

## Analysis of Degenerate Variants of *Clostridium acetobutylicum* ATCC 824

K. P. Stim-Herndon<sup>1</sup>, R. Nair<sup>2</sup>, E. T. Papoutsakis<sup>2</sup> and G. N. Bennett<sup>1</sup>

<sup>1</sup>Department of Biochemistry & Cell Biology, Rice University, 6100 Main Street, Houston TX, 77005-1892 and

<sup>2</sup>Department of Chemical Engineering, Northwestern University, Evanston IL, 60208-3120, U.S.A.

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It is known that degenerate mutants of the solvent producing *Clostridium acetobutylicum* will spontaneously develop during repeated subculturing or continuous fermentation. Several hypotheses have been proposed as to what causes this spontaneous degeneration. One proposed explanation is that aberrations in regulatory proteins result in the failure of the organism to respond to influences causing the switch from the production of acids (acetate and butyrate) to the production of solvents (acetone, butanol, and ethanol). Another possibility is a mutation or rearrangement of the region of the chromosome involved in the production of the enzymes involved in solventogenesis.

To further investigate the processes altered in degenerate mutants, a set of degenerate variants was obtained. This set includes strains obtained from repeated subculturing, and from chemical mutagenesis. All of the strains show decreased or no production of acetone and/or butanol by gas chromatography analysis, and the loss of enzyme activity of one or more of the enzymes involved in solvent production. Experiments indicate that a genetic region encoding an aldehyde/alcohol dehydrogenase (*aad*, *adhE*), the acetoacetyl-coenzyme A:acetate/butyrate:coenzyme A-transferase (*ctfAB*), and the acetoacetate decarboxylase (*adc*) is lost during the degeneration process. This result coupled with previous complementation studies of degenerate mutants with plasmids containing solvent formation genes which yielded a restoration of solvent formation suggest that in full degenerates the defect is not simply a result of a loss of an essential expression factor, but is due to a defect in that region of the chromosome encoding the solvent genes.

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### Introduction

*Clostridium acetobutylicum* ATCC 824 is capable of producing both acids (such as acetate or butyrate), or

Address correspondence to G.N. Bennett, Department of Biochemistry & Cell Biology, Rice University, 6100 Main Street, Houston TX 77005-1892. E-mail: gbennett@bioc.rice.edu

solvents (such as acetone, butanol, or ethanol), during fermentation. This strict anaerobe is a saccharolytic bacterium capable of fermenting a wide variety of sugars, oligosaccharides, and polysaccharides resulting in the production of these acids or solvents. The acetone–butanol fermentation industry using solvent-producing strains of *Clostridium* was of major

importance earlier in this century, before competition from the chemical synthesis of solvents by the petroleum industry and the rising costs of substrates such as molasses and corn rendered it uneconomic. However, with the advent of molecular biology, interest in the commercial production of chemicals using a superior metabolically engineered strain of *C. acetobutylicum* was renewed and has recently led to the cloning and characterization of genes involved in solvent production or its regulation. Several shuttle vectors, an efficient transformation protocol, and gene inactivation systems are now available for work with *C. acetobutylicum*, and metabolic engineering studies have been initiated. These advances have been reviewed recently [1,2].

One problem in long-term culture of *C. acetobutylicum* is the spontaneous degeneration of the culture over time to a non-solvent producing form. The degeneration process [3] and the characteristic morphology of degenerating strains have been described [4]. Solvent producing capability has also been correlated to motility. Highly motile cultures have superior solvent production while non-motile cultures yield low or no solvents [5,6]. The purpose of this study was to initiate an investigation into the degeneration process using degenerate strains obtained by different culture methods. The experiments presented here characterized these degenerate strains by enzyme assays, gas chromatograph analysis, and DNA analysis. The results suggest that more than one mechanism may be involved in the degeneration process.

