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Isolation and Characterization of Mutants of *Clostridium acetobutylicum* ATCC 824 Deficient in Acetoacetyl-Coenzyme A:Acetate/Butyrate:Coenzyme A-Transferase (EC 2.8.3.9) and in Other Solvent Pathway Enzymes

SANDRA W. CLARK, GEORGE N. BENNETT, AND FREDERICK B. RUDOLPH*
Department of Biochemistry, Rice University, P.O. Box 1892, Houston, Texas 77251

Received 14 November 1988/Accepted 19 January 1989

Mutants of *Clostridium acetobutylicum* ATCC 824 exhibiting resistance to 2-bromobutyrate or rifampin were isolated after nitrosoguanidine treatment. Mutants were screened for solvent production by using an automated alcohol test system. Isolates were analyzed for levels of butanol, ethanol, acetone, butyrate, acetate, and acetoin in stationary-phase batch cultures. The specific activities of NADH- and NADPH-dependent butanol dehydrogenase and butyraldehyde dehydrogenase as well as those of acetoacetyl-coenzyme A:acetate/butyrate: coenzyme A-transferase (butyrate-acetoacetate coenzyme A-transferase [EC 2.8.3.9]) (CoA-transferase), butyrate kinase, and phosphotransbutyrylase were measured at the onset of stationary phase. Rifampin-resistant strain D10 and 2-bromobutyrate mutant R were found to be deficient in only CoA-transferase, while several other mutants exhibited reduced butyraldehyde dehydrogenase and butanol dehydrogenase activities as well. The colony morphology of 2-bromobutyrate mutant R was similar to that of the parent on RCM medium; however, it had about 1/10 the level of CoA-transferase and increased levels of butanol dehydrogenase and butyraldehyde dehydrogenase. A nonsporulating, spontaneously derived degenerated strain exhibited reduced levels of butyraldehyde dehydrogenase, butanol, dehydrogenase, and CoA-transferase compared with those of the original strain. When *C. acetobutylicum* ATCC 824 was grown on medium containing low levels of 2-bromobutyrate, an altered colony morphology was observed. Not all strains resistant to 2-bromobutyrate (12 mM) were non-solvent-producing strains.

Although *Clostridium acetobutylicum* has been used for many years in the commercial production of acetone and butanol, the detailed mechanism of induction of solvent production has never been well understood. The genetics and physiology of the organism have been the topics of recent reviews (8, 15), and recent studies of fermentation parameters have allowed a clearer understanding of the requirements and cellular consequences of the conversion to the solvent-producing phase (8, 13).

Classical approaches to obtaining mutants with specific alterations in the solvent production pathway have been difficult to pursue with this organism. Inefficient plating and the difficulty of obtaining highly efficient mutagenesis with most common *C. acetobutylicum* strains have been two restraints on the advance of genetics of this anaerobe. A few successful approaches for obtaining useful solvent pathway mutants have been reported, including the use of alcohol or acid analogs which are toxic upon enzymatic conversion by the cell (4, 10, 16, 19). Growth in the presence of these analogs has given rise to mutants with reduced butanol levels which produce butyraldehyde in addition to normal amounts of acetone (16). Mutants with altered solvent production characteristics have been isolated by using a pyruvate-negative screen in *Clostridium saccharolyticum* (14), fluoroacetate resistance in *Clostridium thermosaccharolyticum* (19), or pH indicator dye screens for lower acid production in *Clostridium thermocellum* (3) and in *C. acetobutylicum* B643 (16). Another approach has been to screen for differences in cell growth or colony morphology (1, 9).

Recent advances in the cloning of genes related to solvent production and the development of improved methods for introducing genes into *C. acetobutylicum* have also raised interest in having a collection of characterized mutants with altered enzymes of the solvent production pathway. One of our goals was to identify and characterize mutants which would be useful in genetic complementation and regulation studies. Our approach has been to use previously demonstrated selective agents to isolate mutants altered in solvent production and then to characterize the levels of several important solvent-related enzymes in those strains. The pattern of enzyme loss may increase the understanding of the specific mechanisms of the selective agents, as well as the coordination of expression of the various enzymatic activities of the solvent pathway.

MATERIALS AND METHODS

Media. Liquid culture medium (RSM) consisted of the following: yeast extract (Y-0375; Sigma Chemical Co., St. Louis, Mo.), 5 g/liter; asparagine, 2 g/liter; cysteine, 0.5 g/liter; $FeSO_4 \cdot H_2O$, 25 mg/liter; KH_2PO_4, 0.752 g/liter; $MgSO_4$, 0.4 g/liter; $MnSO_4$, 10 mg/liter; $(NH_4)_2SO_4$, 2 g/liter; NaCl, 1 g/liter; and glucose, 50 g/liter. Plating of cells was on reinforced clostridial agar (RCM; Difco Laboratories, Detroit, Mich.). Spores were stored in corn mash medium (corn meal, 50 g/liter; glucose, 5 g/liter; cysteine, 0.5 g/liter).

