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Analysis of Tn*916*-induced mutants of *Clostridium acetobutylicum* altered in solventogenesis and sporulation

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SUMMARY

The conjugative transposon Tn*916* was used for mutagenesis of *Clostridium acetobutylicum* ATCC 824. Tetracycline-resistant mutants were screened for loss of granulose synthesis and five classes of granulose mutants, that contained single transposon insertions, were identified on the basis of altered solvent production. Class 1 mutants did not make acetone or butanol, lacked activity of enzymes induced during solventogenesis, and did not sporulate, indicating that they are regulatory mutants. The class 2 mutant strains also did not produce acetone but did form small amounts of butanol and ethanol, while the class 3 mutants produced low amounts of all solvents. Class 4 and 5 mutants produced essentially the same or higher amounts of solvents than the parent strain. Transposon insertions in the class 1 mutants were used as markers for in vitro synthesis of flanking chromosomal DNA using Tn*916*-specific primers. The DNA fragments were labeled to produce specific probes. Transposon insertion sites in the chromosomes of 13 different class 1 regulatory mutants were compared by hybridization of the specific probes to Southern blots of restriction endonuclease-digested parental chromosomal DNA. Insertions in two mutants appeared to be in the same region of the chromosome. These results predict that multiple regulatory elements are required to induce solvent production and sporulation.

INTRODUCTION

The production of acetone and butanol using *Clostridium acetobutylicum* was one of the first large-scale industrial fermentation processes ever developed [1]. The fermentation continued to be the major source of acetone and butanol until after the Second World War when increasing substrate costs and new petroleum-based chemical processes rendered the biological process less competitive. Recent research has helped broaden understanding of the genetics, biochemistry, and physiology of *C. acetobutylicum* [27,30]; however, the molecular mechanisms that regulate solventogenesis and differentiation in this organism are still not well understood.

Strains of *C. acetobutylicum* which have low solvent yields usually sporulate poorly. The relationship between the loss of solvent production and the loss of spore formation has been investigated in batch cultures and the formation of morphologically distinct clostridial forms has been associated with the shift from acid production to solventogenesis [15,18]. The correlation between solvent production and sporulation rests primarily on the selection of *spo* (sporulation) and *cls* (clostridial) mutants following chemical mutagenesis. The early and late *spo* mutants develop normal clostridial forms, granulose, capsules, and produce solvents but do not form spores. The *cls* mutants have been identified as phase-dark rods which do not produce clostridial forms, granulose, capsules, solvents, or endospores [18].

The enterococcal conjugative transposons Tn*916* [11], Tn*925* [5], and Tn*1545* [9] have been transferred to *C. acetobutylicum* in filter-mating experiments using *Enterococcus* and *Bacillus* donors. Transfer was from a chromosomal location in the donor [16,36], or occurred when the transposon was carried on a conjugative plasmid [2,19]. Insertion into multiple chromosomal sites in *C. acetobutylicum* has been demonstrated for Tn*916* and potential regulatory mutants of *C. acetobutylicum* have been isolated [3,19].

We report here, that following mutagenesis with transposon Tn*916*, presumptive regulatory mutants of *C. acetobutylicum* were isolated. These mutants, selected as granulose-negative and sporulation-negative, fell into five classes based on their ability to induce solvent production. The transposon insertion was used as a marker for PCR (polymerase chain reaction) amplification of flanking chromosomal DNA. The DNA fragments amplified from 13 mutants that produced no solvents, were labeled as probes for hybridization experiments in order to roughly map and compare the insertion sites. A preliminary account of some of this data was reported [20].

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1 along with their relevant phenotypic markers or genotype. *C. acetobutylicum* strains were routinely grown

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TABLE 1

Bacterial strains and plasmids[a]

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
Escherichia coli		
CG120	Ap^r, Tet^r (pAM120)	[14]
Enterococcus faecalis		
CF180	Ery^r, Tet^r (pAM180)	[13]
Clostridium acetobutylicum		
824	wild-type strain	ATCC[b]
109	Tet^r (Tn*916*), Gra^-, Sol^-, Spo^-	This study
115	Tet^r (Tn*916*), Gra^-, Spo^-	This study
118	Tet^r (Tn*916*)	This study
154	Tet^r (Tn*916*), Gra^-, Spo^-	This study
158	Tet^r (Tn*916*), Gra^-, Sol^-, Spo^-	This study
163	Tet^r (Tn*916*), Gra^-, Ace^-	This study
173	Tet^r (Tn*916*), Gra^-, Sol^-, Spo^-	This study
174	Tet^r (Tn*916*), Gra^-, Sol^-, Spo^-	This study
179	Tet^r (Tn*916*), Gra^-, Sol^-, Spo^-	This study
202	Tet^r (Tn*916*), Gra^-, Sol^-, Spo^-	This study
212	Tet^r (Tn*916*), Gra^-	This study
220	Tet^r (Tn*916*), Gra^-, Sol^-, Spo^-	This study
221	Tet^r (Tn*916*), Gra^-, Ace^-, Spo^-	This study
224	Tet^r (Tn*916*), Gra^-, Sol^-, Spo^-	This study
229	Tet^r (Tn*916*), Gra^-, Spo^-	This study
231	Tet^r (Tn*916*), Gra^-, Sol^-, Spo^-	This study
233	Tet^r (Tn*916*), Gra^-, Sol^-, Spo^-	This study
234	Tet^r (Tn*916*), Gra^-, Sol^-, Spo^-	This study
240	Tet^r (Tn*916*), Gra^-, Spo^-	This study
243	Tet^r (Tn*916*), Gra^-, Sol^-, Spo^-	This study
244	Tet^r (Tn*916*), Gra^-, Sol^-, Spo^-	This study
254	Tet^r (Tn*916*), Gra^-, Spo^-	This study
Plasmids		
Escherichia coli		
pAM120	Ap^r, Tet^r (Tn*916*)	[14]
Enterococcus faecalis		
pAM180	Ery^r, Tet^r (Tn*916*)	[13]

[a]Ap^r, ampicillin-resistant; Ery^r, erythromycin-resistant; Sp^r, spectinomycin-resistant; St^r, streptomycin-resistant; Tet^r, tetracycline-resistant; Ace^-, no acetone produced; Gra^-, no granulose produced; Sol^-, no acetone or butanol produced; Spo^-, no endospores formed.