## Materials and Methods

### *Bacterial strains and microbiological characterization*

*Clostridium acetobutylicum* ATCC 824 and strain M5 [7] were maintained on Reinforced Clostridial Agar (RCM, Difco Laboratories, Detroit, MI) or Reinforced Soluble Medium (RSM) as described previously [6]. Newly isolated degenerate colonies were maintained on NYG plates [8 g nutrient broth (Difco), 1 g yeast extract (Difco), 4 g glucose and 15 g agar per L water]. CGM [8] contains 0.75 g  $\text{KH}_2\text{PO}_4$ , 0.75 g  $\text{K}_2\text{HPO}_4$ , 0.35 g  $\text{MgSO}_4$ , 0.01 g  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.01 g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 1 g NaCl, 2 g L-asparagine, 5 g yeast extract (Difco), 2 g ammonium sulfate, and 50 g glucose in 1 L.

To examine colony morphology, fresh, logarithmic phase cultures were streaked on NYG plates and grown at 37°C for 3–4 days. Mutant colonies were identified by the classification of Adler and Crow [4]. Subsequent growth and restreaking of individual colonies confirmed that the mutant phenotype was stably maintained with repeated transfer.

To observe cell morphology and motility, phase-contrast microscopy (Nikon Optophot, Melville, NY) was used to examine cell shape and association and the motility of actively growing cultures. Motility was also examined by stabbing a toothpick containing the culture or an individual colony into motility agar plates [15 mL of RCM with 0.35% w/v agar on top of a layer (15 mL) of RCM with 1.5% agar] and measuring the colony diameter after 24–48 h [6].

Sporulation was checked by heat shocking a 7-day-old culture grown in Corn Mash Medium (5 g corn meal, 0.5 g glucose, 0.05 g L-cysteine in 100 mL water, final pH 6.4) [9] and checking for survival after several days incubation [7].

Strains were grown and maintained in an anaerobic chamber (Model 1025 Forma Scientific (Marietta, OH) supplied with 10% hydrogen, 5%  $\text{CO}_2$ , 85% nitrogen (TriGas, Houston, TX) equipped with a temperature-controlled incubator.

### *Determination of solvent production*

Mutant and wild-type cultures grown anaerobically at 37°C in CGM liquid broth [8] were analysed for ethanol, acetone, butanol, and acetic and butyric acids by injecting cell-free supernatants from centrifuged liquid culture samples into a gas chromatograph (Hewlett-Packard Model 5890 with a Porapak Q 100/120 column) equipped with a flame ionization detector and integrator (Hewlett-Packard, Wilmington, DE) [6,7]. The retention times and peak areas were standardized with known amounts of these compounds.

### *Enzyme assays*

Crude cell extracts were prepared from 200 mL CGM cultures [8] by resuspending the centrifuged cell pellet in a solution consisting of 0.5 mM  $\text{ZnSO}_4$ , 500 mM  $\text{NH}_4\text{SO}_4$ , 20% glycerol (v/v), and 50 mM MOPS (pH 7.0). The resuspended cell pellet was sonicated, and cell debris was removed by centrifugation at 15 000 g for 30 min. Spectrophotometric enzyme assays for phosphotransbutyrylase (PTB), butyrate kinase (BK), acetoacetyl-coenzyme A:acetate/butyrate:coenzyme A transferase (CoAT), NADH- and NADPH-dependent butanol dehydrogenase (BDH), and NADH- and NADPH-dependent butyraldehyde dehydrogenase (BAD) were performed as previously described [6,7]. Reagent chemicals and substrates were obtained from Sigma Chemical Co. (St. Louis, MO). Briefly, phosphotransbutyrylase (PTB) was assayed by monitoring the liberation of coenzyme A (CoA) after the addition of butyryl-CoA to the reaction mixture. The product was detected by complexing

with 5,5'-dithio-(2-nitrobenzoic acid) (DTNB). 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  $13.6 \text{ mM}^{-1} \text{ cm}^{-1}$ .

