JOURNAL OF BACTERIOLOGY, Sept. 1997, p. 5442–5447 Vol. 179, No. 17
0021-9193/97/$04.00+0

The Genes for Butanol and Acetone Formation in *Clostridium acetobutylicum* ATCC 824 Reside on a Large Plasmid Whose Loss Leads to Degeneration of the Strain

EMMANUEL CORNILLOT,[1] RAMESH V. NAIR,[2]† ELEFTHERIOS T. PAPOUTSAKIS,[2] AND PHILIPPE SOUCAILLE[1]*

INSA, Centre de Bioingénierie Gilbert Durand, Complexe Scientifique de Rangueil, F-31077 Toulouse Cédex, France,[1] *and Chemical Engineering Department, Northwestern University, Evanston, Illinois 60208-3120*[2]

Received 14 March 1997/Accepted 16 June 1997

Degeneration is the process whereby *Clostridium acetobutylicum* ATCC 824 loses the capacity to produce acetone and butanol after repeated vegetative transfers or in continuous culture. Two degenerate mutants (M5 and DG1) of *C. acetobutylicum* ATCC 824 do not contain the four genes (*ctfA*, *ctfB*, *adc*, and *aad*) for acetone and butanol formation. Strain ATCC 824 contains a 210-kb plasmid (pSOL1) which is absent in M5 and DG1. pSOL1 carries the four acetone and butanol formation genes. A restriction map of pSOL1 was constructed by using *Apa*I, *Sma*I, *Sst*II, and *Nar*I digestions. M5 and DG1 could be complemented for acetone and butanol formation by expressing the corresponding genes (*ctfA*, *ctfB*, and *adc* for acetone; *aad* for butanol) on the plasmid. Degeneration of this strain thus appears to be the result of pSOL1 loss.

The *American Heritage Dictionary* defines the term "degenerate" as "having fallen or descended to a state below what is considered normal or desirable." Within the solventogenic clostridial community, the term "degenerate" is used to describe mutants which have one common trait, namely, that they produce more acids (butyrate and acetate) and little or no solvents (butanol, acetone, and ethanol). Degeneracy as applied to solventogenic clostridia appears to have no implications about the nature or origin of the mutation(s).

It has long been observed that upon serial subculturing or in continuous culture, solventogenic clostridia undergo a degenerative process which affects both morphological and physiological features and leads to mutant strains with impaired solvent formation capabilities (14). Weizmann's original strain of *Clostridium acetobutylicum* was found (20) to degenerate after 10 to 20 transfers when the transfers were made during the acidogenic phase of the fermentation. Solvent formation before the 50th transfer dropped to 0.5 to 2.0% of the starch fermented, and the spore formation capability was almost completely abolished. Attempts to recover "normal" solvent-producing strains from degenerate strains by culture with growth factors and various salts and by inducing sporulation were only partially successful. However, when transfers were made following sporulation and heat shocking, a solvent yield of 24.7% was retained after 150 transfers (more than 2 years).

Jones et al. observed that certain morphological and cytological changes, which could be correlated with growth and physiological changes, occurred in *C. acetobutylicum* P262 during solvent production (13). The number of swollen, cigar-shaped clostridial forms could be directly correlated with the production of solvents. Initiation of solvent production and clostridial-stage formation are essential for sporulation in different *Clostridium* species (12, 18). Thus, degenerate mutants would be expected to be asporogenous. However, the existence of an asporogenous strain which is a good solvent producer (17) should be noted, because it demonstrates that degeneracy (assumed here to imply impaired solvent formation ability) is not necessarily equivalent to an inability to form spores.

There has been an increased interest recently in mutants deficient in solvent formation (the so-called degenerate mutants), because complementation of these mutants provides the strongest possible evidence for the physiological role of novel genes which are related to solvent formation (27). Only a few degenerate mutants have been characterized in terms of in vitro enzymatic activities. The *C. acetobutylicum* ATCC 824 mutant M5 (4), wherein the three inducible enzymes butyraldehyde dehydrogenase (BYDH), acetoacetate decarboxylase (AADC), and acetoacetyl-coenzyme A:acetate/butyrate:coenzyme A-transferase (CoAT) are not detectable in vitro (4, 29), and consequently butanol and acetone are not produced, was isolated by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) mutagenesis. The spontaneous mutant DG1 (25), which lacks in vitro AADC (36) and BYDH activities (25) and does not produce butanol or acetone, was obtained through serial subculturing of strain ATCC 824 for several generations. Mutant M5 has been complemented for acetone (23) and butanol (27) formation by being made to express plasmid-encoded genes for AADC (*adc*) plus CoAT (*ctfA*, *ctfB*) and BYDH (*aad*), respectively.

Preliminary evidence for a possible mechanism for the degeneration process of *C. acetobutylicum* ATCC 4259 (the Weizmann strain) has been recently presented in a letter communication (5). This strain contains a 210-kb extrachromosomal element (pWEIZ) that carries the genes required for the final steps of acetone and butanol formation (5) and is lost in the three degenerate mutants. ATCC 4259 has been poorly studied in term of physiology and genetics, whereas ATCC 824 is now considered worldwide to be the type strain of *C. acetobutylicum* (16) and the model solvent-producing strain important in terms of fundamental research on strictly anaerobic bacteria. We therefore investigated the degeneration mechanism of this strain by molecular analysis of the wild-type and degenerate mutant strains (M5 and DG1). We found that mutants M5 and DG1 do not contain the genes of the solvent formation locus (*sol* locus, consisting of the *sol* operon [*aad*,

* Corresponding author. Phone: 33 561 55 94 52. Fax: 33 561 55 94 02. E-mail: soucaille@insa-tlse.fr.

