APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1993, p. 1077–1081
0099-2240/93/041077-05$02.00/0

Vol. 59, No. 4

In Vivo Methylation in *Escherichia coli* by the *Bacillus subtilis* Phage ϕ3T I Methyltransferase To Protect Plasmids from Restriction upon Transformation of *Clostridium acetobutylicum* ATCC 824

LEE D. MERMELSTEIN AND ELEFTHERIOS T. PAPOUTSAKIS*

Department of Chemical Engineering, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208

Received 13 October 1992/Accepted 25 January 1993

The restriction endonuclease *Cac*824I has been shown to be a major barrier to electrotransformation of *Clostridium acetobutylicum* ATCC 824 (L. D. Mermelstein, N. E. Welker, G. N. Bennett, and E. T. Papoutsakis, Bio/Technology 10:190–195, 1992). Methylation by the ϕ3T I methyltransferase encoded by *Bacillus subtilis* phage ϕ3T was shown to protect plasmid DNA from restriction by *Cac*824I. Expression in *Escherichia coli* of the *ϕ3tI* gene (which encodes the ϕ3T I methyltransferase) from pAN1, which replicates via the p15A origin of replication, was sufficient to completely methylate coresident *E. coli-C. acetobutylicum* shuttle vectors with ColE1 origins of replication. Three shuttle vectors (pIMP1, pSYL2, and pSYL7) methylated in this manner were used to efficiently electrotransform strain ATCC 824. These vectors could not be introduced into strain ATCC 824 when unmethylated because the *E. coli* portions of the plasmids contain a large number of *Cac*824I sites. This method obviates the need to use *B. subtilis-C. acetobutylicum* shuttle vectors with few *Cac*824I sites to introduce DNA into *C. acetobutylicum* ATCC 824.

In the last few years, considerable effort has been directed toward the development of techniques for the genetic manipulation of the solvent (butanol, acetone, and ethanol)-producing bacterium *Clostridium acetobutylicum*. It is expected that the application of such techniques will lead to a more fundamental understanding of the genetic regulation of solventogenesis and to metabolically altered strains with superior solvent-producing abilities. To this end, transformation methods and vectors have been developed for several strains (16, 20, 25). Also, a number of solvent formation genes have been cloned and expressed in *Escherichia coli* (21), and methods of RNA isolation and characterization recently have been developed and applied to the characterization of some of the corresponding transcripts (8, 26). It may be possible to use cloned genes and a vector-transformation system to alter the fermentative characteristics of an organism and determine rate-limiting enzyme activities by (i) the expression of cloned solventogenic genes from multicopy vectors; (ii) the reduction of the rate of translation of chromosomal copies of cloned genes by the formation of plasmid-encoded antisense mRNA; and (iii) the mutation of chromosomal copies of cloned genes by recombination with plasmids containing homologous sequences. Also, plasmid-borne reporter genes under the control of regulatory regions of cloned clostridial solventogenic genes may be introduced into *C. acetobutylicum*. The expression profiles of the reporter genes may help elucidate the transcriptional regulation mechanisms of the solventogenic genes.

Physiological studies of transcriptional regulation and rate-limiting enzyme activities with cloned clostridial genes should be carried out with the strain from which the genes were cloned. The majority of solvent formation genes have been cloned from strain ATCC 824. ATCC 824 is also the best-characterized strain physiologically and the only strain in which the expression of plasmid-borne solvent formation genes has been reported (16). However, there are difficulties with cloning in and transformation of strain ATCC 824 (16).

We previously showed that strain ATCC 824 contains a restriction endonuclease, *Cac*824I, that is a major barrier to electrotransformation (16). The *Cac*824I restriction endonuclease recognition sequence, 5′-GCNGC-3′, where N can be any nucleotide, occurs infrequently in *C. acetobutylicum* DNA because of the high A + T DNA content of the organism (72% A + T [10]) but frequently in *E. coli* plasmids (16). *Cac*824I restriction, in general, prevents the transformation of ATCC 824 with shuttle vectors that function in *E. coli* (16). The only reported exceptions are with pMU1328, which exists in ATCC 824 as a derivative in which the *E. coli* DNA has been specifically deleted (16), and with pSYL2 which, in one experiment, transformed ATCC 824 with a low efficiency but subsequently did not transform the strain (12).