[b]American Type Culture Collection, Rockville, Maryland.

in an anaerobic chamber in a yeast extract medium (YE) and YE with 1.5% agar for plating as described previously [31]. *Clostridium* strains were maintained as spore stocks by plating on YE agar plates or were stored by freezing at −70 °C in YE broth that contained 10% (v/v) glycerol. For the preparation of chromosomal DNA, *C. acetobutylicum* strains were grown overnight in clostridium basal medium (CBM) [24]. Filter matings using *Enterococcus* donors and *Clostridium* recipients (see below for procedure) were incubated on Reinforced Clostridial Medium (RCM) (Difco Laboratories, Detroit, MI, USA) agar plates before plating on minimal medium (MM) agar plates for selection of transconjugants. The MM was that used by Oultram and Young [25], with the following additions per liter: asparagine, 1.0 g; cysteine HCl, 0.5 g; para-amino benzoic acid, 1 mg; thiamine HCl, 1 mg; and biotin, 12 μg. *Enterococcus faecalis* strains were grown aerobically at 37 °C in Todd Hewitt Broth (THB) (Difco Laboratories). Prior to use as the donor strain in conjugation experiments, *Enterococcus* was grown in an anaerobic chamber overnight at 37 °C. *E. faecalis* strains were stored by freezing at −70 °C in THB broth that contained 10% (v/v) glycerol. *Escherichia coli* strains were routinely grown aerobically in Luria-Bertani (LB) medium [32], and were stored by freezing at −70 °C in LB broth that contained 10% (v/v) glycerol. Antibiotics were added as required to growth and selection media in the following

concentrations for respective organisms: *C. acetobutylicum*, 20 μg erythromycin (ery) ml^{-1}; 10 μg tetracycline (tet) ml^{-1}; *E. faecalis*, 20 μg ery ml^{-1}, 10 μg tet ml^{-1}; *E. coli*, 50 μg ampicillin (ap) ml^{-1}, and 5 μg tet ml^{-1}.

Plasmid and chromosomal DNA isolation and manipulation

Molecular biology reagents were obtained from Boehringer Mannheim (Indianapolis, Ind, USA), New England Biolabs (Beverly, MA, USA), and Life Technologies (Gaithersburg, MD, USA) and were all used according to the manufacturer's instructions with buffers provided by the manufacturer. Plasmids were isolated from *E. coli* using Qiagen columns (Qiagen, Inc., Chatsworth, CA, USA) according to the manufacturer's instructions. Qiagen columns were also used to isolate plasmids from *E. faecalis* with slight modifications of the manufacturer's instructions. Lysozyme was added to buffer P1 to a final concentration of 1 mg ml^{-1} and mutanalysin (Sigma Chemical Co., St Louis, MO, USA) was added to a final concentration of 100 units ml^{-1}. Samples were incubated for 1 h at 37 °C in reagent P1 before adding reagents P2 and P3 and continuing with the column elution of plasmid DNA as per the manufacturer's directions. Chromosomal DNA was isolated from *Clostridium* strains using a modification of a previously published technique [8]. During treatment of cells with lysozyme to produce protoplasts, heat-treated RNase A was added to 100–200 μg ml^{-1}. The DNA content of each sample was determined by adding ethidium bromide and the fluorescence intensity was compared with samples of known DNA [32].

Filter mating, selection of tetracycline-resistant mutants, and screening for granulose production

Filter mating was performed in an anaerobic chamber, essentially as described by Oultram and Young [25], using *E. faecalis* strain CG180 as the donor and *C. acetobutylicum* strain ATCC 824 as the recipient. *E. faecalis* CG180 contained the plasmid pAM180 which consisted of the erythromycin-resistant conjugative plasmid pAM81 into which the tetracycline-resistant conjugative transposon Tn*916* had spontaneously inserted [11]. Briefly, 1 ml of the donor strain, that had been grown overnight to stationary phase, was mixed with 4 ml of the recipient strain, at mid-exponential growth, to give a donor : recipient ratio of about 2 : 1. The cell mixture was deposited on a 2.5-cm diameter Millipore® filter membrane (0.45-μm pore size, Millipore Corp., Bedford, MA, USA) using a syringe and Swinnex® disc filter holder (Millipore Corp.). The filter membrane was placed cells down on a RCM agar plate followed by anaerobic incubation at 37 °C for 4 h. The bacteria were recovered from the filter by briefly vortexing the filter in 5 ml of anaerobic holding buffer (HB) made up of 1 mM magnesium sulfate and 25 mM potassium phosphate, pH 7.0. Transconjugants were selected by plating 0.2 ml of the cells in HB onto MM agar plates that contained either 20 μg ery ml^{-1} (MMery), 10 μg tet ml^{-1} (MMet), or both antibiotics (MMerytet). The MM selection plates, which the donor *E. faecalis* strain was not able to grown on, were observed for the appearance of tetracycline-resistant (Tet^r) or erythromycin-resistant (Ery^r) colonies. Transconjugants were rarely observed when selection plates included both antibiotics. Tet^r colonies were transferred to MMtet and MMerytet plates, and the Ery^sTet^r colonies were selected and transferred to MMtet plates. Granulose-negative (Gra^-) mutants were identified by staining with iodine vapors after growth on YEtet plates that contained 4% (w/v) sucrose which enhances granulose formation [28]. The Tet^r, Gra^- *C. acetobutylicum* transconjugants that were identified were stored by freezing at −70 °C in YE plus 10% (v/v) glycerol for subsequent characterization of solvent production, sporulation, and Tn*916* insertions.

