehyde.

During the past several years, there has been an increase in research and development efforts involving clostridial fermentations, namely, the branched fermentative pathways of butyric group acid bacteria (11). Among them, *Clostridium butyricum* metabolizes glycerol into end products involving two branch points, the first dividing the carbon flux toward 1,3-propanediol (1,3-PD) and pyruvate biosynthesis and the second dividing the carbon flux toward butyrate and acetate.

In a previous investigation, we found *C. butyricum* mutants producing less total acid than the wild-type strain but with a decrease in the acetate-to-butyrate ratio (2). The amount of hydrogenase in the mutants was markedly decreased, and hydrogen production was partially suppressed (4), allowing NADH formation at the expense of hydrogen production. However, the results for conversion of glycerol to 1,3-PD were almost the same for the wild type and for the mutants. This demonstrated that excess NADH was used only to produce butyrate and that only the branch point implicated in acetate-butyrate formation was used in the regulation of carbon flux (2). To understand the signals that regulate the pathways in this fermentation, the aim of this work was to select mutants affected in the path leading to 1,3-PD production and to determine how the carbon flux would be affected. 1,3-PD biosynthesis occurs via glycerol dehydrase and 1,3-PD dehydrogenase with 3-hydroxypropionaldehyde as an intermediate compound. Treatment with allyl alcohol (AA) as a selective agent has been used to obtain mutations in the structural genes for alcohol dehydrogenase. AA is oxidized by alcohol dehydrogenases to a toxic aldehyde, acrolein. Thus, mutant organisms that synthesize altered forms of alcohol dehydrogenase or that are deficient in this enzyme survive. Such mutants have been isolated from yeasts with partial deficiency in alcohol dehydrogenase (7) and, recently, from *Clostridium acetobutylicum* (6, 9). Here, we report the isolation and the properties of four AA-resistant mutants of *C. butyricum*.

Organism. *C. butyricum* E5 was used (8) and was grown as described previously (2).

Analytical methods. Glycerol, fermentation products, and calculations were determined as reported elsewhere (2).

Mutagenesis and mutant selection. For mutagenesis, 9 ml of exponential-phase-cell culture of *C. butyricum* E5 was treated in Hungate tubes with 1 ml of 0.1% (wt/vol) *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) at 34°C for 60 min. NTG is the mutagen most commonly used to obtain clostridial mutants (10), and, under the conditions that we used, approximately 1 of 100 cells survived. After centrifugation and two washings with fresh sterile reinforced clostridial medium (RCM) (Oxoid Ltd., Basingstoke, England), cell samples were inoculated into RCM supplemented with 108 mM glycerol (10 g/liter) and were grown overnight at 34°C. Samples of this culture (0.1 ml) were plated and spread on selective medium. For selection of AA-resistant mutants, exponential-phase cells were spread onto petri plates containing the culture medium with 10 g of glycerol per liter supplemented with L-cysteine hydrochloride (0.75 g/liter) and agar (15 g/liter) and were exposed to 50 to 100 μ l of AA added to paper disks on the agar surface (diameter, 9 mm). After 48 to 72 h of incubation anaerobically (GasPak; BBL Microbiology systems, Cockeysville, Md.) at 34°C, small colonies which appeared in the clear zones were picked into RCM medium supplemented with 1% glycerol. Grown cells were selected again by plating onto petri plates as described above but were supplemented with different concentrations of AA. Concentrations of 0.5, 1, and 2 g/liter allowed cell growth, whereas a concentration of 4 g/liter was totally inhibitory. Selective pressure was obtained with 3 g/liter. Acrolein and AA tolerance were determined as described elsewhere (8). Cell extracts were prepared and enzyme activities were determined by the procedure described by Abbad-Andaloussi et al. (2). Intracellular concentrations of NAD⁺, NADH, and acetyl coenzyme A were determined as reported previously (1).

Selection and properties of AA-resistant mutants. A total of 15 colonies which appeared in the clear zones were picked and purified onto petri plates supplemented with 3 g of AA per liter. Addition of acrolein or AA on the growth medium of the wild type and of the mutants showed that they were more resistant than strain E5. In the case of the mutant E5-MD, the addition of 2 mM acrolein allowed full growth, whereas the wild type exhibited a growth inhibition that was approximately 50%. At 175 mM AA, the inhibition of growth for *C. butyricum*

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TABLE 1. Fermentation data for *C. butyricum* E5 and mutants grown on glycerol (673 mM) at pH 7.0

Strain	Biomass (g/liter)	Concn of products (mM) ^a				Acetate/butyrate ratio	Y _{1,3-PD} ^b	Carbon recovery (%)	Concn of NADH (mmol) ^c		NADH recovery (%) ^d
		Acetate	Butyrate	1,3-PD	Total acids				Minimum	Maximum	
Wild type	1.62	60.7 (5)	63.6 (5.4)	423 (31)	106.6	0.95	0.609	90.8	248.6	436.5	96.9
E5-MB	1.15	147.7 (14)	33.3 (3)	415 (44)	181.0	4.43	0.616	93.5	362	576.3	72
E5-MC	1.19	163.3 (12)	34.1 (4)	425 (32)	197.4	4.79	0.631	97.5	394.8	626.3	67.8
E5-MD	1.26	166.1 (10)	43.1 (4)	422 (39)	209.2	3.85	0.627	100.2	418.4	670.7	62.9
E5-MG	1.21	165.6 (12)	35.8 (3)	413 (35)	201.4	4.62	0.613	96.6	402.8	640	64.5

^a Values are the averages of three determinations at the end of the fermentation, i.e., at approximately 25 to 30 h. Standard deviations are given in parentheses.