RCM used for screening of mutants was supplemented with the following analogs: 4-chlorobutyrate (3.95 ml/liter), 4-bromobutyrate (4.26 ml/liter), 2-bromobutyrate (2-BB) (0.64 ml/liter), allyl alcohol (27.2 ml/liter), and 3 buten-1-ol (8.6 ml/liter) (all analogs from Aldrich Chemical Co., Milwaukee, Wis.). Selection plates for rifampin resistance contained final concentrations of 0.002, 0.02, 0.2, 2.0, 20, and

* Corresponding author.

100 μg/liter. Stock rifampin (Sigma), 4 mg/ml, was dissolved in dimethyl sulfoxide, filter sterilized, and added to the medium after autoclaving.

Mutagenesis and screening procedures. With the exception of the heat-shock treatment (described below), all operations were carried out in an anaerobic chamber (model 1025; Forma Scientific, Marietta, Ohio). The first mutagenesis method was an adaptation of that of Murray et al. (14). Exponential-phase cultures from heat-shocked spores (*C. acetobutylicum* ATCC 824) were concentrated approximately sixfold by centrifugation in Eppendorf tubes and suspension in RSM without glucose. After incubation for 2 h at 37°C, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NMNG; Sigma) was added (final concentration, 100 μg/ml) and the cultures were incubated an additional hour. The cells were then washed two times with RSM and grown overnight at 37°C. The outgrown cultures were plated and spread on selective medium.

A second method (10) was to plate an exponential-phase culture directly on selective medium and place a filter paper disk, impregnated with 75 μl of a saturated solution of NMNG, on the center of the plate.

Colonies were picked from selective plates and inoculated into microdilution plates containing 200 μl of RSM per well. After incubation (24 to 40 h), each plate was tested for solvent production by the tetrazolium salt method; 10 μl from each well was transferred to a microdilution plate containing 170 μl of assay mix (NAD, 25 μg/ml; phenazine methosulfate, 8 μg/ml; Nitro Blue Tetrazolium, 172 μg/ml; horse liver alcohol dehydrogenase [Sigma], 100 μg/ml; 100 mM Tris chloride [pH 8.6]) per well and incubated at room temperature for 10 min. The reaction was stopped by the addition of 1 M KH_2PO_4 (pH 6) at 36 μl per well. These assays were performed using the Pro/pette liquid handling system (Pro/pette Plus Express, Perkin-Elmer-Cetus Corp., Norwalk, Conn.). Reaction plates were read with an automated microplate reader (lambda reader, Perkin-Elmer-Cetus). Cultures which yielded a substantially reduced blue color in the tetrazolium assay were termed non-solvent-producing cultures.

Non-solvent-producing cultures were reserved for further study. Maintenance of these strains was primarily on RCM and RCM-plus-analog plates, since the strains were usually nonsporeforming as well. Spore production was determined by heat shocking 1- to 2-week-old cultures at 70°C for 10 min, reinoculating into fresh medium, and incubating at 37°C for 1 to 3 days. Mutants resistant to rifampin were maintained on RCM containing 20 or 100 μg of rifampin per ml.

Enzyme assays and analytical methods. Strains were grown in 600 ml of RCM at 37°C. The pH was not controlled. Samples (200 ml) were taken at three 4- to 6-h intervals in order to obtain data corresponding to the maximal solvent-producing stage, which occurs soon after the onset of the stationary phase. The appropriate harvest time was determined initially by measuring pH and A_{600}. The efficiency of sonication and the appearance of the subsequent extract were additional parameters. Lighter-colored pellets derived from cultures beyond the early stationary phase were difficult to sonicate and produced pale extracts with very low enzyme levels. The change in enzyme activities over time indicated which extracts were representative of this stage.

Supernatants of the harvested cells were analyzed for the presence of acetate, acetone, acetoin, butanol, butyrate, and ethanol by gas chromatography. The analyses were performed on a model V6000 gas chromatograph (Varian, Walnut Creek, Calif.) as previously described (13, 17).

Crude cell extracts were prepared by sonication of the frozen pellet in a solution consisting of 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 0.5 mM $ZnSO_4$, 500 mM NH_4SO_4, and 20% glycerol (vol/vol) (pH 7.0). Since the acetoacetyl-coenzyme A:acetate/butyrate:coenzyme A-transferase (butyrate-acetoacetate coenzyme A-transferase [EC 2.8.3.9]) (CoA-transferase) assay was thought not to be reliable in the presence of dithiothreitol, a sample of the extract was removed prior to its addition (final concentration, 2.5 mM) after sonication. The protein concentrations of the crude extracts were estimated by the method of Lowry et al. (12) with lysozyme as the standard protein.