DNA amplification

Chromosomal DNA was isolated from the transposon mutants and chromosomal fragments that flanked Tn*916* insertions were amplified using an inverse PCR technique (PCR is the subject of US Patent Numbers 4 683 195 and 4 683 202 granted to Cetus Corporation) with the divergent primers Tn*916*tet and Tn*916*L2 [35]. Tn*916*L2 primed extension into the unknown region of *Clostridium* chromosomal DNA that flanked the left end of Tn*916*. The other primer, Tn*916*tet, extended in the opposite direction from Tn*916*L2 and annealed to the 5′ side of the *Hin*dIII site within the *tet*M gene. The sequence for the primer Tn*916*tet, 5′CGC TTC CTA ATT CTG TAA TCG 3′, was taken from the nucleotide sequence of the *tet*M gene of Tn*916* [4], and the sequence for the primer Tn*916*L2, 5′CGA GAA CAC ATA GAA TAA GGC 3′, was taken from the nucleotide sequence of the left end of Tn*916* [7]. The primer sequences were determined on the basis of calculated melting temperatures and the absence of hairpin loop structures using the primer optimization software Oligo™ 4.0 (National Biosciences, Inc., Plymouth, MN, USA). The primers were produced by the Microchemical Facility, University of Minnesota.

One microgram of chromosomal DNA was digested to completion using 10 units of *Hin*dIII in a 20-μl final volume. The digested DNA was extracted once with phenol : chloroform and once with chloroform, and was then precipitated using 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol. The precipitated DNA was centrifuged, washed once with 70% ethanol, dried, and then resuspended in 100 μl of TE to give a final concentration of 10 ng DNA per μl. Ten nanograms of the DNA (1 μl) were ligated overnight at 16 °C using 2 units of T4 DNA ligase in a 100-μl reaction volume to give a final concentration of 0.1 ng DNA ml^{-1}. The very dilute DNA concentration was used to promote circularization of individual fragments and some of the circular products included a fragment of Tn*916* with the primers now oriented to converge on the flanking *Clostridium* chromosomal DNA. The ligation products were precipitated, washed once, dried, and resuspended in 10 μl of TE for a final DNA concentration of 1 ng μl^{-1}. One nanogram (1 μl) of the ligation product was used as template in a 50-μl amplification reaction that contained: 25 pmol each of the primers Tn*916*L2 and

Tn*916*tet; 200 μM each of dATP, dTTP, dCTP, and dGTP; 1.5 mM magnesium chloride; and 2.5 units of *Taq* polymerase (Boehringer Mannheim Biochemicals). The DNA was amplified for 30–35 cycles, in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT, USA), that consisted of: heating to 94 °C for 3 min followed by addition of the polymerase (this technique minimized background amplification reactions); 5 cycles of denaturation of 94 °C for 30 s, primer annealing at 54 °C for 30 s, and extension at 72 °C for 1 min; 25–30 cycles of denaturation at 94 °C for 30 s, primer annealing at 49 °C for 30 s, and extension at 72 °C for 1 min; and a final extension for 10 min at 72 °C. Control samples included wild-type ATCC 824 chromosomal DNA and no added chromosomal DNA. One to 5 μl of the completed reaction was electrophoresed through a 3% or 4% agarose gel and products were visualized by UV-transillumination after staining with ethidium bromide. Bands of interest were isolated by removing a plug of the agarose gel with a sterile Pasteur pipette. Gel plugs were melted by heating to 95 °C for 10 min in 1.0 ml of TE and 1 μl of the melted gel plug was used in a second amplification reaction.

Probe labeling and hybridization

DNA probes that were used in hybridization experiments were labeled with digoxigenin-dUTP by either random primed labeling with the Genius™ nonradioactive DNA labeling kit (Boehringer Mannheim Biochemicals) following the manufacturer's instructions, or by incorporation into DNA during exponential amplification [17]. Amplified hybridization probes were made by recovering inverse PCR-amplified fragments from 3% agarose gels and reamplifying them to incorporate digoxigenin-labeled dUTP into the product. Thirty percent of the dTTP in the reamplification reaction was replaced with digoxigenin-dUTP to achieve sufficient labeling for successful detection. Transfer of DNA fragments from agarose gels to MagnaGraph™ nylon hybridization membranes (Micron Separations, Inc., Westborough, MA, USA) was done according to recommendations for using the Genius™ nonradioactive DNA labeling kit. Membranes were used either immediately for hybridization experiments or were stored at 4 °C in a heat-sealable pouch.

Determination of fermentation products

C. acetobutylicum Tet^r transconjugants, and the wild-type parent, were grown in 20 ml of YE that contained 5% (w/v) glucose; Tet^r strains were grown with 2 μg tet ml^{-1} added to the medium. After 72 h of growth at 37 °C the cells were pelleted by centrifugation and the supernatant medium from each culture was extracted for the determination of solvent and acid concentrations, or the supernatant medium was frozen at −20 °C for later analysis [31]. Gas chromatographic analyses were performed as previously described [8].

Preparation of cell extracts and enzyme assays

Cell extracts were prepared by ultrasonic treatment as previously described [31]. Aliquots of the cell extracts were flash frozen in an ethanol-dry ice bath and were stored at −70 °C until analyzed. Protein content of the samples was determined by a modification of the Bradford technique using the Bio-Rad protein reagent (Bio-Rad Laboratories, Melville, NY, USA) and following the manufacturer's instructions.

Coenzyme A (CoA)-dependent butyraldehyde dehydrogenase (BAD) was assayed for enzyme activity in the reverse direction to reduce NAD^+ using a procedure similar to that used by Yan et al. [37]. Reaction mixtures were 1.0 ml in total volume and consisted of: 50 mM potassium 2-(*N*-cyclohexylamino)ethane sulfonate (CHES) buffer, pH 9.0; 3 mM NAD^+; 0.5 mM CoA, reduced form; 1 mM dithiothreitol; 100 μl of cell extract in buffer W; and 10 mM butyraldehyde. Reaction mixtures were preincubated for 30 min at room temperature before reactions were initiated by adding butyraldehyde. The rate of NADH formation at 340 nm was measured using a LKB Biochrom Ultrospec Plus spectrophotometer (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ, USA) with a kinetics program. The extinction coefficient used for NADH at 340 nm was $\varepsilon = 6300\ M^{-1}\ cm^{-1}$ and one unit of BAD activity was defined as the formation of 1 μmol of NADH per minute.