^b Y_{1,3-PD}, moles of 1,3-PD produced/moles of glycerol consumed.

^c In the catabolism of glycerol, the minimum amount of NADH formed via the acetate pathway was 2 and the maximum amount of NADH was 3, on the assumption that the reduced ferredoxin was entirely reoxidized by the ferredoxin-NAD reductase activity. The minimum and maximum amounts of NADH produced during the butyrate pathway were 2 and 4 (when the two reduced ferredoxins were reoxidized by the ferredoxin-NAD reductase activity), respectively.

^d NADH recovery, the percentage of maximum available NADH used for the formation of 1,3-PD, which was calculated by (mmol of 1,3-PD/mmol of NADH_{max}) × 100, where NADH_{max} is the maximum NADH.

E5 was 80%, whereas it was 20% for the mutant. A survey of the fermentation characteristics of four isolated AA-resistant mutants and the parental strain is shown in Table 1.

All AA-resistant mutants showed patterns of products significantly different from that of the parent strain. The wild type produced almost the same quantities of acetate and butyrate (ratio of 0.95), whereas the mutants produced less butyrate than acetate (acetate-to-butyrate ratios which varied from 3.8 to 4.9). The generation time changed from 1 h and 50 min for the wild type to approximately 1 h and 20 min for the mutants; this difference was probably due to the fact that acetate formation generates more ATP than butyrate formation, allowing a higher rate of growth. Concomitantly, total acid formation was higher for the mutants (Table 1) than for the wild-type strain, explaining the decrease in final biomass formation due to acid toxicity. Surprisingly, the results for conversion of glycerol to 1,3-PD (moles of 1,3-PD per moles of glycerol) were almost the same for the wild type and for the mutants. Whereas 97% of NADH was available for the wild-type strain, only 62 to 72% was available for the mutants (Table 1). This fact means that the NADH not used in butyrate biosynthesis did not lead to greater formation of 1,3-PD. These mutants exhibited higher hydrogenase activity (Table 2) and a considerable increase in hydrogen production (4), indicating that reducing equivalents were channelled toward hydrogen production via the NADH-ferredoxin reductase and the hydrogenase (2).

Analyses of key metabolic enzymatic activities. Mutants that were resistant to AA were selected. The toxicity of this compound depends on the reversibility of the 1,3-PD dehydrogenase; nevertheless, both the mutants and the wild-type strain

oxidize 1,3-PD (Table 2) and AA (Table 3). Although there was a decrease in 1,3-PD dehydrogenase specific activity, AA resistance cannot be attributed to loss of the oxidizing activity of the enzyme. Glycerol dehydrogenase activity in the mutants was also slightly decreased, whereas the values of the diol dehydratase were relatively constant. For the wild type and the mutants, the acetate kinase activities were higher than the butyrate kinase activities.

Kinetic properties of the 1,3-PD dehydrogenase. The apparent K_m values for the various reactions catalyzed by the 1,3-PD dehydrogenase activities of wild-type and mutant E5-MD were calculated from standard Lineweaver-Burk plots and are summarized in Table 3. Because 3-hydroxypropionaldehyde is not commercially available, we used glyceraldehyde, which was shown to be another substrate of the 1,3-PD dehydrogenase (5). Of particular significance was the observation that AA was always oxidized by the 1,3-PD dehydrogenase activities of wild-type and mutant E5-MD cell extracts. The apparent K_m s with NAD⁺ and NADH do not differ greatly but show markedly different apparent K_m s for glyceraldehyde and 1,3-PD. In cell extracts of the mutants, the decrease in K_m for glyceraldehyde and the increase in K_m for 1,3-PD were what would be expected for an enzyme primarily concerned with the reduction of an aldehyde to alcohol, and the kinetic properties of the 1,3-PD dehydrogenase in other mutants have common regulatory features (Table 3). This shift in excretion may be strengthened by a higher intracellular concentration of NADH in the mutants (Table 4). These changes are consistent with mutagenic events leading to an increase aldehyde reduction and to a decrease in alcohol oxidation. In *C. acetobutylicum*, two groups of mutants which were resistant to AA were character-

TABLE 2. Enzymatic activities from cell extracts of 673 mM glycerol batch cultures of *C. butyricum* E5 and mutants