Phosphotransbutyrylase (phosphate butyryltransferase [EC 2.3.1.19]) (PTB) was assayed by monitoring the liberation of coenzyme A (CoA) after the addition of butyryl-CoA to the reaction mixture (2, 6). The product was detected by complexing with 5,5′-dithio-(2-nitro-benzoic acid) (DTNB; Sigma). The assay mixture contained (in a volume of 1 ml): 0.1 M potassium phosphate buffer (pH 7.4), 0.2 mM butyryl-CoA, 0.08 mM DTNB, and crude extract (approximately 1 μg of protein). The reaction was initiated by the addition of diluted extract and monitored at 412 nm. The molar extinction coefficient of the DTNB-CoA-SH complex, E_{405}, is 13.6 mM^{-1} cm^{-1}.

Butyrate kinase (EC 2.7.2.7) (BK) was assayed by following the formation of the hydroxamic acid by butyryl phosphate in the presence of excess hydroxylamine (18). The subsequent formation of a colored ferric-hydroxamate complex in acid solution was quantified. The assay mixture contained (in a volume of 1 ml): 0.77 M potassium butyrate (pH 7.5), 48 mM Tris chloride, 10 mM $MgSO_4$, 0.7 M KOH, 10 mM ATP, and 100 to 400 μg of protein. The reaction was initiated by the addition of ATP, proceeded for 5 min at 29°C, and was stopped by the addition of 1 ml of 10% trichloroacetic acid. The quantity of the end product was determined by the addition of 4 ml of $FeCl_3$ (1.25% in 1 N HCl). The absorbance was read at 540 nm, where the extinction coefficient of the product is 0.691 mM^{-1} cm^{-1}.

CoA-transferase was assayed by following the disappearance of acetoacetyl-CoA at 310 nm (7). A nonspecific reaction occurs in the absence of substrate, and this background change in absorbance must be subtracted from the overall change in absorbance obtained in the reaction. The assay mixture contained (in a volume of 1 ml): 110 mM Tris chloride (pH 7.5), 5.5% (vol/vol) glycerol, 20 mM $MgCl_2$, 0.1 mM acetoacetyl-CoA, crude sonicated cell extract (50 to 100 μg of protein), and 0.32 M potassium butyrate or 0.32 M potassium acetate. This reaction was conducted in the absence of dithiothreitol. The reaction was started by the addition of acetate after a preincubation of about 1 min. The extinction coefficient of acetoacetyl-CoA at 310 nm is 8.0 mM^{-1} cm^{-1}.

Butyraldehyde dehydrogenase (BAD) (similar to acetaldehyde:NAD^+ oxidoreductase [CoA acylating] [acetaldehyde dehydrogenase] [EC 1.2.1.10]) was assayed by following the reduction of NADP (or NAD) to NADPH (or NADH) at 340 nm. Butyraldehyde was dissolved in methanol (50 mM). The assay mixture contained (in a volume of 1 ml): 50 mM glycylglycine buffer (pH 9.0), 50 mM butyraldehyde, 0.5 mM CoA, 0.3 mM NAD(P), 1.0 mM dithiothreitol, and crude extract (20 to 600 μg of protein). The extinction coefficient of NAD(P)H is 6.22 mM^{-1} cm^{-1} (20).

Butanol dehydrogenase (BDH) {butanol:NAD^+ oxidoreductase [alcohol dehydrogenase] [EC 1.1.1.1]; butanol:$NADP^+$ oxidoreductase [alcohol dehydrogenase ($NADP^+$)] [EC 1.1.1.2]} activity was assayed by monitoring the oxida-

TABLE 1. Growth and product analysis of batch cultures of clostridia strains

Strain	Avg time (h) to stationary stage[a]	Final A_{600}	Final pH	Concn (mmol/liter) of fermentation products:					
				Butanol	Ethanol	Acetone	Butyrate	Acetate	Acetoin
C. acetobutylicum ATCC 824									
Parent	18	5.0	3.6	55.0	6.5	19.3	9.0	17.0	10.5
Type IV[b]	19	4.0	3.7	8.1	2.6	1.3	35.6	16.6	4.7
Mutants derived from *C. acetobutylicum* ATCC 824									
M3[c]	19	2.2	3.9	0.0	2.1	0.0	27.6	15.2	1.4
M5[c]	19	5.0	3.9	0.0	3.0	0.0	42.3	8.6	5.6
2-BB R	23	5.9	3.8	18.0	4.3	1.3	9.8	28.0	8.0
2-BB D	23	6.2	3.8	44.3	3.9	13.5	9.8	15.0	12.2
Rif[r] B12[d]	28	1.5	4.0	1.5	1.2	3.7	27.0	11.5	0.5
Rif[r] D10[d]	28	1.0	4.1	0.0	0.5	5.0	17.6	9.9	0.1
Rif[r] F7[d]	28	2.0	3.9	1.3	2.1	3.2	41.0	15.2	2.6
C. butyricum ATCC 860	15	1.4	4.2	0.0	2.3	0.1	9.4	6.4	0.0