Acetoacetyl-coenzyme A : acetate/butyrate : coenzyme A-transferase (ACT) was assayed in the direction of conversion of butyrate to butyryl-CoA by monitoring the decrease in absorbance at 310 nm as an indication of the disappearance of the enolate form of acetoacetyl-CoA [33]. Reaction mixtures were 1.0 ml in total volume and consisted of: 100 mM Tris-HCl buffer, pH 8.1; 15 mM magnesium chloride; 0.1 mM acetoacetyl-CoA; 100 mM potassium butyrate, pH 8.1; and 100 μl of cell extract. The extinction coefficient used for the metal chelate of acetoacetyl-CoA at pH 8.1 and 15 mM Mg^{++}, at 340 nm was $\varepsilon = 15\ 500\ M^{-1}\ cm^{-1}$ [34]. One unit of ACT activity was defined as the disappearance of 1 μmol of the enolate form of acetoacetyl-CoA per minute. Before assaying for ACT activity, the crude extracts were treated by passing them through Bio-gel P10 columns (100–200 mesh) (Bio-Rad Laboratories) to remove components that interfered with the assay [26]. The P10 matrix was hydrated in 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (pH 7.2) that also contained 20% glycerol (v/v) and 1 μg leupeptin ml^{-1} (Sigma Chemical Co.). Crude extract (400 μl) was loaded onto a P10 column (1 ml total bed volume), and protein was eluted with the hydrating buffer.

Spore counts and germination

C. acetobutylicum strains were evaluated for spore formation by determining the percent sporulation of 2- to 4-week-old colonies on YE agar plates. Cells were removed from the plates, mixed into sterile distilled water, and counted microscopically using a Petroff-Hausser bacteria counting chamber (Hausser Scientific, Blue Bell, PA, USA).

Germination ability was assessed by mixing 2- to 4-week-old colonies from YE agar into 0.5 ml of sterile distilled water and heating the samples to 80 °C for 2 min. This treatment killed any vegetative cells that may have remained

and enhanced germination of any spores, after adding YE medium and observing for outgrowth over 48 h.

RESULTS

Selection of tetracycline-resistant, granulose-negative mutants

The conjugative transposon Tn*916* was introduced into *C. acetobutylicum* ATCC 824 by transfer of the plasmid pAM180 in filter-matings using *E. faecalis* CG180 as the donor (see Materials and Methods for procedure). The maximum recovery of Tet^r transconjugants per filter mating was obtained with a donor : recipient ratio of about 2 : 1 and an incubation time of 4 h during filter-mating before spreading on selection plates. The best results (up to 10^{-3} Tet^r transconjugants per recipient) were obtained when the recipients were harvested during early exponential growth. When individual Tet^r transconjugants were tested for Ery^r, about one-half were Ery^s. This observation suggested that Tn*916* had excised from pAM180 and inserted into the *Clostridium* chromosome and that the plasmid that conferred Ery^r had been lost. The apparent instability of the hybrid plasmid pAM180 when in the *C. acetobutylicum* host, suggested the possibility of using filter mating with *E. faecalis* CG180 donors as a means to deliver Tn*916* into *C. acetobutylicum* ATCC 824 for mutagenesis studies.

Colonies identified as Tet^rEry^s were purified on MMtet agar plates to remove *Enterococcus* contaminants. The strains were then tested for granulose production by staining with iodine vapors. A total of 92 Gra^- mutants were chosen from 13 different filter matings. Neither Tet^s nor Gra^+ revertants of any of the mutant strains were ever recovered despite multiple transfers in YE broth that did not contain tetracycline.

*Identification of Tn*916 *chromosomal insertions in the mutants*

Samples of chromosomal DNA from the Gra^-, Tet^r transconjugants and from the wild-type parent were cut with *Hin*dIII which cleaves Tn*916* asymmetrically in the *tet*M gene. The fragments were separated by agarose gel electrophoresis, were transferred to hybridization membranes, and were hybridized to a Genius™ labeled pAM120 or DNA probe. The plasmid pAM120 is a chimeric molecule made up of the *Eco*RI F fragment of *E. faecalis* plasmid pAD1, into which Tn*916* had inserted, ligated into the single *Eco*RI site of the *E. coli* vector pGL101 [12]. No DNA fragments from the wild-type *C. acetobutylicum* ATCC 824 strain hybridized with the Tn*916*-containing probe (Fig. 1, lane 2). Each of the mutant strains, however, possessed two or more positive-hybridizing bands indicating that Tn*916* had inserted into the mutants' chromosomes. A single insertion of Tn*916* would result in two positive-hybridizing *Hin*dIII frgments (Fig. 1, lanes 3–15, and lanes 17–19) and more than two positive-hybridizing bands indicates that multiple transposon insertions had occurred (Fig. 1, lane 16). The variety of sizes of positive-hybridizing bands indicates that the transposon inserted into a number of different locations in the *C. acetobutylicum* chromosome. Of 92 Gra^-, Tet^r mutants that were analyzed, 44 contained single Tn*916* insertions while the remaining 48 strains contained two or more insertions.

Fermentation products of the mutants

Fermentation products of the Gra^-, Tet^r mutants were determined by gas chromatography of extracted fermentation broths and five classes, with respect to solvent production, were differentiated (Table 2). Mutant classes 1, 2, 3, and 5 had altered solvent production when compared to the wild-type parent strain, ATCC 824. Class 1 mutants did not form detectable amounts of butanol or acetone, and produced less ethanol (no more than 3 mM) than the parent strain ATCC 824. The class 2 mutant strains also did not produce detectable amounts of acetone, but did form butanol and ethanol, although in concentrations that were greatly reduced in comparison with the wild-type strain. Acetone, ethanol, and butanol production by the class 3 mutants was attenuated when compared to strain ATCC 824. In contrast to the other Gra^- classes, the single class 4 mutant produced the same amounts of solvents as the wild-type strain, and the class 5 mutant produced an excess of all three solvents. The levels of acetic and butyric acids in the fermentation broths generally were greater for the mutant strains that had impaired solventogenesis (classes 1, 2, and 3). The solvent-deficient mutants were less hardy when grown on YEtet agar plates and had to be transferred to fresh plates after about three days of growth or no viable cells were recovered. Of the 44 Gra^- mutants that appeared to have single Tn*916* insertions, 13 fell into class 1, 12 into class 2, 17 into class 3, and one each into classes 4 and 5.