Enzyme	Sp act (U/mg of proteins) for ^a :					
	E5		E5-MD		E5-MC	
	I	II	I	II	I	II
Glycerol dehydrogenase	0.53 (0.11)	0.48 (0.10)	0.29 (0.06)	0.32 (0.08)	0.21 (0.04)	0.38 (0.07)
1,3-PD dehydrogenase	0.99 (0.22)	0.50 (0.12)	0.32 (0.06)	0.30 (0.05)	0.35 (0.08)	0.31 (0.07)
Diol dehydratase	0.11 (0.03)	0.08 (0.02)	0.09 (0.02)	0.10 (0.03)	0.07 (0.01)	0.13 (0.04)
Hydrogenase	0.36 (0.06)	0.75 (0.11)	0.65 (0.06)	1.51 (0.13)	0.83 (0.06)	1.78 (0.18)
Acetate kinase	11.5 (2.8)	8.9 (2.5)	12.7 (3.1)	13.9 (3.3)	14.3 (3.7)	13.4 (3.5)
Butyrate kinase	2.4 (0.7)	4.2 (0.9)	3.9 (0.8)	3.6 (0.7)	2.4 (0.6)	2.7 (0.7)

^a One unit of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate per min. I, exponential phase of the growth; II, end of the growth. Standard deviations (of three determinations) are given in parentheses.

TABLE 3. Determination of K_m values for substrates and coenzymes of 1,3-PD dehydrogenase

Substrate or coenzyme tested	Other reactant (concn [mM])	K_m (mM) ^a			
		E5	E5-MD	E5-MB	E5-MG
AA	NAD ⁺ (2)	3.9 (0.9)	6.1 (1.1)		
NAD ⁺	AA (100)	0.26 (0.05)	0.29 (0.09)		
1,3-PD	NAD ⁺ (2)	6.8 (1.2)	11.7 (2.5)	11.0 (3)	14.3 (3.8)
NAD ⁺	1,3-PD (100)	0.21 (0.06)	0.22 (0.08)	0.19 (0.04)	0.36 (0.05)
Glyceraldehyde	NADH (0.7)	118.8 (29)	19.0 (3.9)	39.1 (7.8)	34.2 (6.0)
NADH	Glyceraldehyde (500)	0.11 (0.03)	0.10 (0.03)	0.11 (0.05)	0.25 (0.07)

^a K_m values were calculated by nonlinear regression to the Michaelis-Menten equation by using the curve Fit feature of the program Grafit (Erithacus Software). Standard deviations (of three determinations) are given in parentheses.

ized (9). Type I AA mutants produced butyraldehyde instead of butanol and were likely altered alcohol dehydrogenase. Type II AA mutants produced no significant butyraldehyde but produced small amounts of all solvents and contained very little butanol dehydrogenase activity, suggesting that they are regulatory mutants. The AA mutants described here were quite different; all produced normal amounts of 1,3-PD. It is most likely that glycerol catabolism by *C. butyricum* requires 1,3-PD dehydrogenase activity to avoid intracellular accumulation of 3-hydroxypropionaldehyde, which is a very toxic compound (3).

Comparison of NAD⁺, NADH, and acetyl coenzyme A levels. In the AA-resistant strains, the total NAD⁺ plus NADH level increased sharply, but the NADH-to-NAD⁺ ratios were always greater than 1 (Table 4). NADH and acetyl coenzyme A were not limiting substrates in butyrate biosynthesis, since high concentrations of these two compounds were found. These facts involve (i) the presence of mechanisms that precisely partition

the carbon flow between acetate and butyrate formation and (ii) the use of the branch point acetate-butyrate alone in the carbon flux regulation, since that leading to 1,3-PD production was not affected.

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TABLE 4. Nucleotide levels of *C. butyricum* E5 and mutants grown in batch culture on glycerol (673 mM) at pH 7.0^a

Strain and phase	Nucleotide concn ($\mu\text{mol} \cdot \text{g}^{-1}$ [dry mass] of cells)				
	NAD ⁺	NADH	NAD ⁺ + NADH	NADH/NAD ⁺	Acetyl coenzyme A
Wild type					
I	5.8 (0.7)	13.0 (1.5)	18.8	2.3 (0.5)	7.4 (0.9)
II	4.8 (0.5)	15.9 (1.6)	20.7	3.3 (0.7)	4.0 (0.5)
E5-MC					
I	15.3 (1.3)	27.7 (2.5)	43.0	1.8 (0.3)	11.3 (1.3)
II	11.7 (1.1)	21.9 (2.0)	33.6	1.9 (0.3)	11.9 (1.4)
E5-MD					
I	17.0 (0.9)	24.9 (2.3)	41.9	1.4 (0.4)	11.8 (1.4)
II	11.5 (0.5)	18.6 (1.9)	30.1	1.6 (0.2)	14.6 (1.6)

^a Each value is the mean ($\pm$ standard deviation) of six determinations from three separated cultures measured by the fluorometric method. I, exponential phase of the growth; II, end of the growth.