For reduction of the problems with *Cac*824I restriction, a *Bacillus subtilis-C. acetobutylicum* shuttle vector, pFNK1, was previously constructed and used for the cloning and expression of homologous genes in strain ATCC 824 (16). Plasmid pFNK1 contains few *Cac*824I sites because of its high A + T DNA content and small size. Transformants generated with pFNK1 and its derivatives presumably originate when the plasmid is completely methylated by the *Cac*824I methylase before there is any restriction. Cloning with pFNK1 is, however, technically difficult and time-consuming compared with what could be accomplished if *E. coli* shuttle vectors could be efficiently introduced into strain ATCC 824. We therefore sought a means by which this could be accomplished.

No methyltransferase that could be used to protect DNA from restriction by the *Cac*824I restriction endonuclease is commercially available. An in vivo methylation strategy has been reported by De Feyter and Gabriel (5); in this strategy, a methyltransferase cloned on a plasmid is used to increase

* Corresponding author.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
C. acetobutylicum ATCC 824	Contains *Cac*824I	ATCC[b]
E. coli ER2275	*hsdR mcr recA1*	NEB[c]
Plasmids		
pACYC184	Tc^r Cm^r; p15A origin	3
pUC19	Ap^r; ColE1 origin	29
pIMP1[d]	Ap^r MLS^r; ColE1 origin	16
pSYL2[d]	Tc^r MLS^r; ColE1 origin	12
pSYL7[d]	Tc^r MLS^r; ColE1 origin	11
pFNK3[d]	MLS^r *adc*	16
pBN16	Ap^r Cm^r $\phi 3tI$; ColE1 origin	18
pBN18	Ap^r Cm^r $\rho 11B$; ColE1 origin	18
pAN1	Cm^r $\phi 3tI$; p15A origin	This study

[a] *hsdR*, host-specific restriction deficient; *mcr*, methylcytosine-specific restriction abolished; *recA1*, homologous recombination abolished; Tc^r, tetracycline resistant; Ap^r, ampicillin resistant; *adc*, ATCC 824 acetoacetate decarboxylase gene; $\rho 11B$:$\rho 11_b$ methyltransferase gene.

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

[c] New England Biolabs.

[d] The source of the gram-positive origin of replication for this shuttle plasmid is given in Table 2.

the frequency of transfer of a coresident, compatible plasmid from *E. coli* into a restrictive *Xanthomonas campestris* strain. This strategy may be easily used to improve the transformation efficiencies of restrictive strains if an appropriate methyltransferase gene can be expressed in *E. coli*. It is therefore an alternative to the isolation of nonrestrictive mutants. The use of chemical mutagenesis to generate nonrestrictive mutants may result in additional, undesirable mutations and may yield strains that are more susceptible to phage infection (a problem that has plagued industrial *C. acetobutylicum* fermentations [10]). Several mutants of ATCC 824 that are deficient in solventogenic enzyme activities and may therefore be useful in complementation studies exist (4, 7). Mutation of each of these strains to generate nonrestrictive derivatives would be tedious.