Enzymatic analysis of the mutants

Enzymes essential for solvent production, such as BAD and ACT, normally are induced in batch cultures of wild-type *C. acetobutylicum* strains following accumulation of butyric and acetic acid and a drop in the pH]13,18,29]. Class 1 mutants lacked detectable BAD activity (Table 2). Nine of 13 class 1 mutants induced no detectable ACT activity, though four strains had reduced ACT activity. Class 2 mutants, which produced low levels of butanol, and no acetone, produced high levels of both enzymes. Mutants of classes 3, 4, and 5 produced solvents and induced BAD and ACT activities. However, mutant 240 (class 4) did not show BAD activity even though it produced butanol. Since mutant 240 contained two Tn*916* inserts, (Fig. 1), perhaps one of them caused altered stability of BAD.

Sporulation by the mutants

Sporulation is a developmental feature of *C. acetobutylicum* that correlates with solvents production [15]. While the wild-type parent produced nearly 90% spores, after extended growth on YE agar plates, none of the Gra^- mutant strains sporulated. When samples of the mutant strains were tested for viable spores by outgrowth following heat treatment, however, only one mutant, (strain 163), grew after 48 h.

Inverse PCR amplification of transposon-flanking chromosomal DNA and formation of probes

To characterize the transposon insertions in the class 1 mutants and to compare the insertion sites in the mutant

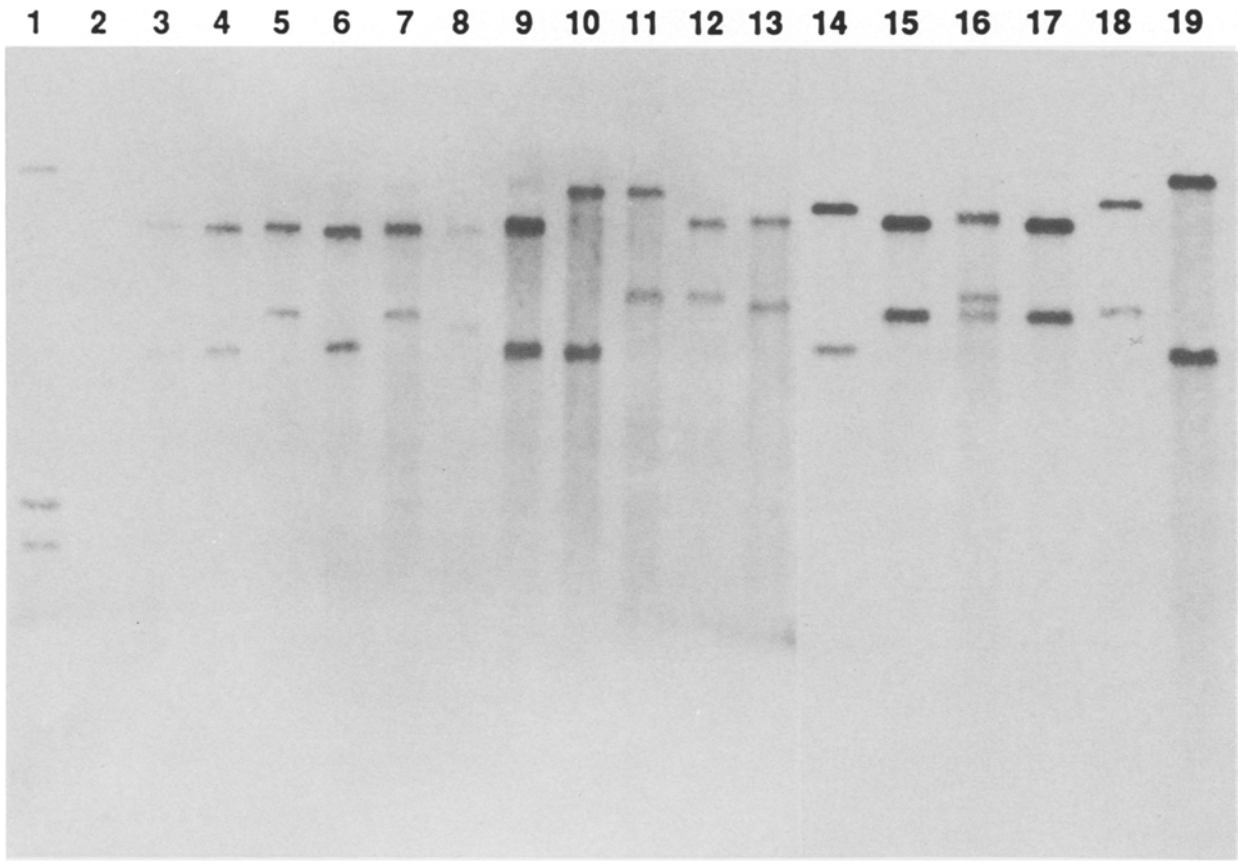

Fig. 1. Hybridization of *Hin*dIII-digested chromosomal DNA from wild-type and tetracycline-resistant mutant strains of *C. acetobutylicum* with probes for Tn*916* (obtained from pAM120. Lanes: 1, 10 ng of *Eco*RI-digested pAM120; 2, the wild-type 824 strain; 3–19, the mutant strains 109, 220, 224, 229, 243, 244, 163, 188, 206, 221, 227, 254, 118, 240, 115, 212, and 154, respectively. DNA size markers are in kilobase pairs.

strains, chromosomal DNA from the mutants was amplified using an inverse PCR procedure that allowed divergent Tn*916*-specific primers to converge across chromosomal DNA that flanked the left end of the transposon (see Materials and Methods). At least one fragment was amplified from each class 1 mutant strain, and fragment sizes ranged from about 150 bp (strain 109) to about 3000 bp (strain 174). Each fragment contained about 140 bp of Tn*916* sequence due to the positioning of the Tn*916*-specific primers. Amplification of chromosomal DNA from the ATCC 824 parent never resulted in fragment products, nor did amplification of a control that lacked chromosomal DNA. DNA fragments that were amplified from the mutants were labeled with digoxigenin either by incorporation of digoxigenin-dUTP into the fragments during a second amplification (strains 109, 202, 220, 229, 231(2), 233, and 234) or by random primed labeling of fragments collected on DEAE paper (strains 158, 173, 174, 179, 224, 231(1), 243, and 244(1 and 2).