[a] Defined as the time at which there is no further increase in A_{600} of the culture.
[b] Type based on morphological characteristics described by Adler and Crow (1).
[c] First isolated from 4-chlorobutyrate plates.
[d] Grown in the presence of rifampin (20 μg/ml). When these strains were grown in the absence of rifampin, the rate of growth was faster.

tion of NADPH (or NADH) at 340 nm. Cuvettes with a path length of 20 mm were used with a reaction volume of 600 μl. The NADPH-dependent reaction was examined at pH 8.0 under the following conditions: 0.4 mM NADPH, 50 mM butyraldehyde, 35 mM Tris chloride (pH 8.0), and crude extract (60 to 600 μg of protein). The NADH-dependent reaction was observed by using 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0) with the other reagents as described above. The change in absorbance of blank reactions without butyraldehyde but with the appropriate quantity of methanol was subtracted from the total. This assay is a modification of Durre et al. (5).

RESULTS

Selection of mutants. A survey of isolated mutants and their properties, along with a comparison with the parent strain and *C. butyricum* ATCC 860, is shown in Table 1. M3 and M5 were isolated from 4-chlorobutyrate selective plates (as non-solvent-producing cultures) by mutagenesis procedure 1 and subsequently were found to be resistant to 2-BB as well. Both M3 and M5 were maintained on RCM, and although a loss of resistance to 2-BB was observed over time, they remained non-solvent-producing strains.

Mutants 2-BB R and D were isolated from 2-BB plates by mutagenesis method 2. They were non-solvent-producing cultures in the microdilution test system. Continued exposure to 2-BB resulted in colonies with increased solvent production. These strains were maintained on 12 mM 2-BB. Allyl alcohol and 3-buten-1-ol are both poor inhibitors of *C. acetobutylicum* ATCC 824 and require very high concentrations to inhibit growth. Mutants were found that produced low amounts of solvents, but they were not further characterized.

Rifampin-resistant strains were isolated from selective plates containing rifampin (2 μg/ml). Subsequently, these strains could be maintained on concentrations as high as 100 μg/ml, but 20 μg/ml was normally used. *C. acetobutylicum* ATCC 824 is sensitive to rifampin at 0.2 μg/ml. The strains analyzed that were resistant to 20 μg of rifampin did not produce significant amounts of solvents.

Colony morphology on RCM. Based on the existence of published data (1) linking colony morphology with solvent production, an attempt was made to do the same with our strains. Additionally, a difference in colony formation was noted within a single strain, depending upon the medium used. Plates were incubated 3 to 5 days to obtain mature colonies. Wild-type colonies similar to the type I of Adler and Crow (1) were found (Fig. 1a). These were spreading, dirty white-to-yellowish colonies with hard, raised centers that stuck to the agar and were not easily removed with a loop. Colonies similar in appearance to the type IV colonies of Adler and Crow (1) were isolated for comparison. These yellowish colonies did not spread and had irregular edges and dense but not hard centers. Intermediate degenerate strains have been isolated by Adler, and he referred to them as types II and III. Variations were observed in the metabolic products of individual colonies of each class. Therefore, the isolate that we have worked with could represent a type III colony by some criteria. M3 (Fig. 1c) gave rounder, whiter colonies with no hard centers. The smooth colonies spread slightly. M5 (Fig. 1d) appeared similar to M3 but had colonies with more-even edges. The colonies of rifampin-resistant mutants were, in general, smaller and spread less than those of the wild-type strain. Even though hard centers were present in some strains, these did not yield heat-resistant spores. The colony appearance of rifampin-resistant strains was not altered by the addition of 20 μg of rifampin per ml to the RCM. The 2-BB mutant R (Fig. 1e) appeared similar to a normal type I colony on RCM, while the 2-BB mutant D (Fig. 1b) appeared more like type IV in colony morphology.

Colony morphology on analog plates. Only a narrow range of concentrations of halogenated analogs of butyric and acetic acids can be used. For example, at 6 mM 2-BB, the wild-type strain forms a "thin lawn of growth" (19) on the plate, and the resistant mutant shows thick growth up to 12 mM and some growth at 24 mM, but none beyond that level.

