A new method for the rapid identification of genes encoding restriction and modification enzymes

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ABSTRACT

We have constructed derivatives of *Escherichia coli* that can be used for the rapid identification of recombinant plasmids encoding DNA restriction enzymes and methyltransferases. The induction of the DNA-damage inducible SOS response by the Mcr and Mrr systems, in the presence of methylated DNA, is used to select plasmids encoding DNA methyltransferases. The strains of *E. coli* that we have constructed are temperature-sensitive for the Mcr and Mrr systems and have been further modified to include a *lacZ* gene fused to the damage-inducible *dinD* locus of *E. coli*. The detection of recombinant plasmids encoding DNA methyltransferases and restriction enzymes is a simple, one step procedure that is based on the induction at the restrictive temperature of the *lacZ* gene. Transformants encoding DNA methyltransferase genes are detected on LB agar plates supplemented with X-gal as blue colonies. Using this method, we have cloned a variety of DNA methyltransferase genes from diverse species such as *Neisseria*, *Haemophilus*, *Treponema*, *Pseudomonas*, *Xanthomonas* and *Saccharopolyspora*.

INTRODUCTION

To date, more than 100 DNA methyltransferase genes have been cloned using one of the following methods: (a) sub-cloning of plasmid genes, (b) selection based on the restriction of incoming DNA, or (c) selection based on the modification of transforming DNA (1). Recently, we described a new method for cloning DNA methyltransferase genes (2). This method was based on the observation that strains carrying temperature-sensitive *mcrB* mutations and cloned DNA methyltransferase genes were able to grow and form colonies at a permissive temperature (42°C) but unable to form colonies at a non-permissive temperature (30°C). The limitation of this method was the necessity to replica plate thousands of colonies. In addition, Mcr restriction might not be completely lethal in all cases at the non-permissive temperature, and some clones would be lost because they grew at both temperatures.

Heitmann and Model (3) have shown that the expression of site-specific DNA methyltransferases induce the SOS response in *Escherichia coli* strains that are *mcrA*+, *mcrB*+ or *mrr*+. Based on this observation, we constructed *E. coli* strains that were temperature sensitive for these genes. This allows for a direct detection of colonies carrying cloned DNA methyltransferase genes among all colonies formed after transformation with appropriate bacterial gene banks. This method is based on the assumption that the *E. coli* cells carrying *mcrA*, *mcrB* or *mrr* ts mutations and SOS inducible promoters fused to the *lac* operon will grow normally at the nonpermissive temperature and that the modification-dependent restriction system will damage DNA sufficiently at the non-permissive temperature to induce *dinD::lacZ*. This should be observed when cells carrying cloned DNA methyltransferase genes are transferred from 42°C to 30°C. Signal inducing the SOS system would be generated in these strains by the expression of the nuclease activity at the non-permissive temperature. Transformation of such cells with the gene bank containing cloned DNA methyltransferase genes should result in the formation of white colonies on X-gal plates incubated at 42°C. Shifting the incubation temperature to 30°C should result in the induction of β -galactosidase activity by those colonies that carry cloned DNA methyltransferase genes.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

E. coli strains MM294 and its *mcrA*, *mcrB* deficient mutant ER1562 were described previously (4) and were kindly provided by E. Raleigh, New England Biolabs. *E. coli* strain RR1 (5) was obtained from W. Arber. *E. coli* strain DH5MCR was obtained from Bethesda Research Laboratories. Plasmids pM.HaeIII, pM.HaeII, pM.MspI, pM.NlaIII, pM.NlaIV, pM.HpaII, and pM.HhaII were obtained from New England Biolabs. Isolation of the plasmid pM.NgoV was described previously (6). Culture media used were Luria-Bertani broth (LB), Luria-Bertani broth supplemented with 1.5% (w/v) or 0.8% (w/v) of agar (7). When necessary these media were supplemented with ampicillin, 50 μ g/ml and 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal), 35 μ g/ml.

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Enzymes and chemicals

Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs and used as recommended by the supplier. All chemicals were reagent grade or better and were purchased from Sigma Chemical Co. (St. Louis, MO).