Butyrate kinase (BK) was assayed by following the formation of the hydroxamic acid by butyryl phosphate in the presence of excess hydroxylamine. 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  $\text{MgSO}_4$ , 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  $\text{FeCl}_3$  (1.25% in 1 N HCl). The absorbance was read at 540 nm, where the extinction coefficient of the product is  $0.691 \text{ mM}^{-1} \text{ cm}^{-1}$ .

Acetoacetyl-coenzyme A: acetate / butyrate: coenzyme A transferase (CoAT) was assayed by following the disappearance of acetoacetyl-CoA at 310 nm. A non-specific 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% (v/v) glycerol, 20 mM  $\text{MgCl}_2$ , 0.1 mM acetoacetyl-CoA, crude sonicated cell extract (50 to 100 µg of protein), and 0.32 M potassium butyrate. 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  $8.0 \text{ mM}^{-1} \text{ cm}^{-1}$ .

Butyraldehyde dehydrogenase (BAD) was assayed by following the reduction of NADP (or NAD) to NADPH (or NADH) at 340 nm. Butyraldehyde was dissolved in methanol. 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 (20–600 µg of protein). The extinction coefficient of NAD(P)H is  $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ .

Butanol dehydrogenase (BDH) activity was assayed by monitoring the oxidation 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 (60–600 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 Dürre *et al.* [10].

The protein content of the extracts was determined using dye reagent method of Biorad with lysozyme as the standard (Bio-Rad, Hercules, CA).

### Southern hybridization

DNA fragments from previously described plasmids were used as probes. The *ptb* and *bk* gene probe was a 2.4 kb *Hind*III fragment of pJC7 encoding PTB and BK [11]. The *aad(adhE)* gene encodes an aldehyde and alcohol dehydrogenase activity, and a probe for this gene was obtained as a 3.3 kb *Eco*RI-*Xba*I fragment of pCAAD [12,13]. The *bdh* probe was prepared from a 3.5 kb *Eco*RI fragment of pBDH51 and contains segments of *bdhA* and *bdhB*, encoding two alcohol dehydrogenase isoenzymes [14,15]. The probe bearing the genes *ctfA ctfB* encoding the two subunits of CoAT was obtained as a 1.5 kb *Bam*HI-*Eco*RI fragment of pSC84, a plasmid bearing a segment of the artificial acetone operon, a construct containing the genes encoding CoAT and acetoacetate decarboxylase (*adc*) described earlier [16]. The acetoacetate decarboxylase probe was derived from pKS17, which contains a 1 kb *Eco*RI fragment bearing the *adc* gene [16]. The thiolase gene probe was a 4.8 kb *Eco*RI fragment of pTECO11 [17,18]. The fragments were isolated from agarose gel and labelled by the random priming method using a kit (Life Technologies Inc., Gaithersburg, MD) and  $\alpha$ - $^{32}\text{P}$ -dATP (ICN Biomedicals Inc., Costa Mesa, CA). The *C. acetobutylicum* chromosomal DNA was isolated from ATCC 824 and from the strain variants using the PureGene DNA Isolation kit for Gram-positive bacteria from Gentra Systems (Research Triangle Park, NC) as described by the manufacturer. Restriction digests of the isolated chromosomal DNA were performed using buffers supplied by the restriction enzyme supplier (Promega Corp., Madison, WI). The restriction enzyme *Sca*I was used in all digests shown in Figure 1. After digestion and separation on agarose gels, the DNA was blotted to Hybond N<sup>+</sup> membrane (Amersham Life Sciences Inc., Arlington Heights, IL) by downward alkaline transfer for 1.5 h using 0.4 M NaOH [19]. The blotted membrane was washed briefly in 2XSSC, prewashed in Rapid Hybridization Buffer (Amersham) at 42°C for 15 min, hybridized with the radiolabeled probe in 5 mL fresh Rapid Hybridization Buffer for 1.5 h and washed three times with 100 mL of wash buffer (40 mM phosphate buffer, pH 7.2, 0.1% SDS) for 30 min at 42°C. The washed

membrane was wrapped in Saran Wrap and exposed to an XAR-5 X-ray film (Eastman Kodak, Rochester, NY) or to a phosphoimaging plate with digital analysis using a phosphoimager (Fuji Medical Systems, Stamford, CT).