Several methyltransferase genes that modify the *Cac*824I recognition sequence have been cloned from *B. subtilis* phages and expressed in *E. coli* (18, 27). In this study, we demonstrate that the ϕ3T I methyltransferase from *B. subtilis* phage ϕ3T protects DNA from cleavage by *Cac*824I. We also demonstrate that when the ϕ3T I methyltransferase gene ($\phi 3tI$) is expressed from a compatible plasmid, coresident *E. coli-C. acetobutylicum* shuttle vectors are completely protected from *Cac*824I restriction and can be efficiently introduced into *C. acetobutylicum* ATCC 824 by electrotransformation. These plasmids are unable to electrotransform ATCC 824 when unmethylated.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and transformation methods. The bacterial strains and plasmids used in this study are listed in Table 1. *C. acetobutylicum* was maintained as spores in corn mash glucose medium (23) or on reinforced clostridial agar (Difco Laboratories, Detroit, Mich.) plates as previously described (16). *C. acetobutylicum* was grown in reinforced clostridial medium (Difco) at pH 5.2 and 37°C in an anaerobic system (model 1024; Forma Scientific, Marietta, Ohio). Spores were activated by heat treatment at 70 to 80°C for 10 min. *E. coli* was grown in Luria-Bertani medium. Media were supplemented with antibiotics as required in the following concentrations: for *C. acetobutylicum*, erythromycin (100 μg/ml); for *E. coli*, erythromycin (250 μg/ml), ampicillin (30 μg/ml), and chloramphenicol (35 μg/ml). Previously published methods were used for the electrotransformation of *E. coli* (6) and *C. acetobutylicum* (16). For electrotransformation of *C. acetobutylicum*, 50 ng of methylated shuttle vector was used. For electrotransformation of unmethylated plasmids, 5 μg of DNA was used. Dilutions were made (when necessary) to obtain 50 to 300 colonies per plate for enumeration. Erythromycin was used to select for the presence of macrolide-lincosamide-streptogramin B resistance (MLS^r) genes.

DNA isolation and manipulation. Previously published methods were used to purify plasmid DNA from *C. acetobutylicum* (16) and *E. coli* (2). An *mcr* mutant strain of *E. coli* was used for the preparation of plasmids or plasmid mixtures that contained the $\phi 3tI$ gene because *E. coli* K-12 (and many common *E. coli* cloning strains) contains a restriction system that cleaves DNA methylated at either of the ϕ3T I methyltransferase target cytosines (22). Stationary-phase cells were used for plasmid preparation to minimize the possibility of partial methylation of a plasmid that might result from newly synthesized DNA. A pZ523 spin column (5 prime to 3 prime Inc., Boulder, Colo.) was used for the large-scale preparation of plasmids that were used to electrotransform *C. acetobutylicum*. DNA concentrations were determined by comparison against standards in agarose gel electrophoresis. Restriction enzymes other than *Cac*824I were purchased from New England Biolabs (Beverly, Mass.) and used in accordance with the supplier's recommendations.

Construction of pAN1. The $\phi 3tI$ gene was previously cloned in pBR328 as a 2.8-kb partial *Hin*dIII fragment (containing one internal *Hin*dIII site) to generate plasmid pBN16 (18). Plasmid pBN16 was partially digested with *Hin*dIII and ligated to *Hin*dIII-linearized pACYC184 (4.2 kb). Chloramphenicol-resistant transformant colonies generated with this ligation mixture were pooled, and plasmids were prepared from them. A sample of this plasmid mixture was digested with *Fnu*4HI (an isoschizomer of *Cac*824I that does not cleave ϕ3T I-methylated DNA [15]) to restrict plasmids that did not contain an intact (expressed) $\phi 3tI$ gene (14). The digestion product was purified and used to transform ER2275(pUC19). Ampicillin- and chloramphenicol-resistant transformants were selected to enrich the *Fnu*4HI digestion product for plasmids that were compatible with pUC19 and therefore contained the p15A replicon from pACYC184. Most of the resulting transformants contained pUC19 and a coresident plasmid with the $\phi 3tI$ gene cloned into pACYC184. One such methyltransferase-containing plasmid, pAN1, with the orientation of fragments shown in Fig. 1, was chosen for further study.