† Present address: Dupont Central Research & Development, Experimental Station, Wilmington, DE 19880-0378.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
C. acetobutylicum strains		
ATCC 824	Wild type	ATCC[b]
M5	BYDH$^-$ AADC$^-$ CoAT$^-$ *spo*	4
DG1	BYDH$^-$ AADC$^-$ *spo*	36
Plasmids		
pAB	Ap^r MLS^r *adc*, *aad ctfA ctfB*	This study
pCAAD	Ap^r MLS^r *aad*	27
pDP2	Ap^r *adc ctfA ctfB*	30
pCOAT4	Ap^r *ctfA ctfB*	2
pFNK6	Ap^r MLS^r *adc ctfA ctfB*	23
pHXS5	Ap^r *aad*, *adc*, *ctfA ctfB orf2*	26
pJCace	Ap^r *ace* MLS^r	10
pMR1	Ap^r Kan^r	This study

[a] Abbreviations: *spo*, asporogenous; Ap^r, ampicillin resistance gene; *aad*, aldehyde/alcohol dehydrogenase gene; *ctfA*, *ctfB*, CoAT genes; MLS^r, macrolide, lincosamide, and streptogramin B resistance gene; *adc*, AADC gene; *orf2*, open reading frame 2, which encodes a putative repressor protein (Fig. 1).

[b] ATCC, American Type Culture Collection, Rockville, Md.

ctfA, *ctfB*] and *adc*), which are responsible for butanol and acetone production. We further show that, like the Weizmann strain, the parent strain ATCC 824 also contains a 210-kb plasmid (pSOL1) which carries the *sol* locus and that neither M5 nor DG1 contains this large plasmid. We also show that M5 and DG1 can be complemented for acetone and butanol formation by expressing the corresponding genes on plasmids. The present investigation makes a compelling case for the proposed mechanism of strain degeneration in this organism, namely, that degeneration of this strain is the result of pSOL1 loss.

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 summarizes all the bacterial strains and plasmids used in this study. *C. acetobutylicum* ATCC 824 was maintained as spores in synthetic medium (34) at −30°C. The spores were activated by heating at 80°C for 10 min. Recombinant clostridial strains were stored frozen in 15% (vol/vol) glycerol at −85°C or as single colonies on reinforced clostridial agar (pH 6.8) (RCA; Difco Laboratories, Detroit, Mich.). *C. acetobutylicum* was grown under anaerobic conditions at 37°C in 2XYTG (28), reinforced clostridial medium (RCM; Difco Laboratories), or clostridium growth medium (CGM) (31). Recombinant *C. acetobutylicum* cells (carrying a plasmid with a macrolide, lincosamide, and streptogramin B resistance [MLS^r] gene) were cultured in the above media supplemented with 40 μg of erythromycin per ml on plates and 100 μg of erythromycin per ml in liquid culture. For the 5-liter batch fermentations, 75 μg of clarithromycin per ml was used instead of erythromycin. Agar (Sigma Chemical Co., St. Louis, Mo.) at 15 g/liter was used in all plate preparations.

DNA isolation and manipulation. Agarose plugs of intact *C. acetobutylicum* DNA were prepared by the procedure of Wilkinson and Young (39). Restriction endonucleases were purchased from New England Biolabs (NEB), Beverly, Mass., and used as specified by the manufacturer. Contaminating RNA was removed from all DNA preparations with RNase Plus (5 Prime → 3 Prime Inc., Boulder, Colo.) as specified by the manufacturer.

PFGE. DNA pulsed-field gel electrophoresis (PFGE) was carried out in the contour-clamped homogeneous electric field mode with the Bio-Rad (Richmond, Calif.) apparatus (CHEF-DRII). For most experiments, a 0.5× Tris-borate-EDTA buffer (TBE) and a 1% (wt/vol) agarose (Bioprobe, Montreuil-sous-Bois, France) gel were used. The gel was run for 20 h at 200 V (6 V/cm) with a pulse time of 18 s, the buffer temperature was stabilized at 9 to 10°C, and concatemers of lambda DNA *c*I857 *ind*1 *S*am7 (NEB) were used as molecular weight standards.

DNA labeling and Southern blots. For Southern blots, the two probes on both sides of the *Nar*I restriction site of the *sol* operon locus (prepared from *Hin*dIII-*Nar*I-digested pCOAT4 and *Ban*II-*Nar*I-digested pDP2) (see Fig. 1) were purified from the agarose gel with the Geneclean II kit (Bio 101 Inc., La Jolla, Calif.) labeled by random priming with [α-^{32}P]dATP (Amersham, Les Ulis, France) and purified on a Bio-Gel P10 (Bio-Rad) column. Plasmid pHXS5 (26) was nick translated (32) with [α-^{32}P]dATP (NEN Research Products, Wilmington, Del.) by using a nick translation system (BRL Life Technologies Inc., Gaithersburg, Md.), and unincorporated radionucleotides were removed on a Select-D G-25 spin column (5 Prime → 3 Prime Inc.).

Southern blotting (35) was performed as previously described (30).