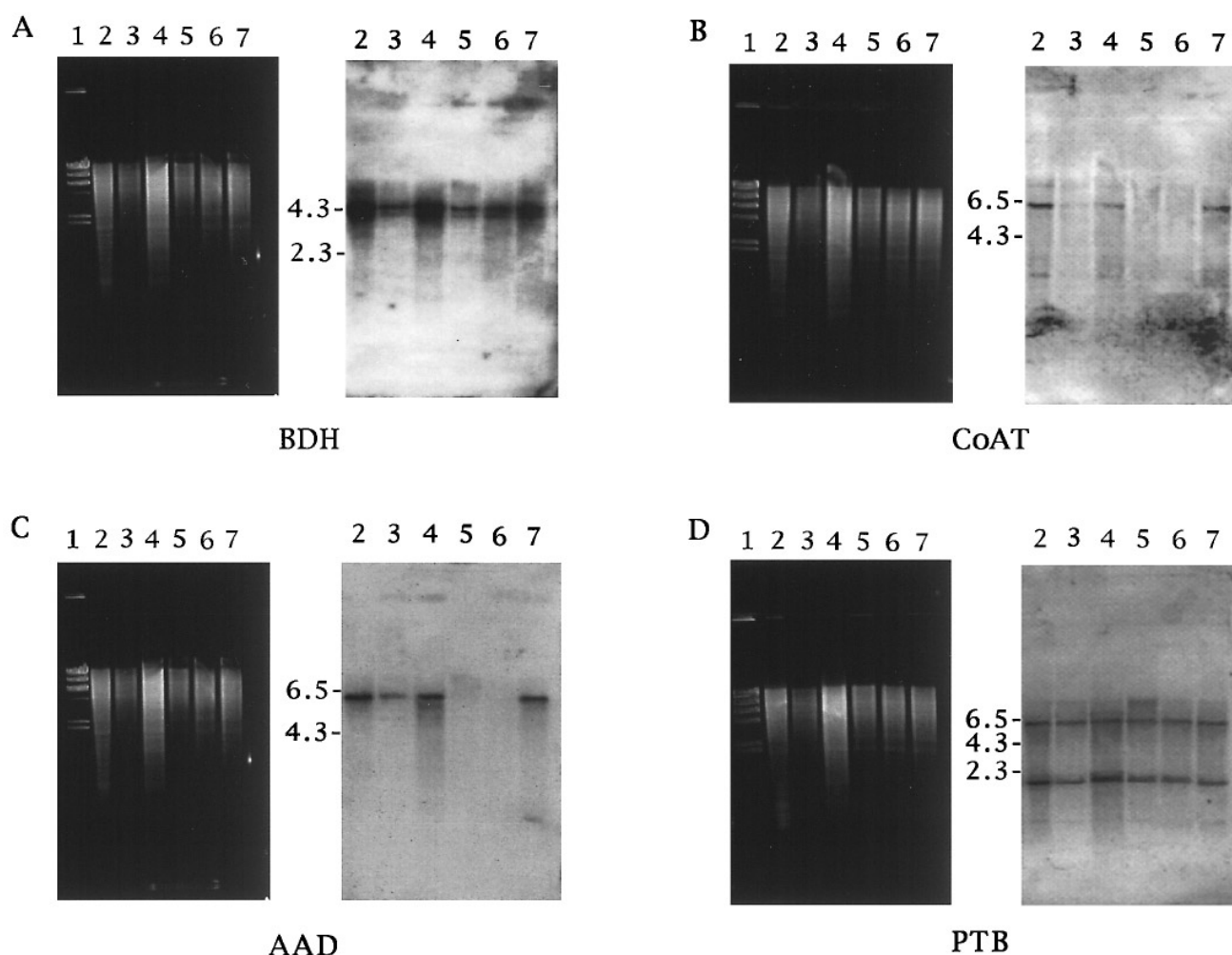
## Results

### *Isolation and morphology of strains*

Selection of degenerate strains was performed by serial subculturing followed by growth on NYG plates (strains 1NYG, 4NYG and 5NYG). Strain M5 was