Digestions with extracts containing *Cac*824I activity. Protoplast extracts containing *Cac*824I activity were prepared from *C. acetobutylicum* ATCC 824 and stored as previously described (16). Digestions with protoplast extracts were done at 37°C in a buffer containing 10 mM $MgCl_2$ (pH 7.5)–150 mM NaCl. One unit of activity was defined as the amount of extract that would completely digest 1 μg of pMU1328 DNA (prepared from *E. coli*) in a 20-μl volume in 1 h, as determined by agarose gel electrophoresis. Plasmid pMU1328 generates a characteristic, well-resolved banding pattern because of the presence of A + T-rich DNA; this pattern can be used to easily distinguish among specific, nonspecific, and partial *Cac*824I digestions (16). *Cac*824I

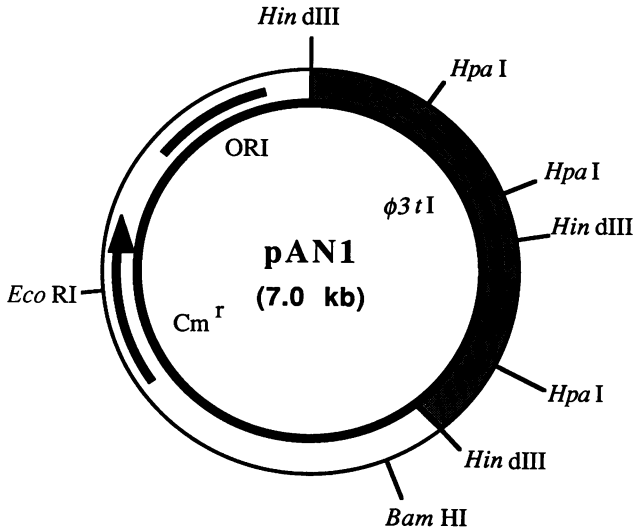

FIG. 1. Schematic representation of plasmid pAN1, used for the in vivo methylation of ColE1 plasmids. Arrows designate structural genes and their transcriptional directions. *B. subtilis* phage ϕ3T DNA is indicated by the shaded region. DNA derived from pACYC184 is indicated by the unshaded region. The p15A origin of replication (ORI), chloramphenicol resistance gene (Cm^r), and *ϕ3tI* gene are also indicated.

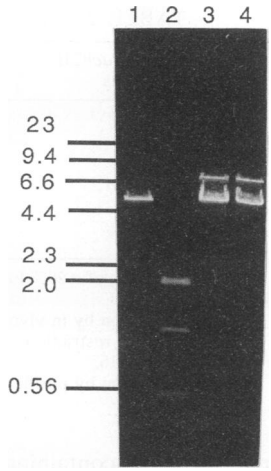

FIG. 2. Comparison of plasmid DNA digests electrophoresed on 0.8% agarose and stained with ethidium bromide. Lanes: 1, pIMP1 linearized with *Eco*RI; 2, pIMP1 digested with *Eco*RI and *Cac*824I; 3, pIMP1 and pAN1 linearized with *Eco*RI; 3, pIMP1 and pAN1 digested with *Eco*RI and *Cac*824I. The majority of the fragments in lane 2 are too small to be visible. Plasmid mixtures used in lanes 3 and 4 were purified from a single *E. coli* strain containing both plasmids. Size standards, indicated at left in kilobase pairs, were lambda DNAs digested with *Hin*dIII.

digestion of plasmids, such as pUC19, that do not contain A + T-rich DNA generates many fragments smaller than 0.5 kb, which are difficult to resolve by conventional agarose gel electrophoresis.

RESULTS

Resistance of ϕ3T I-methylated DNA to *Cac*824I restriction. The ϕ3T I methyltransferase enzyme has two target recognition domains, one of which recognizes the sequence 5′-GGCC-3′ and another of which recognizes the sequence 5′-GCNGC-3′ (24). The product of the methyltransferase-catalyzed reaction is a 5-methylcytosine at the internal cytosine of each of these recognition sequences. The residue that is methylated in *C. acetobutylicum* to protect against *Cac*824I restriction has not been determined. However, since the *Cac*824I recognition sequence does not necessarily contain any adenine residues and bacterial DNA methyltransferases modify adenine or cytosine residues, we predicted that methylation at one of the two cytosine residues in the recognition sequence would block *Cac*824I restriction. Furthermore, strain ATCC 824 genomic DNA is resistant to cleavage by the *Cac*824I isoschizomer *Fnu*4HI (16), and methylation at either of the cytosines in the *Cac*824I recognition sequence is sufficient to block cleavage by *Fnu*4HI (15).