PCR. DNA fragments were amplified by PCR in a 100-μl reaction mixture as described previously (26) with total DNA from strains ATCC 824, M5, and DG1 as template DNA. A total of 15 cycles were performed in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.), with each cycle consisting of denaturation at 94°C for 1 min, annealing at 40°C for 1 min, and extension at 72°C for periods depending on the lengths of the DNA fragments that were amplified (2 min 15 s for the 1,979-bp DNA fragment containing the *aad* promoter region and *orf2*; 2 min 45 s for 2,619-bp DNA fragment containing *aad*; and 1 min for the 975-bp DNA fragment containing *adc*).

DNA fragments amplified by PCR. The DNA fragment containing *orf2* and the promoter region of *aad* was amplified by PCR with total DNA from strains ATCC 824, M5, and DG1 as templates. The upstream primer DAP-UP (5′-ATGGTCGGCGTGAATTCGTGAACAATTG-3′) and the downstream primer DAP-N (5′-TGCTGCCATTGCTGCAGTTCTAAAGATT-3′) on the complementary strand were used based on sequence information reported previously (26).

The *aad* structural gene was amplified by PCR with total DNA from strains ATCC 824, M5, and DG1 as templates. To achieve this, we used two previously described primers (26), the upstream primer PAAD-UP at the N-terminal end of *aad* and the downstream primer PAAD-DN on the complementary strand at the C-terminal end of *aad.*

The *adc* gene region was amplified by PCR with total DNA from strains ATCC 824, M5, and DG1 as templates. To achieve this, the upstream primer PADC-UP (5′-GAATTCATAAAAACACCTCCACATAAGT-3′) starting 240 bp upstream (29) of the N-terminal end of *adc* and the downstream primer PADC-DN (5′-TTACTTAAGATAATCATATATAACTTCA-3′) starting at the very C-terminal end of *adc* on the complementary strand were used.

Construction of plasmid pAB. The 5.9-kb plasmid pCADEX1 (26) containing the *aad* gene was linearized by *Acc*I digestion and made blunt ended with the DNA polymerase I large (Klenow) fragment (NEB) as specified by the manufacturer. The 7.0-kb plasmid pFNK6 (23) was digested with *Pvu*II, and the resulting 4.7-kb fragment, containing the *ace* operon (*adc*, *ctfA*, and *ctfB* genes), the MLS^r gene, and the gram-positive origin of replication, was purified from a 0.8% LMP agarose gel (SeaPlaque GTG; FMC, Rockland, Maine). The *aad*-containing fragment (5.9 kb) and the *ace* operon-containing fragment (4.7 kb) were ligated to yield the 10.6-kb plasmid pAB, which carries the *aad* and the *ace* operon in a convergent fashion.

Construction of plasmid pMR1. The *repL* gene from pIM13 (24) was introduced into pBluescript I SK (−) as a 0.9-kb *Cla*I-*Hin*dIII fragment to give plasmid pSC2. The kanamycin resistance cassette from pIC28 (37) was introduced into pSC2 as a 1.3-kb *Sma*I fragment to give plasmid pMR1. After methylation and introduction by electroporation into *C. acetobutylicum* DG1, pMR1 leads to resistance to kanamycin at concentrations up to 600 μg/ml.

Conjugation experiment. Filter matings between *C. acetobutylicum* ATCC 824 (pJCace integrant on pSOL1 [10]) and DG1 (pMR1) were performed by the procedure of Oultram et al. (28), with donor-to-recipient ratios ranging from 1:10 to 10:1. pJCace carries the *ace* operon (23) and a MLS^r gene. Selection for transconjugants was done on RCA plates with 40 μg of erythromycin per ml and 250 μg of kanamycin per ml.

RESULTS AND DISCUSSION

To investigate the molecular basis of degeneration in *C. acetobutylicum* ATCC 824, we have examined two distinct (25, 36) degenerate mutants of this strain. Neither mutant produces any detectable amounts of butanol or acetone, and both have been phenotypically stable since their isolation several years ago. The genes coding for the enzymes required for acetone and butanol production are clustered (7, 8, 26, 30) in the *sol* locus (Fig. 1). To check if these genes are still present in M5 and DG1, Southern hybridizations were carried out on *Nco*I-digested total DNA of DG1, M5, and ATCC 824 with a probe containing *orf2*, *aad*, *ctfA*, *ctfB*, and part of *adc* (Fig. 1). Hybridization signals were seen only with ATCC 824 DNA and the positive control (Fig. 2A). We confirmed these findings by attempting PCR amplification, from M5, DG1, and ATCC 824 DNA templates, of several DNA fragments from the *sol* locus: the 1,979-bp DNA fragment containing *orf2* (26) (Fig. 1) and the *aad* promoter, the 2,619-bp DNA fragment containing the *aad* gene (Fig. 1), and the 975-bp DNA fragment containing the *adc* gene (Fig. 1). The resulting bands were visualized in a 0.8% (wt/vol) agarose gel (Fig. 2B). Amplified fragments were detected for ATCC 824 but not for M5 or DG1. These results