There was minimal growth of the wild type on butyric acid analog plates. All strains exhibited similar colony morphology on these plates, but no cross resistance has been found so far. On 2-BB (6 mM for wild-type *C. acetobutylicum* ATCC 824, 12 mM for mutant strains) plates, very small, round, white colonies were present (Fig. 2). When the colonies were magnified, however, differences could be seen

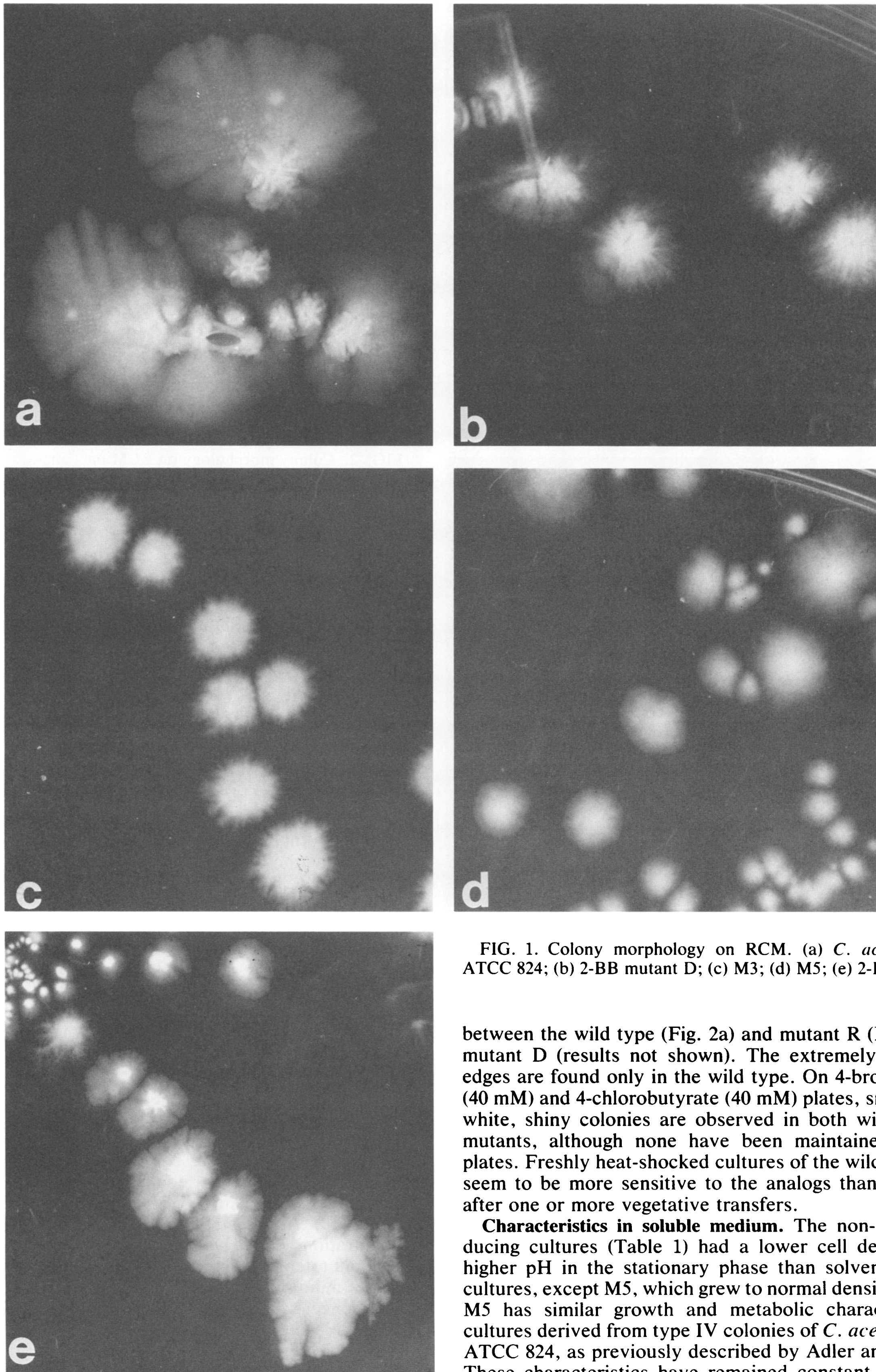

FIG. 1. Colony morphology on RCM. (a) *C. acetobutylicum* ATCC 824; (b) 2-BB mutant D; (c) M3; (d) M5; (e) 2-BB mutant R.

between the wild type (Fig. 2a) and mutant R (Fig. 2b) and mutant D (results not shown). The extremely tight, rigid edges are found only in the wild type. On 4-bromobutyrate (40 mM) and 4-chlorobutyrate (40 mM) plates, small, round, white, shiny colonies are observed in both wild type and mutants, although none have been maintained on these plates. Freshly heat-shocked cultures of the wild-type strain seem to be more sensitive to the analogs than cells taken after one or more vegetative transfers.