The *ϕ3t*I gene is well expressed in *E. coli* from plasmid pBN16 by use of its natural promoter (18). The plasmid is therefore methylated and could be used to determine the susceptibility of ϕ3T I-methylated DNA to *Cac*824I cleavage. Plasmids pBN16, pUC19, and pACYC184 (100 ng) were overdigested with an ATCC 824 protoplast extract containing approximately 10 U of *Cac*824I activity. The products were analyzed by agarose gel electrophoresis along with undigested control samples of each plasmid. Plasmid pBN16 was found to be completely resistant to *Cac*824I digestion, whereas the unmethylated control plasmids pUC19 and pACYC184 were digested into many small (<0.5-kb) fragments (results not shown). Therefore, methylation at the internal cytosine in the *Cac*824I recognition sequence prevents *Cac*824I restriction, and the *ϕ3t*I gene is sufficiently well expressed in *E. coli* to completely methylate plasmid DNA in vivo. We therefore reasoned that a coresident shuttle vector would also be protected against *Cac*824I restriction. Plasmid pBN18, which contains the gene for the $\rho 11_b$ methyltransferase (which also methylates the sequences 5′-GCNGC-3′ and 5′-GGCC-3′) from *B. subtilis* phage ρ11, was also resistant to *Cac*824I cleavage but was not used further in this study.

Protection of coresident shuttle vectors from *Cac*824I restriction by growth in the presence of pAN1. The potential *E. coli-C. acetobutylicum* ATCC 824 shuttle vectors that we wished to introduce into *C. acetobutylicum* ATCC 824 (pIMP1, pSYL2, and pSYL7) replicate in *E. coli* via the ColE1 origin. The *ϕ3t*I gene was previously cloned into ColE1 plasmid pBR328 (18). Therefore, for in vivo methylation of the shuttle vectors, the *ϕ3t*I gene was cloned into a compatible plasmid. The resulting plasmid, pAN1 (Fig. 1), was constructed by inserting the *ϕ3t*I gene into plasmid pACYC184, which replicates via the p15A origin and is compatible with ColE1 plasmids. Plasmid pAN1 also contains the pACYC184 chloramphenicol resistance gene, a selectable marker distinct from those used in the *E. coli-C. acetobutylicum* shuttle vectors. Therefore, it is possible to maintain selective pressure for the presence of both the shuttle vectors and pAN1 in *E. coli*. As was the case for pBN16, pAN1 was completely resistant to *Cac*824I cleavage (results not shown). Plasmid mixtures were purified from *E. coli* that had been transformed with both pAN1 and one of three potential *E. coli-C. acetobutylicum* shuttle vectors, pIMP1, pSYL2, and pSYL7. Each of these plasmid mixtures was found to be completely resistant to *Cac*824I restriction, as shown for pIMP1 in Fig. 2.

Electrotransformation of methylated shuttle vectors into

TABLE 2. Electrotransformation efficiencies and characteristics of shuttle plasmids

Plasmid	Size (kb)	No. of *Cac*824I sites	Gram-positive origin of replication	Source of MLS^r gene	No. of transformants/μg of DNA[a]
pIMP1	4.8	20[b]	*B. subtilis* pIM13	*B. subtilis* pIM13	5.0×10^5
pIMP1	4.8	20[c]	*B. subtilis* pIM13	*B. subtilis* pIM13	<0.2
pSYL2	8.7	37[b]	*Clostridium butyricum* pCBU2	*Enterococcus faecalis* pAMβ1	2.7×10^5
pSYL7	9.2	38[b]	*C. perfringens* pJU122	*E. faecalis* pAMβ1	6.0×10^4
pFNK3[d]	4.5	2[e]	*B. subtilis* pIM13	*B. subtilis* pIM13	1.7×10^5
pFNK3[d]	4.5	2[c]	*B. subtilis* pIM13	*B. subtilis* pIM13	1.6×10^1

[a] Determined with average accuracies of ±20% for pIMP1, pSYL2, and pSYL7, ±15% for pFNK3 prepared from *B. subtilis*, and ±50% for pFNK3 prepared from *C. acetobutylicum*.
[b] Protected against *Cac*824I restriction by in vivo methylation in *E. coli*.
[c] *Cac*824I sites not protected against restriction.
[d] Efficiency data are from reference 16.
[e] Protected against *Cac*824I restriction by purification from strain ATCC 824.