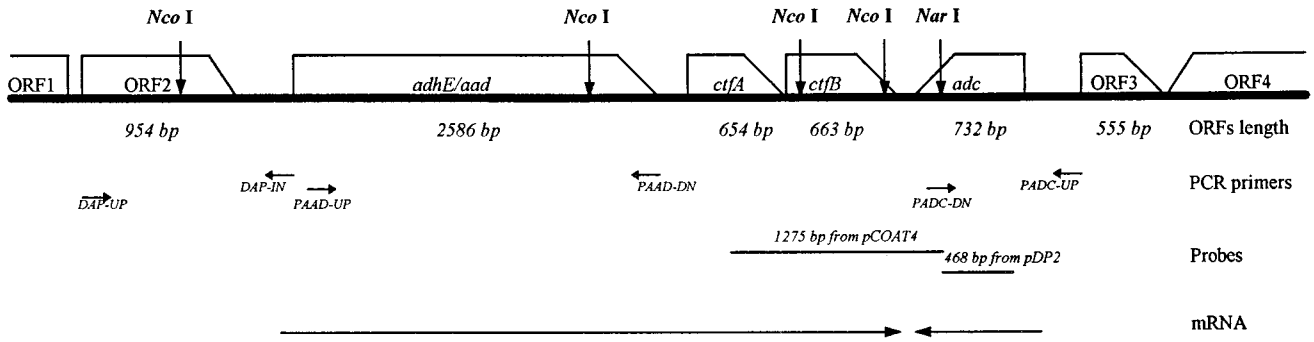

FIG. 1. The *sol* operon gene locus (ca. 9.5 kb). *adhE*/*aad* codes for a bifunctional aldehyde-alcohol dehydrogenase (7, 26). *ctfA* and *ctfB* code for the two coenzyme A transferase (7, 8, 30) subunits. *adc* codes for the acetoacetate decarboxylase (8, 30). The functions of ORF1 and ORF3 have not been identified. ORF4 shows some homology to the genes encoding α-amylases (8). *orf2* codes for a putative repressor protein (25). The positions of the different primers used for PCR amplification in Fig. 2B and the two probes, one on each side of the *Nar*I site, used for mapping pSOL1 in Fig. 6 are also shown.

show that M5 and DG1 contain neither the *sol* operon nor the *adc* gene.

To investigate this loss of large DNA segments from the two degenerate strains, DNA from ATCC 824, M5, and DG1 was prepared in agarose plugs and subjected to analysis by PFGE. For unrestricted DNA preparations, below a band of sheared chromosomal DNA, an extra band that migrates at 210 kb was observed for the wild-type strain but was absent in M5 and DG1 (Fig. 3A). This result is identical to the one observed for the Weizmann strain (5). We therefore decided to confirm the structure and topology of this extrachromosomal element. In PFGE, migration of supercoiled plasmids is independent of the switch time, while large relaxed plasmids do not enter the gel (1). To establish the nature of the 210-kb band, the PFGE conditions (amplitude and slope of the pulse time ramp) were varied, showing that this band always migrates at 210 kb (data not shown), excluding the possibility that it corresponds to a supercoiled plasmid and ascertaining that it is linear DNA. PFGE of DNA digested by *Sst*II, *Sma*I, or *Apa*I (Fig. 3B and C; results are presented for the *Sst*II and *Sma*I digests only) shows that the 210-kb band is of higher intensity than the band from unrestricted ATCC 824 DNA and there is no 210-kb band for either DG1 or M5. Except for this 210-kb band, the macrorestriction profiles of the three strains were identical. As large (>100-kb) relaxed circular DNA cannot enter the gel in PFGE (38), our data show that strain ATCC 824 harbors a 210-kb plasmid, which was absent in M5 and DG1. In our DNA preparations, this plasmid was present in its relaxed circular form and not as a supercoiled form, which was either absent or below the limit of detection. The plasmid was linearized by *Apa*I, *Sma*I, and *Sst*II. A small portion of this plasmid was linear in our uncut-DNA preparations. The 210-kb linear DNA was isolated from a preparative gel and cut in the agarose gel by *Sst*II. After PFGE, a broad smear with an average size of 90 kb was obtained (data not shown) which indicated that the linear form of the plasmid is the result of a nonspecific cleavage (or breakage).