Hybridization of probes to restriction enzyme-digested wild-type chromosomal DNA

Samples of parental ATCC 824 chromosomal DNA were digested separately with seven different restriction enzymes that preliminary experiments had shown to be medium to high frequency cutters of the *Clostridium* chromosome. Replicate Southern blots of panels of chromosomal DNA digests were hybridized with the probes derived from the mutant strains; results from four of the probes are shown in Fig. 2. In the absence of a physical map for *C. acetobutylicum*, a comparison of the Tn*916* insertions between the mutant strains was made by evaluating the unordered patterns of restriction fragments in the wild-type parent that hybridized to the mutant-derived probes (Table 3). The probes hybridized to restiction fragments with a wide range of sizes with very little similarity among most of the mutant strains, which indicated that each transposon insertion was unique. However, Southern blots that were hybridized with probes derived from mutants 224 and 244(2) suggested Tn*916* insertion sites are in the same region since the probes hybridized to the same size fragments from six of seven restriction digests (Fig. 3 and Table 3). The probes derived from mutants 233 and 234 also hybridized to similar restriction fragments (data not shown). In that case, for each restriction digest, a fragment that hybridized strongly to one probe comigrated with a fragment that hybridized weakly to the other probe.

TABLE 2

Product formation and enzyme activities of Tn*916*-induced granulose mutants of *C. acetobutylicum* ATCC 824

Class[a]	Strain	Fermentation products[b]					Enzyme activities[d]	
		Solvents (mM)			Acids (mM)		Units mg^{-1} protein	
		Acetone	Ethanol	Butanol	Acetic	Butyric	BAD[e]	ACT[f]
Parent	824	38	10	65	19	14	5.7	15.3
1	109	0[c]	2	0	31	55	nd[g]	1.1
	158	0	1	0	27	59	nd	nd
	173	0	1	0	29	45	nd	3.8
	174	0	1	0	20	45	nd	nd
	179	0	2	0	26	55	nd	nd
	202	0	2	0	33	60	nd	nd
	220	0	1	0	21	44	nd	nd
	224	0	2	0	14	26	nd	nd
	231	0	3	0	24	52	nd	3.1
	233	0	1	0	11	19	nd	nd
	234	0	2	0	14	21	nd	4.9
	243	0	2	0	35	65	nd	nd
	244	0	2	0	32	65	nd	nd
2	163	0	3	2	30	38	11.8	8.9
	221	0	2	3	28	45	3.8	17.3
3	154	4	4	6	27	38	1.2	8.5
	229	1	2	2	36	51	7.5	1.1
4	115	23	11	76	32	22	3.1	10.4
	240	20	7	56	39	25	nd	9.2
5	254	30	20	99	22	3	9.5	8.5

[a]Mutant classes were differentiated by comparing solvent production.
[b]Solvent and acid concentrations were determined by gas chromatographic analysis of extracted fermentation beers, after 72 h growth (see Materials and Methods section).
[c]The amount of solvent was either undetectable or less than 0.5 mM.
[d]Enzyme activities were determined using cell extracts prepared from cells of 20-h cultures after solventogenesis was induced (see Materials and Methods section). Activities are expressed as enzyme units per mg cell protein.
[e]One unit of BAD (butyraldehyde dehydrogenase) activity was defined as the formation of one μmol of NADH per min measured at 340 nm.
[f]One unit of ACT (acyl coenzyme A transferase) activity was defined as the disappearance of one μmol of the enolate form of acetoacetyl-CoA per min measured at 310 nm.
[g]Not detectable.

DISCUSSION

The use of Tn*916* as an insertional mutagen in several different organisms suggested the potential utility of this transposon in studies on the regulation of solventogenesis and sporulation in *C. acetobutylicum* [23]. Tn*916* was delivered into *C. acetobutylicum* ATCC 824 by transfer of the transposon-bearing conjugal plasmid pAM180 from an *Enterococcus* donor at frequencies per recipient up to 10^{-3} using selection for Tet^r that were similar to reports of transfer of several other transposon-bearing conjugal plasmids into *C. acetobutylicum* strains P262 and DSM792 [2,3]. Bertram and Dürre reported, however, that no transconjugants had been recovered following conjugal transfer of pAM180 into the DSM792 strain [2].

The selection of transposon-induced solventogenesis mutants reported here and by others demonstrate the potential for Tn*916*-mediated mutagenesis in *C. acetobutylicum*. The five classes of solventogenesis mutants isolated in this study were first screened for loss of granulose synthesis (Gra^-) and correspond to some of the categories of mutants that were isolated previously by selection for resistance to growth-inhibiting compounds [6,10,15,31]. Since granulose formation is an early event that precedes solventogenesis and sporulation, there was a greater probability of isolating pleiotropic regulatory mutants in our experiments rather than mutants of individual solvent genes.

In addition to being granulose-deficient, our class 1 mutants did not make acetone or butanol, were attenuated for ethanol synthesis, and did not sporulate. Clearly,