Construction of Bacterial Strains

Two *E. coli* strains were used in these studies: AP1-200 (F-*endA1 thi-1 supE44 hsdR17 mcrB251 mrr253 lacZ::Tn10 [dinD2::Mu dII734 (kan lac)] [F' lacI^q lacZ::Tn5]*) and AP1-200-9 (F-*endA1 thi-1 supE44 hsdR17 mcrB251 mrr253 mcrA252 lacZ::Tn10 [dinD1::Mu dII734 (Kan lac)] [F' lacI^q lacZ::Tn5]*). The isolation of *E. coli* strain AP1-10 carrying a temperature-sensitive *mcrB* mutation has been described previously (2). To assay SOS induction in the presence of plasmids carrying DNA methyltransferase genes, derivatives of this strain were constructed as follows: a *dinD2::Mu dII734(Kan lac)* fusion from *E. coli* strain JH140 (obtained from J. Heitman) was introduced into strain AP1-10 by P1 transduction with selection for kanamycin resistance. One transductant, AP1-100, was chosen for further analysis. The isolation of the *mcrA* temperature-sensitive mutant AP1-100-9 was described previously (8). Spontaneous *lac* mutants of these strains were isolated (AP1-111 and AP1-100-91 respectively) and mated with GC2438 (9) to transfer in an F episome carrying *lacI*, to yield AP1-112 and AP1-100-92, respectively. Since both AP1-111 and AP1-100-91 revert to the Lac positive phenotype at a high frequency, and this would inhibit our ability to distinguish clones carrying cloned DNA methyltransferase gene from the Lac positive revertants, the *lacZ98::Tn10* chromosomal mutation from *E. coli* strain N3033 (CGSC 6660) (10) was introduced into AP1-112 and AP1-100-92 by P1 transduction with selection for tetracycline resistance. The resulting transductants were tested for the Lac phenotype, McrB or McrA temperature-sensitive phenotype and the induction of the SOS system after transformation with plasmids carrying cloned DNA methyltransferase genes. From 40 transductants derived from each of these strains, two clones were isolated and designated AP1-200 and AP1-200-9 respectively. Both of these clones were

no longer resistant to tetracycline, and the reason for the loss of this marker is not known and was not examined any further.

Preparation of cell extracts and DNA methylation assay

Appropriate *E. coli* cells carrying cloned DNA methyltransferase genes were grown overnight at 37°C in 50 ml of LB medium containing ampicillin (50 µg/ml), centrifuged and suspended in 0.5 ml of 50 mM Tris-Hydrochloride (pH 8.0), 14 mM 2-mercaptoethanol and 0.01% PMSF. Lysozyme was added to a final concentration of 2 mg/ml and the cells were incubated on ice for 3 h followed by disruption by sonication. After centrifugation, the supernatant was stored at 4°C. The methylation reaction mixture contained in a total volume of 20 µl to 50 µl 50 mM Tris-HCl (pH 8.0) 10 mM EDTA, 7.0 mM 2-mercaptoethanol, 2 µM [H]³ AdoMet, 2 µg of lambda DNA and 5 µl of enzyme extract. Unincorporated label was removed from the sample by extraction with phenol and separation on a 5 ml Sephadex G-100 column. If possible, the presence of the cloned DNA methyltransferase gene was also verified by testing the resistance of the plasmid DNA carrying cloned genes to digestion by the appropriate restriction enzyme.

DNA procedures

Plasmid DNA was isolated by the procedure of Birnboim and Doly (11) followed by cesium chloride isopycnic density centrifugation. General molecular biology techniques used were as described previously (7). Competent *E. coli* cells were prepared by the CaCl₂ procedure described previously (7). When transformations were performed at different temperatures, the cells were grown before CaCl₂ treatment at the appropriate temperature (42°C or 30°C) followed by a 2 min heat shock at 42°C. Expression at the appropriate temperature was allowed for 60 min before plating on selective media. Transformants were selected by adding aliquots of the transformation mixture into 3 ml of semisolid LBA medium, mixing and pouring onto prewarmed plates containing 10 ml of solidified LBA medium followed by an 18 h incubation at the appropriate temperature. When necessary, both the top and bottom agars contained X-gal. To test for the induction of the SOS response by cells

Table 1. Properties of *Escherichia coli* AP1-200 and AP1-200-9

Bacterial Strains	Plasmid DNA	Relative Efficiency ^a of transformation at		Growth of the ^b transformants		SOS induction after ^c transformation with	
		30°C	42°C	30°C	42°C	30°C	42°C
AP1-200	pM.HaeIII	1 × 10 ⁻⁴	1	—	+	+	—
	pM.NgoMI	1 × 10 ⁻³	1	—	+	+	—
	pM.HaeII	1 × 10 ⁻⁴	1	—	+	+	—
	pM.MspI	1 × 10 ⁻⁴	1	+/-	+	+	—
	pM.NgoV	1 × 10 ⁻³	1	—	+	+	—
	pM.NlaIV	1 × 10 ⁻³	1	+/-	+	+	—
	pM.NlaIII	1 × 10 ⁻³	1	+/-	+	+	—
	pBR322	1	1	+	+	—	—
AP1-200-9	pM.HaeIII	1 × 10 ⁻⁴	1	—	+	+	—
	pM.MspI	1 × 10 ⁻³	1	+/-	+	+	—
	pM.HpaII	0.8	1	+/-	+	+	—
	pBR322	1	1	+	+	—	—