To confirm the presence of a plasmid in ATCC 824, we used

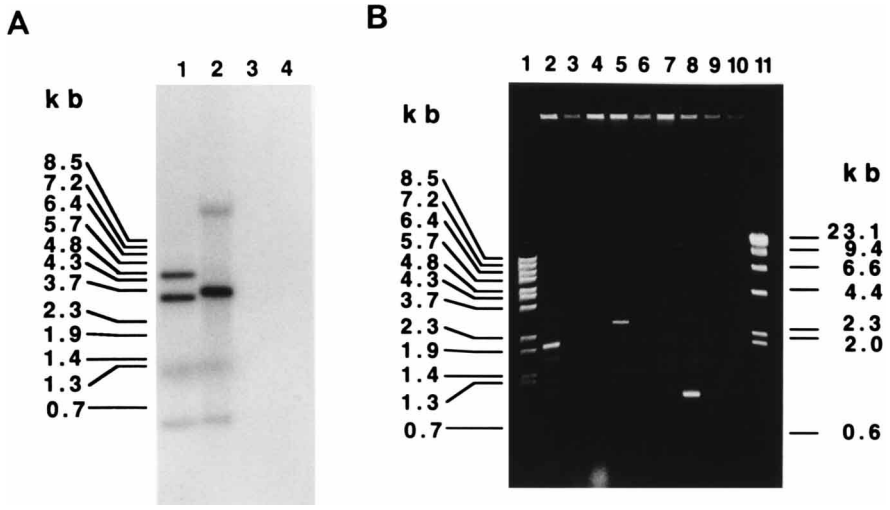

FIG. 2. Strains DG1 and M5 do not contain the genes for butanol and acetone formation. (A) Southern analysis of the *sol* operon locus in ATCC 824, M5, and DG1. Total DNA of ATCC 824 (lane 2), M5 (lane 3), and DG1 (lane 4) were digested with *Nco*I and hybridized with pHXS5 (26), which contains part of the *sol* operon locus going from the left end to the *Nar*I site of the DNA fragment shown in Fig. 1. *Nco*I-digested pHXS5 (lane 1) was used as a positive control. (B) PCR amplification from the *sol* operon locus. Amplification of a 1,979-bp (with primers DAP-UP and DAP-IN) DNA fragment containing the *aad* promoter region and *orf2* (lanes 2 to 4), a 2,619-bp (with primers PAAD-UP and PAAD-DN) DNA fragment containing *aad* (lanes 5 to 7), and a 975-bp (with primers PADC-DN and PADC-UP) DNA fragment containing *adc* (lanes 8 to 10) was performed by PCR from ATCC 824 (lanes 2, 5, and 8), M5 (lanes 3, 6, and 9), and DG1 (lanes 4, 7, and 10) total DNA, respectively, as templates (see Fig. 1 for oligonucleotide names). Postamplification samples were electrophoresed in a 0.8% (wt/vol) agarose gel. λ DNA size markers, either *Bst*EII digested (lane 1) or *Hin*dIII digested (lane 11), are shown alongside.

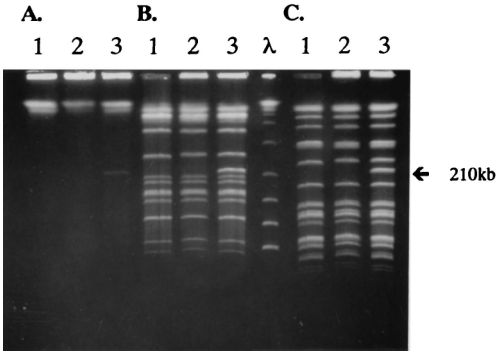

FIG. 3. Strain ATCC 824 contains an extrachromosomal element which is missing in DG1 and M5. Ethidium bromide-stained unrestricted total DNA (A) from M5 (lane 1), DG1 (lane 2), and ATCC 824 (lane 3) or total DNA digested by *Sst*II (B) or *Sma*I (C) after separation by PFGE is shown. Lane λ contains concatemers of lambda DNA *c*I857 *ind*1 *S*am7 (NEB) used as molecular size standards.

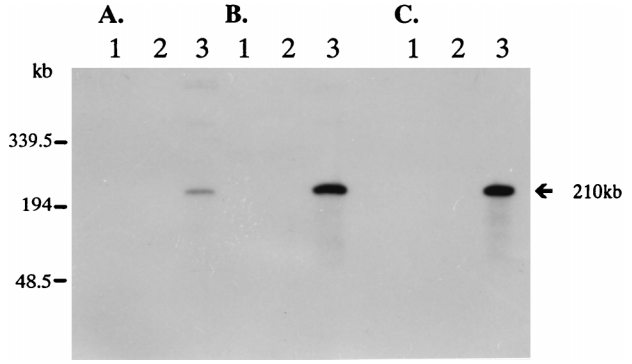

FIG. 5. The 210-kb plasmid carries the genes of the *sol* operon locus. Hybridization with pCOAT4 (which carries the *ctfA* and *ctfB* genes) of unrestricted total DNA (A) from M5 (lane 1), DG1 (lane 2), and ATCC 824 (lane 3) or total DNA digested by *Sst*II (B) or *Sma*I (C) after separation by PFGE is shown.

several methods (3, 6, 15) described for large-plasmid extraction. However, due to the high nuclease activity of this strain, we always obtained smears after conventional electrophoresis on agarose gels. To unambiguously confirm the presence of a relaxed circular plasmid in ATCC 824, we carried out the following experiments. DNA plugs from the three strains were digested by *Eag*I, an enzyme which does not cut the 210-kb DNA element (data not shown). In these digestions, the chromosomal DNA would be cut but the plasmid would remain circular in the plugs. PFGE was then performed on the digested plugs. Linear DNA fragments should be removed from the plugs, while the large relaxed circular plasmid should remain in the plugs. The plugs were then digested by *Sst*II, an enzyme which cuts once and linearizes the plasmid, and analyzed by PFGE. The results, presented in Fig. 4, show a single 210-kb band for ATCC 824 and no bands for either M5 or DG1, thus confirming the presence of a 210-kb circular plasmid in ATCC 824 and its loss in M5 and DG1.

In view of the finding that M5 and DG1 do not contain either the *sol* operon locus or the 210-kb plasmid, we used Southern hybridization on the gel in Fig. 3 to examine the possibility that this DNA element contains the genes of the *sol* operon locus. As a probe, we used the *ctfA* and *ctfB* genes (on pCOAT4) of ATCC 824 (2). A hybridization signal was obtained with the 210-kb linearized plasmid form of *C. acetobutylicum* ATCC 824 (Fig. 5), but no signal was obtained with either M5 or DG1 (Fig. 5). As this 210-kb plasmid carries the *sol* locus, it was named pSOL1. Based on the size and the genetic marker, pSOL1 should be very similar to pWEIZ (5). When the *bdhA* and *bdhB* genes were used as a probe, no hybridization signal was obtained with pSOL1, but in the three strains, the same restriction fragments gave a positive signals (data not shown), indicating that these genes are located on the chromosome.