ATCC 824. Plasmid mixtures containing pAN1 and one of the shuttle vectors, pIMP1, pSYL2, or pSYL7, were used to electrotransform *C. acetobutylicum* ATCC 824 to erythromycin resistance. The efficiencies of electrotransformation and the characteristics of these plasmids are given in Table 2. Methylation plasmid pAN1 cannot transform ATCC 824 because it lacks a proper origin of replication and an antibiotic resistance marker. No transformants were obtained with these shuttle vectors when they were not methylated by the ϕ3T I methyltransferase, even with as much as 5 μg of plasmid DNA per electrotransformation.

With pIMP1 and pSYL2, ATCC 824 transformants were visible approximately 18 h after plating. Transformants obtained with pSYL7, however, took approximately 35 h to become visible. Plasmid DNA from clostridial transformants in several independent electrotransformation experiments was physically mapped. Restriction patterns of plasmids pIMP1, pSYL2, and pSYL7 purified from ATCC 824 were identical to those of these plasmids purified from *E. coli* (results not shown). The yield of pSYL7 prepared from *C. acetobutylicum* was, however, considerably lower than that of pIMP1 or pSYL2, a result suggesting that this plasmid exists at a lower copy number. Plasmid DNA prepared from *C. acetobutylicum* could also be used to retransform *E. coli*, with selection for all antibiotic resistances initially present on the plasmid. These data suggest that the plasmids did not undergo deletions or rearrangements in *C. acetobutylicum* ATCC 824.

DISCUSSION

In a previous study in which a methyltransferase-containing plasmid was used to methylate a compatible vector so that it could be transferred to a restrictive strain, the enzyme recognized a 6-bp target sequence that only occurred a few times in the vector (5). In the present study, the method was shown to work for plasmids that require more extensive methylation because of the presence of up to 38 *Cac*824I recognition sites. The inability to detect any digestion of methylated plasmids by *Cac*824I and the efficient electrotransformation of *C. acetobutylicum* with these plasmids suggest that the in vivo methylation method may have broad applications in circumventing restriction barriers for strains that are difficult or otherwise undesirable to mutagenize. Furthermore, methyltransferase genes with a wide variety of specificities have been cloned and expressed in *E. coli* (27). If necessary, an appropriate methyltransferase gene may be cloned from the restrictive strain. This procedure is usually straightforward when the method of Mann et al. (14), in which a plasmid (or cosmid) library is enriched for methyltransferase clones prior to transformation of *E. coli* by digestion with the corresponding restriction endonuclease (or an appropriate isoschizomer), is used.

In vivo methylation by the pAN1-encoded methyltransferase was used to protect *E. coli-C. acetobutylicum* shuttle vectors with ColE1 origins of replication from *Cac*824I restriction but in principle may be used to protect any *E. coli* plasmid that is compatible with p15A-based plasmids. If it is necessary to methylate a p15A-based plasmid, pBN16, a ColE1-based plasmid that contains the ϕ*3t*I gene, may be similarly used. In the future, it may be necessary to protect vectors that cannot replicate in *E. coli* from *Cac*824I restriction. It should be possible to methylate such plasmids in vitro by use of extracts from strains of *B. subtilis* or *E. coli* in which the ϕ*3t*I gene is expressed from a strong promoter (9).