Characteristics in soluble medium. The non-solvent-producing cultures (Table 1) had a lower cell density and a higher pH in the stationary phase than solvent-producing cultures, except M5, which grew to normal density (Table 1). M5 has similar growth and metabolic characteristics to cultures derived from type IV colonies of *C. acetobutylicum* ATCC 824, as previously described by Adler and Crow (1). These characteristics have remained constant for over 10 months.

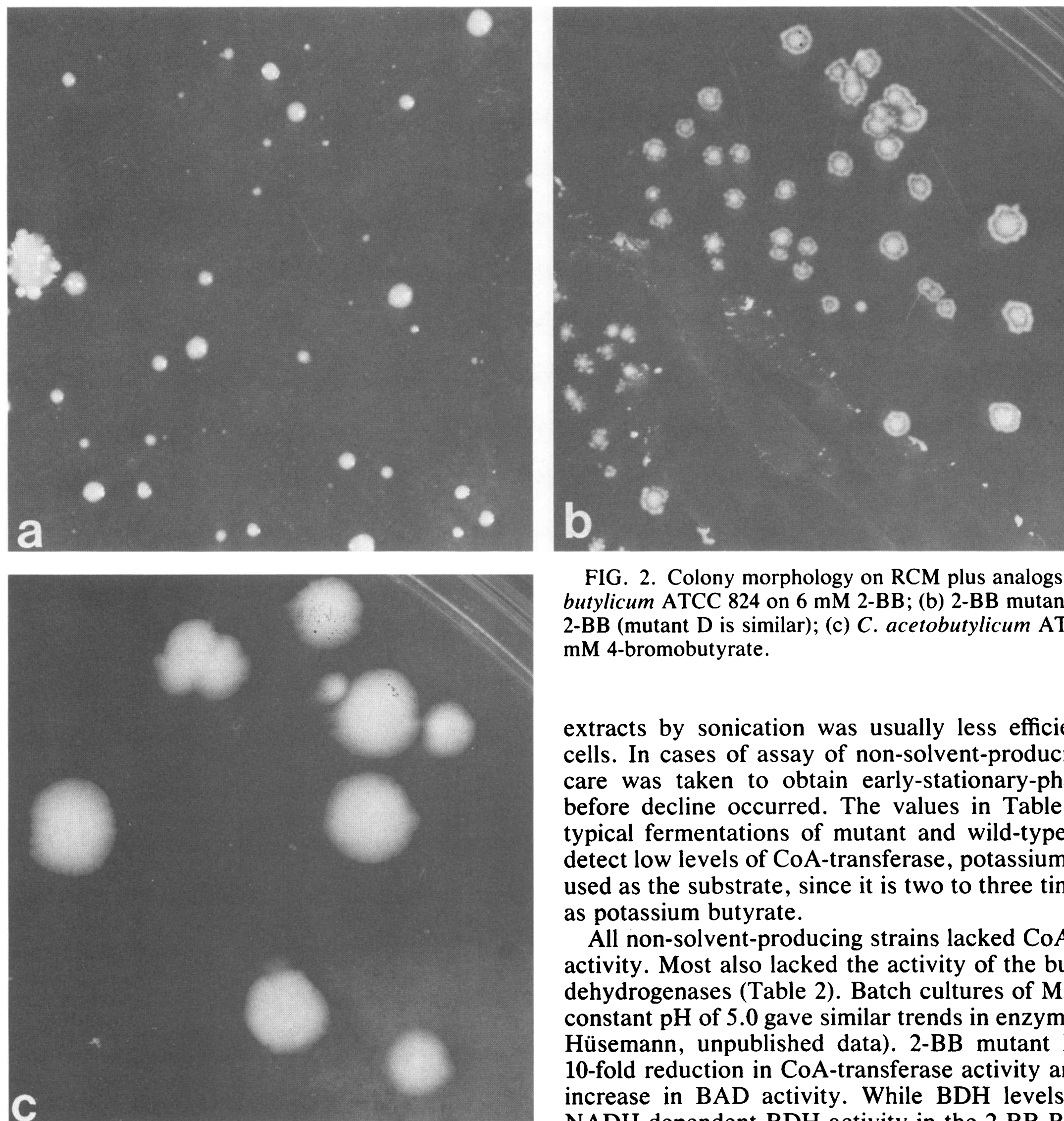

FIG. 2. Colony morphology on RCM plus analogs. (a) *C. acetobutylicum* ATCC 824 on 6 mM 2-BB; (b) 2-BB mutant R on 12 mM 2-BB (mutant D is similar); (c) *C. acetobutylicum* ATCC 824 on 40 mM 4-bromobutyrate.

The low final absorbance of M3 is typical of non-solvent-producing strains. Other strains with this property include *C. butyricum* ATCC 860 and ATCC 19398. The Rif^r mutants grow slower in the presence of rifampin (20 μg/ml) than they do in RSM and require at least 4 more h to reach the stationary phase. These mutants grew at the same rate as the wild-type strain in the absence of rifampin in RSM. None of our non-solvent-producing strains made heat-resistant spores.