reported earlier [7], it was originally maintained on RSM plates. Strain M5 was originally isolated as a non-solvent producing strain after N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis as a colony on 4-chlorobutyrate plates. It also showed resistance to 2-bromobutyrate. M5 had been previously analysed and exhibited no detectable enzyme activity for acetoacetyl-coenzyme A: acetate / butyrate: coenzyme A-transferase or butyraldehyde dehydrogenase [7] or acetoacetate decarboxylase [6]. Strain DG1 was obtained from serial subculture from a fermentation experiment [20].

The colony appearance of these strains allowed them to be classified according to the types described by Adler and Crow [4]. Strains M5 and DG1 appeared as type IV colonies, while 1NYG, 4NYG and 5NYG



**Figure 1.** Southern hybridization of *C. acetobutylicum* strains. Chromosomal DNA was isolated from the strains described in Tables 1 and 2. The DNAs were digested with *Sca*I, separated by electrophoresis and stained with ethidium bromide and photographed. This photograph is shown in the left side of each panel. The gels were then blotted as described by Koetsier *et al.* [19]. The membrane was probed with a radiolabeled DNA fragment prepared as described in Materials and Methods. Hybridization with probes corresponding to the butanol dehydrogenase A and B genes, BDH, panel A; the coenzyme A transferase genes (*ctfAB*), CoAT, panel B; the alcohol/aldehyde dehydrogenase gene *adhE* (*aad*), AAD, panel C; and the phosphotransbutyrylase-butyrate kinase genes, PTB-BK, panel D. Lane 7 in each panel contains DNA isolated from *Clostridium acetobutylicum* ATCC 824 and the other lanes contain DNA from the variant strains as follows: lane 2, 5NYG; lane 3, 4NYG; lane 4, 1NYG; lane 5, M5; lane 6, DG1. In lane 1, the  $\lambda$  *Hind*III fragment standards were loaded and are visible in the ethidium bromide stained gel. The sizes of  $\lambda$  *Hind*III fragment standards are shown in kb at the left of each autoradiograph.

appeared as type II or III intermediate degenerate strains with 4NYG having more characteristics of a type II strain. *C. acetobutylicum* ATCC 824 exhibited the appearance and outgrowths typical of type I colonies. Microscopic examination revealed frequent filamentous structures in 1NYG, 4NYG and 5NYG while *C. acetobutylicum* ATCC 824, M5 and DG1 showed predominately rod-type cells. Sporulation tests were positive only for the wild-type parent and 4NYG. Motility tests indicated a normal motility only for 4NYG in addition to ATCC 824.

#### Enzyme and metabolite analysis

Large cultures (600 mL) of each of the strains were grown in CGM [8] and samples removed at various times for GC and enzyme assays. Samples of these same cultures were also stored and examined by DNA techniques subsequently. Several enzymes indicative of solvent formation or acid formation were assayed for each of the strains. The growth rates and final cell density differed for the strains (see Table 1). Cultures of the degenerate strains exhibited a lower final absorbance and a lower final pH than those of the wild-type ATCC 824. Slower growth was also observed for the strain variants. The enzyme assays (Table 1) indicated an absence of CoAT in M5, DG1, 1NYG and 5NYG. No BAD activity was detected in M5 or DG1. Overall BDH activities were reduced in the later culture stages in all degenerate strains compared to the activity from the wild-type cultures.

Analysis of the metabolites produced by the strains

during the above culture experiments were determined by gas chromatography. The results are shown (Table 2). The strains DG1 and M5 produce no detectable butanol or acetone, while 1NYG and 5NYG produce low levels of acetone and butanol and about the same level of butyrate and acetate as the wild-type culture. Strain 4NYG produces almost normal levels of butanol but little acetone over the 23 h incubation period analysed.