The electrotransformation efficiencies of the ϕ3T I-methylated *E. coli-C. acetobutylicum* shuttle vectors are similar to that of the *B. subtilis-C. acetobutylicum* ATCC 824 shuttle plasmid, pFNK3, that was prepared from *C. acetobutylicum* ATCC 824 (16). This result confirms that *Cac*824I is the primary barrier to the electrotransformation of strain ATCC 824 and suggests that the fortuitous methylation of the sequence 5′-GGCC-3′ by the ϕ3T I methyltransferase did not have a deleterious effect on transformation efficiency, as might occur if there were a clostridial methylcytosine-specific restriction system. We will report shortly the use of this in vivo methylation method to introduce and express several primary metabolic genes (including the acetone pathway genes and butyrate pathway genes) in ATCC 824 (unpublished data).

The three vectors introduced into *C. acetobutylicum* ATCC 824 after in vivo methylation have diverse gram-positive origins of replication. They may therefore also have diverse stability properties (at a normal or elevated temperature), copy numbers, and host-plasmid interactions that may be exploited in future applications. A very stable vector may be used to express cloned genes throughout the course of a large-scale batch fermentation without the need for the maintenance of plasmid selective pressure. Unstable vectors may be useful for the selection of transpositions from plasmid to chromosome (30) or for the creation of stable null mutants by double recombination between a chromosomal gene and a cloned, mutated, plasmid-borne derivative of the gene (28). A thorough investigation of the properties of the shuttle plasmids introduced into ATCC 824 should therefore be undertaken. The only published study to this effect, for *C. acetobutylicum*, concerns the stability of shuttle vectors

with origins of replication from *B. subtilis* plasmid pIM13 (with the same replicon as that present in pIMP1) or *C. butyricum* plasmid pCB101 (1).

The in vivo methylation system may also be useful for generating gene-specific mutants by Campbell recombination (17) or developing plasmids for chromosomal integration (suicide plasmids) (13) in strain ATCC 824. The transformation efficiencies attained in this study with methylated vectors are approximately those necessary for the selection of integration events with other organisms (13, 19). The method described in this study for protecting DNA from *Cac*824I restriction upon transformation of *C. acetobutylicum* ATCC 824 should therefore greatly simplify metabolic engineering and genetic analysis studies of this organism.

ACKNOWLEDGMENTS

We thank M. Noyer-Weidner and T. A. Trautner for providing the methylase clones and N. Pagratis for suggesting the use of in vivo methylation.

This research was supported by a grant from the National Science Foundation (BCS-9210108).