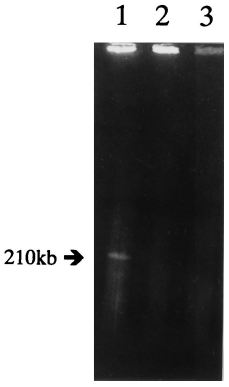

FIG. 4. Strain ATCC 824 contains a circular 210-kb plasmid which is missing in DG1 and M5. Total DNA plugs were digested by *Eag*I, linear fragments were then removed from the plugs by PFGE and treated with *Sst*II, and residual DNA fragments from ATCC 824 (lane 1), DG1 (lane 2), and M5 (lane 3) were separated by PFGE and stained by ethidium bromide.

A restriction map of pSOL1 was constructed with *Apa*I, *Sma*I, *Sst*II, and *Nar*I. We identified the *Apa*I, *Sma*I, and *Sst*II restriction sites by double digestion of total ATCC 824 DNA with the *ctfA* and *ctfB* genes as probes. One *Nar*I-cutting site was previously identified within the *adc* gene (Fig. 1). We developed a modified indirect end-labelling-based strategy (9, 33) with *Nar*I partial digestions and two probes, one from each side of the *Nar*I site of the *sol* operon locus. Specifically, partial *Nar*I digestions without and with a complete *Apa*I digestion were used to map these sites (Fig. 6A). Some of the digestion fragments in Fig. 6A could be seen only after overexposure of the film. The results are summarized on the plasmid map (Fig. 6B). The *Nar*I site between the *Sma*I and *Sst*II sites is cut preferentially, and partial digests of this site could rarely be obtained. It should be pointed out here that *Nar*I partial digestions always gave hybridizing bands of a maximal size of 210 kb, in agreement with the extrachromosal location of the *sol* locus. Consistent with our findings, it was recently reported (39) that in ATCC 824 the cluster of genes around the *sol* operon (Fig. 1) are carried on a *Sma*I or *Apa*I 205-kb restriction fragment, although these genes were not identified as plasmidic.

To investigate the possibility that pSOL1 was a conjugative plasmid, several matings between *C. acetobutylicum* ATCC 824 (pJCace integrant on pSOL1 [10]) and DG1 (pMR1) were done. pJCace carries the *ace* operon (23) and a MLS^r gene. Plasmid pMR1 is an *E. coli-C. acetobutylicum* shuttle vector that carries a Km^r gene and the gram-positive replicon from pIM13. No transconjugants were obtained on RCA plates plus erythromycin and kanamycin. This cannot be explained by a problem of resistance of the transconjugants to both antibiotics, since it was possible to electrotransform *C. acetobutylicum* ATCC 824 (pJCace integrant on pSOL1) by pMR1 to obtain recombinant strains resistant to both antibiotics (data not shown). These results indicate that pSOL1-pJCace cannot conjugate under the conditions used in the mating experiments.

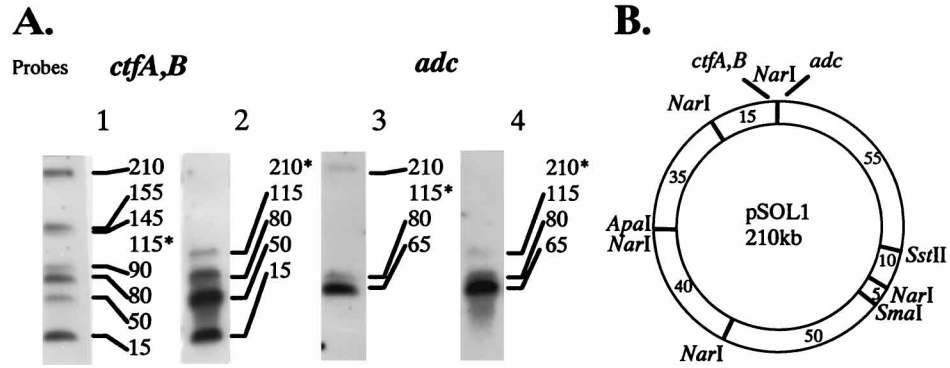

FIG. 6. Map of the 210-kb pSOL1 plasmid of *C. acetobutylicum* ATCC 824. (A) Southern analysis after partial *Nar*I digestion alone (lanes 1 and 3) and in association with complete *Apa*I digestion (lanes 2 and 4) of total DNA prepared in an agarose plug. The two probes on each side of the *Nar*I restriction site of the *sol* operon locus (Fig. 1) were prepared from *Hin*dIII-*Nar*I-digested pCOAT4 (2) and *Nar*I-*Ban*II-digested pDP2 (30). The sizes of the different hybridizing fragments are given on the sides of each Southern blot. Some of the digestion fragments (asterisk) could be seen only after film overexposure. (B) Physical and genetic map of pSOL1. The *Apa*I and one of the *Nar*I restriction sites are close to each other.

Because it is not possible to isolate large amounts of intact circular pSOL1 to electrotransform *C. acetobutylicum*, we restored the ability of the degenerate mutants to produce acetone or butanol (separately or together) by expressing the corresponding genes (*ctfA*, *ctfB*, and *adc* for acetone, *aad* for butanol) on plasmids in M5 and DG1 (Table 2). The amounts of the solvent(s) produced with the recombinant M5 and DG1 strains are not as large as those obtained with the wild-type strain, thus suggesting that other genes carried on pSOL1 might be important in solvent formation.