#### DNA analysis

Analysis of chromosomal DNA of the strains was conducted by Southern hybridization using probes from various solvent-stage-induced or acid production genes (Figure 1). Blots revealed the presence of the normal pattern of bands in the case of the phosphotransbutyrylase and butyrate kinase probe (6.0 kb and 1.5 kb *ScaI* fragments). The thiolase A gene probe also displayed the same sized *ScaI* bands (5.8 kb, 2.3 kb, 0.5 kb) in all strains tested (data not shown). Probes containing the two butanol dehydrogenase genes also produced identical patterns for all strains tested. In this case, a *ScaI* fragment of ~3.8 kb is expected based on the sequences analysed [15] and on the map reported [14]. A *ScaI* site has been reported ~0.4 kb from the end preceding these fragments [14], suggesting an incomplete digestion at the site nearest the *bdh* genes. Hybridization tests with DNA fragments arising from genes encoding CoAT (*ctfAB*), acetoacetate decarboxylase (*adc*) or the alcohol/aldehyde dehydrogenase produced by *aad(adhE)* showed

**Table 1.** Growth and enzyme activity of *C. acetobutylicum* strains

Culture	Enzyme specific activity										
	Strain	Time (h)	OD	pH	PTB	BK	NADH BDH	NADPH BDH	NAD BAD	NADP BAD	CoAT
Wild-type		16.5	4.59	3.35	12440	2120	12	34	59	49	300
		19.75	5.22	3.30	10710	2640	21	55	50	39	740
		22.5	5.35	3.54	10190	1540	27	49	69	43	1160
M5		16.5	2.01	3.32	14990	2600	13	48	<2	<3	<3
		19.75	2.28	3.33	10050	3850	30	19	<2	<3	<3
		22.5	2.55	3.38	11980	3980	25	6	<2	<3	<3
1NYG		16.5	1.75	3.25	14780	3580	23	19	24	33	<3
		19.75	2.40	3.22	10950	3550	6	16	28	28	<3
		22.5	3.04	3.22	9870	3040	<1	2	44	41	<3
4NYG		16.5	2.89	3.19	17400	2210	15	6	38	44	12
		19.75	3.50	3.16	11890	2610	14	17	47	50	110
		22.5	3.82	3.14	12780	2540	12	28	37	43	390
5NYG		16.5	1.71	3.25	22650	3900	11	14	34	35	<3
		19.75	2.39	3.24	11800	3410	10	24	27	28	<3
		22.5	2.67	3.23	10500	2990	<1	5	34	34	<3
DG1		16.5	1.96	3.27	28000	6560	4	11	<2	<3	<3
		19.75	2.04	3.11	18680	3730	4	15	<2	<3	<3
		22.5	2.24	3.06	12810	5250	17	9	<2	<3	<3

All enzyme specific activity values are in nmol/min/mg protein. Cells were grown in CGM (Clostridial Growth Medium; Roos *et al.*, [8]) and inoculated from 24 h culture growth in a tube containing 10 mL CGM. The enzymes were assayed as described [7]. Abbreviations are as follows: PTB=Phosphotransbutyrylase; BK=Butyrate Kinase; BDH=Butanol Dehydrogenase; BAD=Butyraldehyde Dehydrogenase; CoAT=Acetoacetyl-coenzyme A:acetate/butyrate: coenzyme A transferase.