REFERENCES

1. **Azeddoug, H., J. Hubert, and G. Reysset.** 1992. Stable inheritance of shuttle vectors based on plasmid pIM13 in a mutant strain of *Clostridium acetobutylicum*. J. Gen. Microbiol. **138:** 1371–1378.
2. **Birnbaum, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
3. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:** 1141–1156.
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. **De Feyter, R., and D. W. Gabriel.** 1991. Use of cloned DNA methylase genes to increase the frequency of transfer of foreign genes into *Xanthomonas campestris* pv. malvacearum. J. Bacteriol. **173:**6421–6427.
6. **Dower, W. J., J. F. Miller, and C. W. Ragsdale.** 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16:**6127–6145.
7. **El Kanouni, A., A.-M. Junelles, R. Janati-Idrissi, H. Petitdemange, and R. Gay.** 1989. *Clostridium acetobutylicum* mutants isolated for resistance to the pyruvate halogen analogs. Curr. Microbiol. **18:**139–144.
8. **Gerischer, U., and P. Dürre.** 1992. mRNA analysis of the *adc* gene region of *Clostridium acetobutylicum* during the shift to solventogenesis. J. Bacteriol. **174:**426–433.
9. **Günthert, U., L. Reiners, and R. Lauster.** 1986. Cloning and expression of *Bacillus subtilis* phage DNA methyltransferase genes in *Escherichia coli* and *B. subtilis*. Gene **41:**261–270.
10. **Jones, D. T., and D. R. Woods.** 1986. Acetone-butanol fermentation revisited. Microbiol. Rev. **50:**484–524.
11. **Lee, S. Y., G. N. Bennett, and E. T. Papoutsakis.** 1992. Construction of *Escherichia coli-Clostridium acetobutylicum* shuttle vectors and transformation of *Clostridium acetobutylicum* strains. Biotechnol. Lett. **14:**427–432.
12. **Lee, S. Y., L. D. Mermelstein, G. N. Bennett, and E. T. Papoutsakis.** 1992. Vector construction, transformation, and gene amplification in *Clostridium acetobutylicum* ATCC 824. Ann. N.Y. Acad. Sci. **665:**39–51.
13. **Leenhouts, K. J., J. Kok, and G. Venema.** 1989. Campbell-like integration of heterologous plasmid DNA into the chromosome of *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. **55:**394–400.
14. **Mann, M. B., R. N. Rao, and H. O. Smith.** 1978. Cloning of restriction and modification genes in *E. coli*: the *Hha*II system from *Haemophilus haemolyticus*. Gene **3:**97–112.
15. **McClelland, M., and M. Nelson.** 1988. The effect of site-specific DNA methylation on restriction endonuclease and DNA modification methyltransferases—a review. Gene **74:**291–304.
16. **Mermelstein, L. D., N. E. Welker, G. N. Bennett, and E. T. Papoutsakis.** 1992. Expression of cloned homologous fermentative genes in *Clostridium acetobutylicum* ATCC 824. Bio/Technology **10:**190–195.
17. **Niaudet, B., A. Goze, and S. D. Ehrlich.** 1982. Insertional mutagenesis in *Bacillus subtilis*: mechanism and use in gene cloning. Gene **19:**277–284.
18. **Noyer-Weidner, M., S. Jentsch, J. Kupsch, M. Bergbauer, and T. A. Trautner.** 1985. DNA methyltransferase genes of *Bacillus subtilis* phages: structural relatedness and gene expression. Gene **35:**143–150.
19. **O'Kane, C., M. A. Stephens, and D. McConnell.** 1986. Integrable α-amylase plasmid for generating random transcriptional fusions in *Bacillus subtilis*. J. Bacteriol. **168:**973–981.
20. **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.
21. **Papoutsakis, E. T., and G. N. Bennett.** Cloning, structure and expression of acid and solvent pathway genes. *In* D. R. Woods (ed.), The clostridia and biotechnology, in press. Butterworths, Boston.
22. **Raleigh, E. A., and G. Wilson.** 1986. *Escherichia coli* K-12 restricts DNA containing 5-methylcytosine. Proc. Natl. Acad. Sci. USA **83:**9070–9074.
23. **Roos, J. W., J. K. McLaughlin, and E. T. Papoutsakis.** 1984. The effect of pH on nitrogen supply, cell lysis, and solvent production in fermentations of *Clostridium acetobutylicum*. Biotechnol. Bioeng. **27:**681–694.
24. **Tran-Betcke, A., B. Behrens, M. Noyer-Weidner, and T. A. Trautner.** 1986. DNA methyltransferase genes of *Bacillus subtilis* phages: comparison of their nucleotide sequences. Gene **42:**89–96.
25. **Truffaut, N., J. Hubert, and G. Reysset.** 1989. Construction of shuttle vectors useful for transforming *Clostridium acetobutylicum*. FEMS Microbiol. Lett. **58:**15–20.
26. **Walter, K. A., G. N. Bennett, and E. T. Papoutsakis.** 1992. Molecular characterization of two *Clostridium acetobutylicum* ATCC 824 butanol dehydrogenase isozyme genes. J. Bacteriol. **22:**7149–7158.
27. **Wilson, G. G.** 1991. Organization of restriction-modification systems. Nucleic Acids Res. **19:**2539–2566.
28. **Wu, L., and N. E. Welker.** 1991. Cloning and characterization of a glutamine transport operon of *Bacillus stearothermophilus* NUB36: effect of temperature on regulation of transcription. J. Bacteriol. **173:**4877–4888.
29. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
30. **Youngman, P. J., J. B. Perkins, and R. Losick.** 1983. Genetic transposition and insertional mutagenesis in *Bacillus subtilis* with *Streptococcus faecalis* transposon Tn*917*. Proc. Natl. Acad. Sci. USA **80:**2305–2309.