Bacterial plasmids have been found to carry genetic information that is typically not considered essential for survival, such as genes for antibiotic and heavy metal resistance (22), conjugation, utilization of various substrates (21), and degradation of compounds (19). What is unique about the pSOL1 plasmid in *C. acetobutylicum* ATCC 824 is that it carries primary metabolic genes, namely, all the genes necessary for production of acetone and butanol, which phenotypically distinguish this strain from the non-solvent-producing butyric acid clostridia (which produce butyrate and acetate as their main products).

TABLE 2. Solvent and acid production by various strains of *C. acetobutylicum*

Strain	Product concn (mM):				
	Acetate	Butyrate	Ethanol	Acetone	Butanol
5-Liter batch fermentations[a]					
ATCC 824	115	78	18	75	130
M5	107	169	6	0	0
M5(pCAAD)	101	99	8	0	84
M5(pFNK6)	60	181	4	44	0
DG1	113	171	5	0	0
DG1 (pCAAD)	84	90	24	0	90
10 ml tube cultures[b]					
ATCC 824	33	19	8	46	73
M5	12	46	4	0	0
M5(pAB)	15	34	5	16	25
DG1	19	59	3	0	0
DG1(pFNK6)	12	40	2	5	0

[a] Except for the M5(pFNK6) and ATCC 824 fermentations performed at pH 5.5, all other fermentations were performed at pH 5.0. For experimental details, see reference 27.

[b] Product concentrations in ~72-h cultures.

The *C. acetobutylicum* degeneration process typically involves a gradual decrease in the amounts of solvents produced upon serial subculturing or in continuous culture. Our findings with mutants M5 and DG1, which show that degeneration is caused by pSOL1 loss, explain the apparent characteristics of the degeneration process: segregational pSOL1 loss by all or a fraction of the cell population leads to decreased solvent production until all cells in the population lose pSOL1, in which case solvent production cannot be restored.

However, these results do not explain why cells grown in phosphate-limited continuous culture remain stable (or why pSOL1 is stably maintained in these cultures). More information regarding the genetic regulations that occur in this organism under conditions of certain substrate limitations is needed. Our study has enabled a new genetic model to be presented which explains the degeneration process in the solventogenic clostridia. The 210-kb plasmid defines a discrete region in the genome of *C. acetobutylicum*. The degenerated strain no longer possesses this region. Genetic tools are now available to study interactions between this plasmid and the chromosome by complementation of the degenerated strain or by gene disruption (11). Such techniques will also enable the functional analysis of the entire sequence of the 210 kb of pSOL1, which is expected to be available soon.

ACKNOWLEDGMENTS

This work was supported in part by grant BES-9632217 to E.T.P by the National Science Foundation. Additional support was provided by a NATO grant to P.S. and E.T.P.

REFERENCES

1. **Beverley, S. M.** 1988. Characterization of the 'unusual' mobility of large circular DNAs in pulsed field-gradient electrophoresis. Nucleic Acids Res. **16:**925–939.
2. **Cary, J. W., D. J. Petersen, E. T. Papoutsakis, and G. N. Bennett.** 1990. Cloning and expression of *Clostridium acetobutylicum* ATCC 824 acetoacetyl-coenzyme A:acetate/butyrate:coenzyme A transferase in *Escherichia coli*. Appl. Environ. Microbiol. **56:**1576–1583.
3. **Casse, F., C. Bouche, J. S. Julliot, M. Michel, and J. Dénarié.** 1979. Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. J. Gen. Microbiol. **113:**229–242.
4. **Clark, S. W., G. N. Bennett, and F. B. Rudolph.** 1989. 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. Appl. Environ. Microbiol. **55:**970–976.
5. **Cornillot, E., and P. Soucaille.** 1996. Solvent-forming genes in clostridia. Nature **380:**489.