**Table 2.** Levels of metabolites in the broth of *C. acetobutylicum* cultures

Culture	Metabolite	Time		
		23 h	19 h	16 h
Wild type	Ethanol	5.0	4.6	4.6
	Acetone	6.8	4.5	2.4
	Acetate	26.7	41.5	38.2
	Butanol	34.6	29.2	23.2
1NYG	Butyrate	24.5	28.2	31.3
	Ethanol	3.1	2.8	1.7
	Acetone	0.4	0.3	0.4
	Acetate	36.5	29.4	30.0
5NYG	Butanol	6.9	5.3	2.5
	Butyrate	28.9	24.2	13.5
	Ethanol	3.0	2.6	1.9
	Acetone	0.3	0.3	0.2
M5	Acetate	26.0	25.2	31.2
	Butanol	7.4	5.1	2.6
	Butyrate	25.2	21.5	15.2
	Ethanol	2.4	2.6	2.5
4NYG	Acetone	<0.1	0.2	0.1
	Acetate	26.0	11.6	6.3
	Butanol	<0.1	<0.1	0.1
	Butyrate	21.9	21.1	16.0
DG1	Ethanol	5.4	4.4	3.4
	Acetone	1.2	1.0	0.5
	Acetate	36.1	38.6	28.3
	Butanol	29.0	20.5	10.5
4NYG	Butyrate	26.1	23.8	18.0
	Ethanol	2.1	2.0	18.0
	Acetone	<0.1	<0.1	<0.1
	Acetate	10.1	14.9	13.9
DG1	Butanol	<0.1	<0.1	<0.1
	Butyrate	32.9	29.9	22.5

The level of the metabolite is given in mM. The cultures were grown at 37°C in CGM and aliquots removed at the times indicated after inoculation. Cultures were those assayed in Table 1. Supernatant broths were acidified and analysed by GC. Concentrations were calculated by comparing integrated peak areas with those of standards.

no bands in the case of M5 and DG1. This gene cluster, located on a *ScaI* fragment of 6 kb, was analysed using three separate probes corresponding to the *ctfAB*, *adc* and *aad* genes. Those for *ctfAB* and *aad* are shown in Figure 1. A similar result for the *adc* probe was observed (data not shown). The 6 kb *ScaI* fragment found in *C. acetobutylicum* ATCC 824 for each of these three gene probes (*ctfAB*, *adc* and *aad*) was observed in the case of strains 1NYG, 4NYG and 5NYG. Experiments with other restriction enzymes (e.g., *NcoI* and *EcoRI*) digestions supported the results. Some of these have been reported [20].

## Discussion

A loss of solvent-forming ability of various *Clostridium acetobutylicum* strains has been noted and recently reviewed [3,4]. In *C. acetobutylicum* ATCC 824 four types of colonies were described by Adler and Crow

[4]: type I cells, which appeared as colonies with dark, dense centers with many large outgrowths and which exhibited vigorous solvent formation and sporulation; type II cells gave somewhat larger colonies with a less dark center and fewer smaller outgrowths, and few spores were obtained from these cells. Production of acetone and butanol was generally reduced to 25–50% of the normal values; type III cells gave larger colonies than type II cells with a mostly smooth perimeter and rare outgrowths and spores. They generally produced a lower amount of solvents than type II cells; type IV colonies were equal to or larger than type III colonies with indented edges and no outgrowths or spores and did not produce any detectable acetone or butanol. The type IV cells were larger than type I cells when analysed by a flow cytometric system [4].

Other reports have noted a correlation between high motility and ability to produce solvents with a non-motile phenotype associated with low solvent production [5,6]. A lack of motility could reduce the ability of the colony to form outgrowths on the surface of rich broth plate rapidly. In one strain, DP4-X [6], a non-motile, non-solvent producing mutant was found to have an altered flagellin protein. In this analysis, several variants of *C. acetobutylicum* ATCC 824 were isolated which produced reduced or undetectable levels of solvents. They were analysed and compared with those strains previously described [4,7]. From the overall results strains M5 and DG1 would appear to correspond well to the type IV strains of Adler and Crow [4]. While 1NYG and 5NYG produce almost no acetone and low levels of butanol, and in general appearance would most closely resemble characteristics of type III cells. The strain 4NYG would appear to be in an early stage of degeneration, most similar to one of the sporulation proficient type II isolates described by Adler and Crow [4]. The level of degeneration also seemed to correlate with motility.