Enzyme activities in various mutants and the wild type. To assess the presence of various enzymes of the solvent production pathway in the mutants, cultures of each were grown for assay. Cells were harvested at 4- to 6-h intervals to ensure obtaining a sample from cells corresponding to the optimal solvent-producing stage. The proper harvest time which would correspond to the same stage of growth in the other strains was determined by cessation of increasing A_{600} and decreasing pH. Non-solvent-producing cells reached a declining state sooner; the cell pellet appeared lighter, and the enzyme activities declined markedly. Preparation of cell extracts by sonication was usually less efficient with old cells. In cases of assay of non-solvent-producing cultures, care was taken to obtain early-stationary-phase cultures before decline occurred. The values in Table 2 represent typical fermentations of mutant and wild-type strains. To detect low levels of CoA-transferase, potassium acetate was used as the substrate, since it is two to three times as active as potassium butyrate.

All non-solvent-producing strains lacked CoA-transferase activity. Most also lacked the activity of the butyraldehyde dehydrogenases (Table 2). Batch cultures of M5 grown at a constant pH of 5.0 gave similar trends in enzyme values (M. Hüsemann, unpublished data). 2-BB mutant R showed a 10-fold reduction in CoA-transferase activity and a twofold increase in BAD activity. While BDH levels varied, the NADH-dependent BDH activity in the 2-BB R mutant was at least twofold higher than has been found in the wild type. The 2-BB R mutant also showed considerably reduced acetone production. The 2-BB D mutant exhibited solvent-producing properties similar to those of the wild-type strain, except for its resistance to 2-BB. The rifampin-resistant mutants appeared to have elevated butyrate kinase activities (certain cultures have yielded as high as 6 U/mg).

DISCUSSION

Our experiments have sought to isolate and characterize clostridial mutants with resistance to various inhibitors for future biochemical and genetic studies. 2-BB has been reported to allow isolation of mutants which did not produce acetone (10). In our studies, only a narrow range of 2-BB concentrations could be used and no mutants were found that could tolerate high doses (>24 mM) of the analog. The resistant mutants were able to grow on 6 to 12 mM 2-BB, a higher concentration than the 0.06 mM used in the previous report of resistance in *C. acetobutylicum* 824 (10). In plating experiments with these analogs, a different colony morphology of the stock *C. acetobutylicum* ATCC 824 wild type was observed on a low concentration of the analog. We also have

TABLE 2. Enzyme activities of mutants of *C. acetobutylicum* ATCC 824

Strain	Enzyme activity (U/mg of protein)[a] for:						
	BDH		BAD		CoA-transferase	BK	PTB
	NADH dependent	NADPH dependent	NAD dependent	NADP dependent			
C. acetobutylicum ATCC 824							
Parent	29.4	85.5	53.7	26.1	2,520	2,000	16,800
Type IV[b]	4.7	33.7	4.7	4.5	230	2,700	15,200
Mutants derived from *C. acetobutylicum* ATCC 824							
M3[c]	17.2	18.3	0.0	0.0	0	1,700	11,300
M5[c]	3.9	14.2	0.0	0.0	0	1,400	13,100
2-BB R	92.5	49.9	92.9	97.5	190	1,800	14,600
2-BB D	42.3	85.6	25.7	39.9	2,500	1,500	12,400
Rif^r B12[d]	19.3	28.9	5.9	0.0	0	3,000	15,300
Rif^r D10[d]	25.3	28.1	20.4	20.4	0	4,700	24,800
Rif^r F7[d]	8.6	78.1	0.0	5.3	0	3,200	17,600

[a] These measurements represent the value obtained on cultures soon after the onset of solvent production. The extracts used were generally from the cultures from which the fermentation product determinations were made (Table 1). Units for each enzyme are defined as nanomoles of product formed per milligram of protein in crude extracts. The assay procedures are described in Materials and Methods.
[b] Type based on morphological characteristics described by Adler and Crow (1).
[c] First isolated from 4-chlorobutyrate plates.
[d] Grown in the presence of rifampin (20 μg/ml).

found a mutant that is resistant to 12 mM 2-BB (mutant D [Fig. 2b]) and still produces solvents. The solvent pathway enzymes all seem normal in this strain and correlate with its solvent-producing capacity. These results indicate that mechanisms other than the loss of butanol pathway enzymes can lead to increased tolerance to 2-BB. This finding and the altered morphology of the wild-type strain on low levels of the analogs may suggest that an alteration in surface structure or transport properties could be involved.