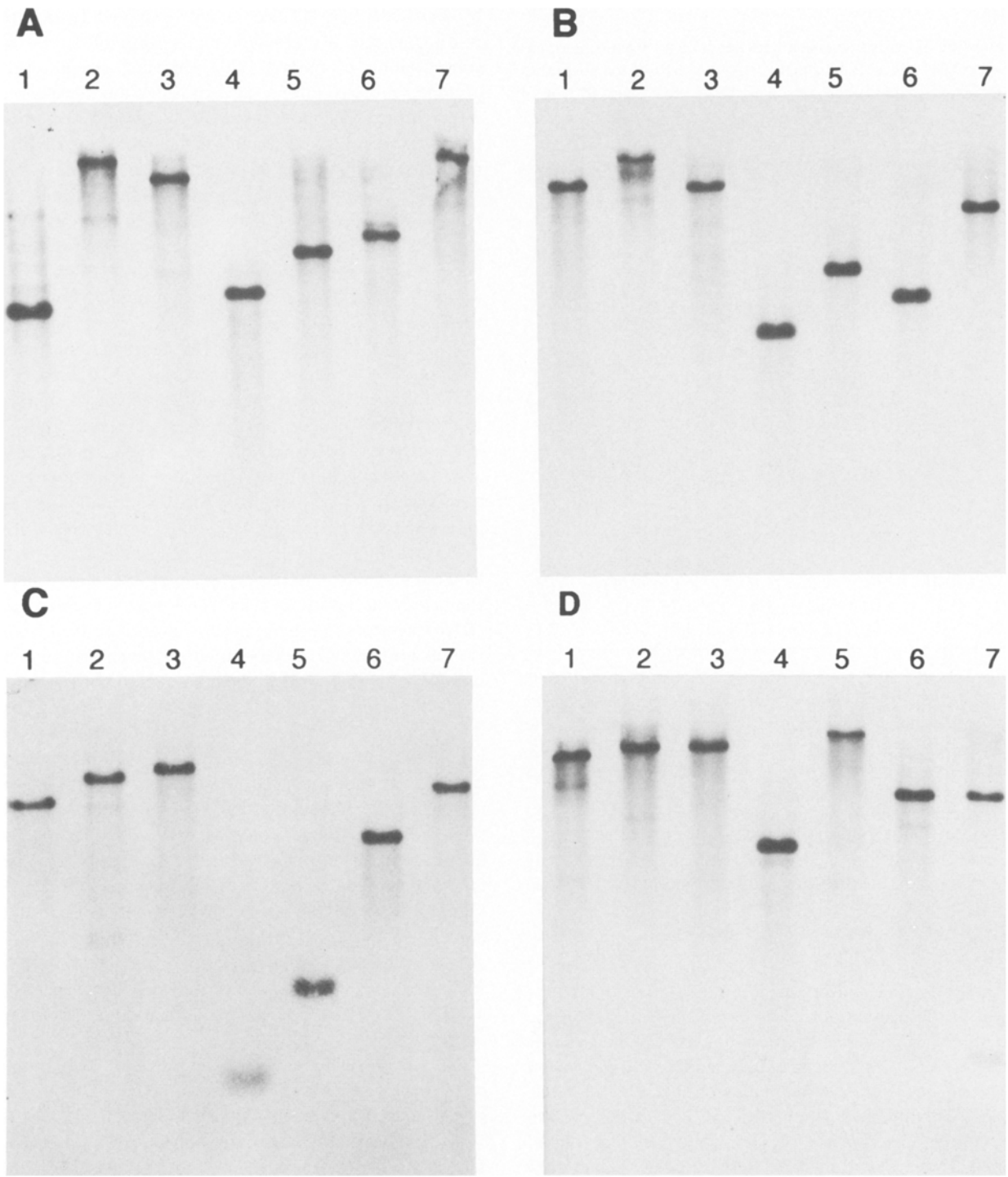

Fig. 2. Hybridization of *C. acetobutylicum* ATCC 824 chromosomal DNA restriction fragments with probes made from amplification products from Tn*916*-induced mutants. Samples of ATCC 824 chromosomal DNA were digested separately with seven different restriction enzymes, fragments were separated by agarose-gel electrophoresis, and were transferred to hybridization membranes by the Southern technique. Lanes 1–7 for each panel contain wild-type chromosomal DNA samples that were digested with, respectively, *Sca*I, *Sac*I, *Hpa*I, *Hin*dIII, *Eco*RV, *Eco*RI, and *Cla*I. Panel A was hybridized with a probe derived from mutant 173, panel B with a probe from mutant 179, panel C with a probe from mutant 220, and panel D with a probe from mutant 231 (1).

TABLE 3

Identification of restriction enzyme fragment bands from *C. acetobutylicum* ATCC 824 chromosomal DNA that hybridized to probes derived from mutant chromosomal DNA that flanked Tn*916* insertions

Probe[b]	Chromosomal DNA fragment size in preparations digested with:[a]						
	*Sca*I	*Sac*I	*Hpa*I	*Hin*dIII	*Eco*RV	*Eco*RI	*Cla*I
109	16.5[c]	4.3	8.5	<0.5	8.2	0.6	4.2
158	6.5	>23.1	5.5	1.2	12.2	6.4	23.0
173	3.7	>23.1	16.9	4.1	5.8	6.8	>23.1
174	8.8	>23.1	8.2	3.5	3.9	2.8	18.5
179	12.2	>23.1	12.0	2.6	4.5	3.6	11.0
202	2.8	20.7	>23.1	1.5	3.4	6.0	>23.1
220	11.0	16.5	22.0	0.5	1.8	6.6	14.5
224	8.4	11.0	4.3	1.7	4.7	9.7	17.1
229	4.0	21.0	1.6	<0.5	4.9	8.6	23.1
231(1)	13.5	14.9	15.4	4.5	>23.1	7.7	7.7
231(2)	5.7	>23.1	2.3	0.7	6.6	8.9	>23.1
233	>23.1	16.5	15.0	1.4	21.4	8.0	11.0
234	21.4	5.9	11.8	0.7	11.0	1.2	5.5
243	>23.1	15.0	2.2	1.6	6.0	22.0	17.8
244(1)	3.3	5.8	16.2	1.0	3.3	1.7	>23.1
244(2)	8.4	11.0	5.2	1.7	4.7	9.7	17.1

[a]One-μg samples of *C. acetobutylicum* ATCC 824 chromosomal DNA were digested with different restriction enzymes, the fragments were separated by agarose gel electrophoresis and transferred to hybridization paper. The blots were hybridized with digoxigenin-labeled probes that were deriven from mutant chromosomal DNA that flanked Tn*916* insertions.

[b]The probes were numbered according to the mutants that they were derived from. The probes were made from PCR-amplified chromosomal DNA fragments; for mutant strains from which more than one fragment was amplified the largest fragment was designated '(1)' and the next largest '(2)'.

[c]Fragment sizes were determined in kilobase pairs.

the regulatory mechanisms that normally control multiple features of the response of *C. acetobutylicum* to a changing environment are altered in these mutants. The enzymes BAD and ACT, which are normally induced during solvent production, were not detected in nine of the class 1 mutants. No BAD activity and low levels of ACT were observed in four mutants. The extracts of class 2 mutants, like four of the class 1 mutants, had ACT activity even though they produced no acetone. Possibly the acetoacetate decarboxylase enzyme (ADC), required for acetone formation, is not induced in any of these strains; however, this enzyme activity was not determined.