In this analysis, we show that the most commonly lost enzyme activity is the CoA transferase (Table 1). This was also found in earlier work on strains arising from halogen substrate analogue selections [7]. In most cases, butyraldehyde dehydrogenase activity is also lost, while some butanol dehydrogenase activity is retained. The complete loss of butanol and acetone production and the lack of solventogenic enzyme activity correlated with a loss of ability to detect the presence of genes encoding the solvent-stage-induced enzymes CoAT, butyraldehyde dehydrogenase, and acetoacetate decarboxylase. These genes are arranged adjacent to each other in a closely packed cluster containing the *sol* (solvent) operon [12,21–24]. In the Southern hybridization experiments performed, all other genes examined are still retained including the butanol dehydrogenase genes, *bdhA* and *bdhB*. This result correlates with the ability of the degenerates to produce small amounts of ethanol and is consistent

with the role of the *aad* (*adhE*) gene which encodes aldehyde and alcohol dehydrogenase enzymatic activities in butanol production [13].

Mutants selected for resistance to allyl alcohol or halogen analogues or sporulation-deficient mutants have been reported to have lost several enzyme activities [25,26] and some of the mutants described by Clarke *et al.* [7] appear to have some attributes of degenerate strains. In the course of the selection or maintenance of these strains, degenerate strains or degenerate derivatives of the original strain may be isolated and propagated.

Previous suggestions that degenerate mutants contained solvent genes were based on preliminary hybridization experiments [6] using labeled whole plasmids and were undertaken before the plasmid was completely characterized. Subsequent analysis [24] indicated that hybridization due to extraneous sequences existing on the plasmid may have contributed similarly sized visible but fainter bands originally attributed to the solvent locus. Reinterpretation considering only small internal fragments derived solely from the acetoacetate decarboxylase encoding gene, *adc* [23,24] indicate the presence of little if any of this segment in DNA preparations of the earlier-analysed non-solvent producing strains.

The presence of genes encoding solvent formation enzymes in those strains exhibiting partial degeneration (analogous to type II or type III of Adler and Crow [4]) indicates a possible second type of degeneration or the presence of a mixture of degenerate and normal cells in the colony cell population. Perhaps such degeneration could be a result of a loss of a regulatory response or a mutation in or near the solvent locus affecting its expression. Such a possibility could potentially explain the strains which still produce one solvent but fail to produce both in normal quantities [7,25]. Such variants have been isolated through mutagenesis [7, C. Sass unpublished] as well as the strain 4NYG reported here.

The recent observation of a mutant of *C. beijerinckii* [27] that exhibits reduced degeneration might be explained (if the mechanism is similar between these two clostridia) as a mutation in a gene which is involved in genetic rearrangement via effects on replication or recombination processes. Such a mutation could affect the rate of loss of DNA elements. However, the ability of the degenerate strains derived from NCIMB 8052 to revert to solvent producers [28] would be hard to reconcile with a mechanism involving complete loss of the solvent locus and that result would therefore favor involvement of a regulatory mechanism.

The reports of complementation of strain M5 for acetone production using an artificial operon consisting of the *ctfAB* genes and the *adc* gene under control of the promoter for the acetoacetate decarboxylase

(*adc*) gene [16] suggest that essential positive regulators are not completely absent in this degenerate strain. This idea is also supported by the enzyme expression and butanol production found in M5 bearing a plasmid containing the *aad/adhE* gene encoding the aldehyde-alcohol dehydrogenase [13]. These findings indicate that when a degenerate strain, which seems to have lost the genes for solvent production, is provided with solvent-stage genes on a plasmid, the solvent-stage genes are expressed. Such a result suggests that the essential regulatory apparatus for expressing solvent-stage genes still exists in the degenerate strains. This result is consistent with our results indicating a loss of these genes in the complete (type IV) degenerates rather than a regulatory alteration.

In our analysis we have not defined the extent of the DNA lost in the degenerate mutants. In some bacterial systems large elements have been found to be lost or rearranged in the normal course of shift from one growth stage to another [29–31]. Such analysis of the mechanisms and extent of the genetic alteration will depend on the further analysis of the overall clostridial genome and its DNA interacting proteins.

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