The 2-BB mutant R exhibits a low production of acetone, as was found for a 2-BB mutant in the earlier report (10). From assays of enzymes of the solvent pathway in this strain, it appears that CoA-transferase is the only solvent pathway enzyme present in significantly reduced quantity. The activities of this enzyme were not reported among the enzyme activities assayed in the studies of allyl alcohol mutants of *C. acetobutylicum* B643 (16). Thus, to our knowledge, this report is the first description of a strain deficient in only CoA-transferase. It is reasonable that this enzyme be absent in a 2-BB-resistant mutant, since conversion of 2-BB to 2-BB-CoA could lead to increased toxic effects.

The other mutants isolated on halogenated butyrate analogs, M3 and M5, have lost other enzymes as well as CoA-transferase. The loss of other solvent pathway enzyme activities in allyl alcohol-resistant mutants was also observed (16). There are two possible explanations for such a result, one related to degeneration and one related to regulatory processes. Perhaps, once solvent-producing ability is lost through one mutation (e.g., butanol dehydrogenase or CoA-transferase), changes occur which lead to loss of other activities through a degenerative process akin to the formation of colonies of altered morphology. The potential for secondary mutations to arise in a mutant strain is significant, especially when the strain does not sporulate well. Alternatively, the mutations which affect the expression of several enzyme activities could be regulatory in nature, abolishing some essential regulatory factor or genetic arrangement needed for expression. Although it is difficult to say whether the multiply deficient mutants are regulatory in nature or whether they arose through accumulation of secondary mutations, two factors can be considered with regard to this question. The findings that (i) similar metabolic and enzyme properties have been obtained for these strains over a period of months and (ii) increased levels of other enzymes are present in certain mutant strains reported here and previously (15) suggest that positive effects on various enzymes can occur. This would be consistent with properties of regulatory mutations.

The relationship between solvent production and early events in sporulation has been considered (11) and the rifampin resistance of various nonsporeforming strains (9) has been used to isolate nonsporeforming and non-solvent-producing strains. The rifampin-resistant strains we have isolated seem typical of the oligosporogenous or *cls* type of Rif^r strain (9). The non-solvent-producing, asporogenous strain 6A (16) was reported to have very little BAD and BDH activity but elevated levels of PTB, phosphotransacetylase, BK, and acetate kinase. The metabolic product profiles of our rifampin-resistant mutants (which do not sporulate) are similar to those reported for the Spo$^-$ mutant 6A by Rogers and Palasaari (16), and our result is similar in that an increased level of BK is found. However, we found only minor differences between the rifampin-resistant and wild-type strains in PTB activity, and the reduction of the butanol dehydrogenases was not as severe. The major change in solvent pathway enzyme activity in all our rifampin-resistant mutants was loss of CoA-transferase activity. Rifampin-resistant mutant D10 is especially interesting in that it has very low CoA-transferase activity while retaining near-normal activities for the other solvent enzymes. One factor to be considered is the effect of rifampin on the expression of the enzymes of the solvent pathway. Our preliminary experiments have suggested that induction of CoA-transferase activity during late exponential phase is very readily blocked by addition of rifampin. It is possible that certain mutations leading to rifampin resistance may yield an altered RNA polymerase which cannot transcribe this gene well.

The similarity of enzyme patterns between the rifampin-resistant mutants and the 2-BB mutants is interesting, particularly the Rif^r mutant D10 and the 2-BB mutant R. Both of these strains exhibited a deficiency in only CoA-transferase

activity. However, they had significantly different properties. In 2-BB mutant R, the levels of BAD and BDH were elevated with respect to the wild type, whereas in Rif[r] mutant D10, these levels were lower. On the other hand, acid enzymes BK and PTB were higher than normal in 2-BB D10 and about normal in mutant R. The metabolic products formed by the two are quire different. 2-BB mutant R can make considerable amounts of butanol, ethanol, and acetoin despite its low level of CoA-transferase, presumably due to the compensatory elevated levels of BAD and BDH. The Rif[r] mutant D10, a poor solvent producer, had two- to fourfold-lower levels of these enzymes and almost no CoA-transferase.

The enzyme complement of various types of non-solvent-producing strains may suggest critical regulatory roles. In our isolates, the most frequently lost enzyme was CoA-transferase, followed by butyraldehyde dehydrogenase. Of the two butanol dehydrogenase activities assayed, the NADH-dependent enzyme was induced two- to threefold above wild-type levels (as was the butyraldehyde dehydrogenase activity) in 2-BB mutant R. This result could suggest that it may have a similar regulation to BAD. In strains in which several solvent enzymes are lost, the NADH-dependent BDH declines as much as or more than the NADPH-dependent form, suggesting a role in solvent formation. The recent reports of cloning of the genes for an NADPH-dependent alcohol dehydrogenase (21) and the butyraldehyde dehydrogenase (P. R. Contag and P. Rogers, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, H-146, p. 169) should contribute to long-range studies of the function of various forms of these enzymes in solvent production.

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