The variety of solvent classes we observed (Table 2) suggests several levels of control of solventogenesis induction. A model has been proposed for the shift to solventogenesis in *C. acetobutylicum* that includes two separate signals required for induction [13]. The model proposes that 'signal 1' is generated by a decreased ATP to ADP ratio caused by increased F_0F_1-ATPase activity required to maintain a transmembrane pH gradient in the face of an increasing concentration of butyric acid. This first signal results in acetone synthesis and net acid consumption as cells recycle acids through the acetoacetyl-CoA : acyl-CoA transferase enzyme. 'Signal 2', according to this model, responds to a need for additional acceptors of reducing equivalents to maintain the redox balance during glycolysis. Signal 2 could be generated by some sensor molecule, in redox equilibrium with NAD(P)H that results in the induction of enzymes for butanol synthesis. According to this model, class 1 mutants would be unable to generate or respond to both signals, while class 2 mutants would be deficient for signal 1 but still sensitive to signal 2, inducing enzymes that produce butanol. The remaining mutant classes 3, 4, and 5 were altered in the regulatory mechanism common to sporulation and granulose production, but not solventogenesis. Very low and very high levels of solvent production (classes 3 and 5) may indicate altered expression of genes that regulate transition-state functions just prior to sporulation as well as preventing sporulation itself as found for *Bacillus* [14].

Inverse PCR amplification of chromosomal DNA that flanked a Tn*916* insertion was used for recovering and characterizing insertion sites. The sizes of chromosomal DNA fragments that hybridized to probes derived from the various mutants were determined for seven different single retriction enzyme digests (Fig. 2). The unordered list of fragment sizes obtained with each probe serves as a unique 'signature' of restriction sites that surrounded Tn*916* insertions in the mutants and provides a rough means of mapping and comparing the insertions in the different mutants [22]. The most striking similarity of unordered fragment sizes was found using the probes from mutants 224 and 244 (Fig. 3). The hybridizing-fragment sizes matched for six of seven enzyme digests (except for *Hpa*I), which suggests that the insertion sites are within 1 kb of each other, in a 1.7 kb *Hin*dIII fragment, and might be as close as 200 bp. Amplification products from mutants 233 and 234 also have some sequence similarity since bands that hybridized to the probes comigrated in each restriction digest (data not shown).

Our data indicate that there are twelve or more chromosomal sites in *C. acetobutylicum*, disrupted by Tn*916* insertions, which normally express the developmental signals which induce solvent production, sporulation and related processes. These results are consistent with the observation that initiation of sporulation and other developmental events in *Bacillus subtilis* is a process requiring expression of 15 or more genes, [reviewed in 14]. We are in the process of cloning parental *C. acetobutylicum* chromosome fragments that have been identified by probes obtained from our Tn*916* mutants. Electrotransformation [21] of the *C. acetobutylicum* class 1 mutants with plasmids carrying the cloned wild-type DNA will be used to complement our mutants and investigate regulatory genes.

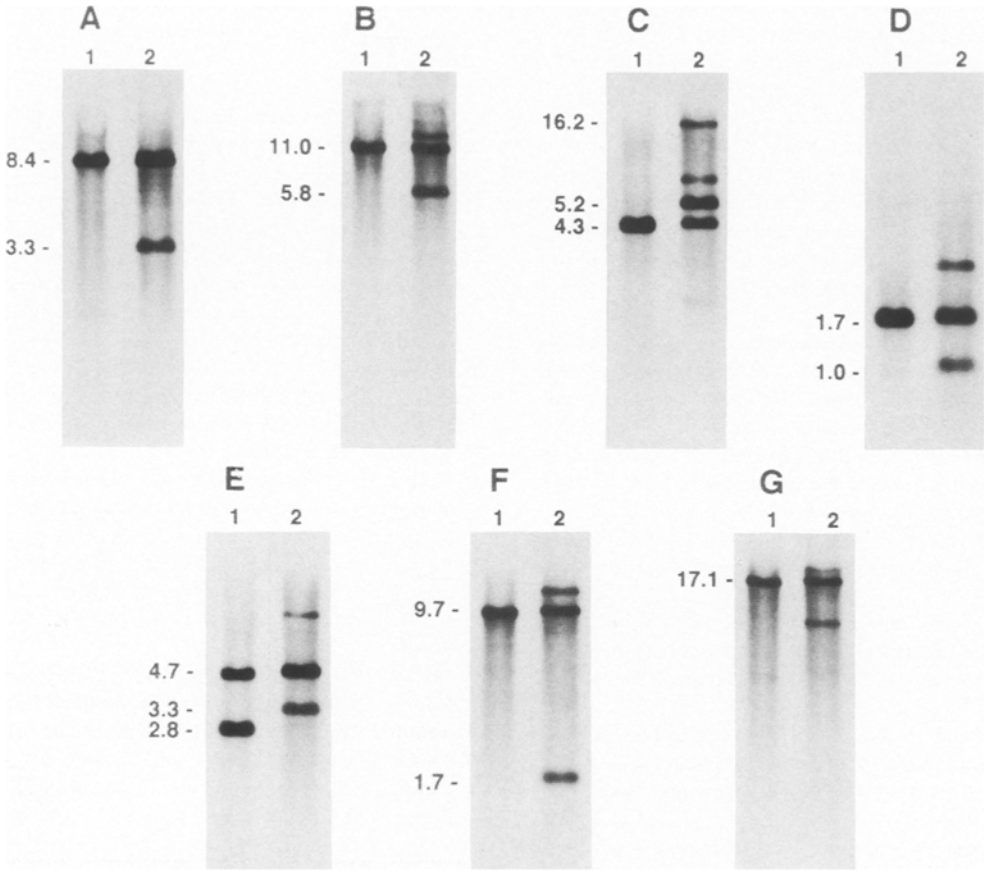

Fig. 3. Hybridization of *C. acetobutylicum* ATCC 824 chromosomal DNA restriction fragments with probes made from amplification products from mutants 224 and 244. Samples of ATCC 824 chromosomal DNA were digested separately with seven different restriction enzymes, fragments were separated by agarose-gel electrophoresis, and were transferred to hybridization membranes by the Southern technique. Panels A–G correspond to samples of 824 chromosomal DNA that were digested with, respectively, *Sca*I, *Sac*I, *Hpa*I, *Hin*dIII, *Eco*RV, *Eco*RI, and *Cla*I. In each panel, lane 1 was hybridized with a probe from mutant 224 and lane 2 was hybridized with the smaller probe from mutant 244. DNA size markers are given in kilobase pairs.

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