6. **Eckhardt, T.** 1978. A rapid method for the identification of plasmid desoxyribonucleic acid in bacteria. Plasmid **1:**584–588.
7. **Fischer, R. J., J. Helms, and P. Dürre.** 1993. Cloning, sequencing and molecular analysis of the *sol* operon of *Clostridium acetobutylicum*, a chromosomal locus involved in solventogenesis. J. Bacteriol. **175:**6959–6969.
8. **Gerischer, U., and P. Dürre.** 1990. Cloning, sequencing, and molecular analysis of the acetoacetate decarboxylase gene region from *Clostridium acetobutylicum*. J. Bacteriol. **172:**6907–6918.
9. **Graham, M. Y., T. Otani, I. Boime, M. V. Olson, G. F. Carle, and D. D. Chaplin.** 1987. Cosmid mapping of the human chorionic gonadotropin beta subunit genes by field-inversion gel electrophoresis. Nucleic Acids Res. **15:** 4437–4448.
10. **Green, E. M., and G. N. Bennett.** Personal communication.
11. **Green, E. M., Z. L. Boynton, L. M. Harris, F. B. Rudolph, E. T. Papoutsakis, and G. N. Bennett.** 1996. Genetic manipulation of acid formation pathways by gene inactivation in *Clostridium acetobutylicum* ATCC 824. Microbiology **142:**2079–2086.
12. **Hsu, E. J., and Z. J. Ordal.** 1970. Comparative metabolism of vegetative and sporulating culture of *Clostridium thermosaccharolyticum*. J. Bacteriol. **102:** 369–376.
13. **Jones, D. T., A. van der Westhuizen, S. Long, E. R. Allcock, S. J. Reid, and D. R. Woods.** 1982. Solvent production and morphological changes in *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **43:**1434–1439.
14. **Jones, D. T., and D. R. Woods.** 1986. Acetone-butanol fermentation revisited. Microbiol. Rev. **50:**484–524.
15. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
16. **Keis, S., C. F. Bennett, V. K. Ward, and D. T. Jones.** 1995. Taxonomy and phylogeny of industrial solvent-producing clostridia. Int. J. Syst. Bacteriol. **45:**693–705.
17. **Lemme, C. J., and J. R. Frankiewicz.** June 1985. U.S. patent 4,521,516.
18. **Long, S., D. T. Jones, and D. R. Woods.** 1984. Initiation of solvent production, clostridial stage, and endospore formation in *Clostridium acetobutylicum* P262. Appl. Microbiol. Biotechnol. **20:**256–261.
19. **Marques, S., and J. L. Ramos.** 1993. Transcriptional control of the *Pseudomonas putida* TOL plasmid catabolic pathways. Mol. Microbiol. **9:**923–929.
20. **McCoy, E., and E. B. Fred.** 1941. The stability of a culture for industrial fermentation. J. Bacteriol. **41:**90–91.
21. **McKay, L. L., K. A. Baldwin, and J. D. Efstathiou.** 1976. Transductional evidence for plasmid linkage of lactose metabolism in *Streptococcus lactis* C2. Appl. Environ. Microbiol. **32:**45–52.
22. **Mergeay, M., D. Nies, H. G. Schlegel, J. Gerits, P. Charles, and F. van Gijsegem.** 1985. *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J. Bacteriol. **162:**328–334.
23. **Mermelstein, L. D., E. T. Papoutsakis, D. J. Petersen, and G. N. Bennett.** 1993. Metabolic engineering of *Clostridium acetobutylicum* ATCC 824 for increased solvent production by enhancement of acetone formation enzyme activities using a synthetic acetone operon. Biotechnol. Bioeng. **42:**1053–1060.
24. **Monod, M., C. Denoya, and D. Dubnau.** 1986. Sequence and properties of pIM13, a macrolide-lincosamide-streptogramin B resistance plasmid from *Bacillus subtilis*. J. Bacteriol. **167:**138–147.
25. **Nair, R. V.** 1995. Ph.D. dissertation. Northwestern University, Evanston, Ill.
26. **Nair, R. V., G. N. Bennett, and E. T. Papoutsakis.** 1994. Molecular characterization of an aldehyde/alcohol dehydrogenase gene from *Clostridium acetobutylicum* ATCC 824. J. Bacteriol. **176:**871–885.
27. **Nair, R. V., and E. T. Papoutsakis.** 1994. Expression of plasmid-encoded *aad* in *Clostridium acetobutylicum* M5 restores vigorous butanol production. J. Bacteriol. **176:**5843–5846.
28. **Oultram, J. D., M. Loughlin, T.-J. Swinfield, J. K. Brehm, D. E. Thompson, and N. P. Minton.** 1988. Introduction of plasmids into whole cells of *Clostridium acetobutylicum* by electroporation. FEMS Microbiol. Lett. **56:**83–88.
29. **Petersen, D. J.** 1991. Ph.D. dissertation. Rice University, Houston, Tex.
30. **Petersen, D. J., J. W. Cary, J. Vanderleyden, and G. N. Bennett.** 1993. Sequence and arrangement of genes encoding enzymes of the acetone-production pathways of *Clostridium acetobutylicum* ATCC 824. Gene **123:** 93–97.
31. **Roos, J. W., K. McLaughlin, and E. T. Papoutsakis.** 1985. The effect of pH on nitrogen supply, cell lysis, and solvent production in fermentations of *Clostridium acetobutylicum*. Biotechnol. Bioeng. **27:**681–694.
32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Smith, H. O., and M. L. Birnstiel.** 1976. A simple method for DNA restriction site mapping. Nucleic Acids Res. **3:**2387–2398.
34. **Soni B. K., P. Soucaille, and G. Goma.** 1987. Continuous acetone butanol fermentation: influence of vitamins on the metabolic activity of *Clostridium acetobutylicum*. Appl. Microbiol. Biotechnol. **27:**1–5.
35. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
36. **Stim-Herndon, K. P., R. Nair, E. T. Papoutsakis, and G. N. Bennett.** 1996. Analysis of degenerate variants of *Clostridium acetobutylicum* ATCC 824. Anaerobe **2:**11–18.
37. **Trieu-Cuot, P., A. Klier, and P. Courvalin.** 1985. DNA sequences specifying the transcription of the streptococcal kanamycin resistance gene in *Escherichia coli* and *Bacillus subtilis*. Mol. Gen. Genet. **198:**348–352.
38. **Wang, M., and E. Lai.** 1995. Pulsed field separation of large supercoiled and open-circular DNAs and its application of bacterial artificial chromosome cloning. Electrophoresis **16:**1–7.
39. **Wilkinson, S. R., and M. Young.** 1993. Wide diversity of genome size among different strains of *Clostridium acetobutylicum*. J. Gen. Microbiol. **139:**1069–1076.