^aRelative efficiency of transformation is the number of transformants obtained divided by the number of transformants obtained in the permissive strain ER1562. All numbers were normalized to pBR322.

^bTransformants were initially identified by growth on selective media at 42°C prior to replica plating and analysis for growth at 30°C.

^cA+ indicates that all colonies induced the SOS response while a- indicates that none of the colonies induced the SOS response.

encoding cloned DNA methyltransferases, cells that were incubated for 18 hr at 42°C were transferred to 30°C, and incubated for an additional 8–16 hr. The development of a blue color in these colonies, as compared to the white color of transformant colonies carrying appropriate plasmid vector DNA was taken as a positive result.

RESULTS AND DISCUSSION

Properties of the *E. coli* AP1-200 and AP1-200-9 strains

To test the usefulness of the constructed strains for the cloning of DNA methyltransferase genes, the properties of these strains were tested in the following manner: (a) *McrA* and *McrB* temperature-sensitive phenotype, as measured by the relative frequency of transformation at permissive and nonpermissive temperature using plasmids carrying known DNA methyltransferase genes, (b) the ability of transformants, containing cloned DNA methyltransferase genes, to grow at permissive and non-permissive temperature after replica plating on selective media, and (c) the induction of the SOS response in transformants carrying known DNA methyltransferase genes. Our data (Table 1) indicated that AP1-200 retained its *McrB* temperature sensitive phenotype since all plasmids carrying cloned DNA methyltransferase genes used in this experiment (pM.HaeIII, pM.HaeII, pM.MspI, pM.NgoV, pM.NlaIII, and pM.NlaIV) were unable to transform this strain at 30°C. We have shown that when transformant colonies grown on X-gal containing medium at 42°C are shifted to 30°C they made a blue color. This indicates the induction of the SOS response after transfer of the colonies from 42°C to 30°C. On the other hand, the presence of plasmids carrying some of the cloned DNA methyltransferase genes, like *mspIM* and *nlalIIM*, although unable to transform these cells at 30°C, were able to grow slightly when cells containing these plasmids were first identified at 42°C, and then replica plated at 30°C (See table 1 for data). The fact that these clones were able to grow at 30°C indicates that simply screening for the lack of growth at 30°C would miss these clones. This indicates that the previous method that we described for the identification of DNA methylase clones might not identify all clones that are sensitive to the *Mcr* and *Mrr* nucleases (2).

We have previously shown that AP1-100-9 lacks the ability to restrict T4gt phage but still shows *McrB* ts phenotype when tested for the restriction of plasmids carrying cloned DNA methyltransferase genes (8). The same *McrB* phenotype is also observed for its derivative AP1-200-9 (data not shown). We have also shown that AP1-100-9 is unable to restrict T6gt phage and plasmid carrying the *hpaIIM* gene indicating a *McrA* negative phenotype for this strain (8). AP1-200-9, although unable to restrict plasmid carrying *hpaIIM* at either 42°C or 30°C, still induces the SOS response (See table 1). These results can be explained if AP1-200-9 produced a very low level of *McrA* nuclease, where the level is too low for the efficient restriction of plasmids encoding *hpaIIM*, but high enough for the induction of the SOS system.

E. coli MM294 is the parent of AP1-200 and AP1-200-9 and carries a wild-type *mrr* allele that is able to restrict plasmids encoding *HhaIIM* (Piekarowicz-unpublished observations). However, we have shown that both AP1-200 and AP1-200-9 have acquired a temperature-sensitive *mrr* mutation, since these strains can be transformed with pM.HhaII at 42°C but not 30°C (data not shown). Furthermore, the presence of this plasmid in these

two strains induces the SOS response. The origin of this temperature sensitive mutation is unknown. Under the same transformation and growth conditions, pBR322 and pUC18 fail to induce the SOS response in these strains.

Fate of plasmids encoding DNA methyltransferase genes when grown at the restrictive temperature

If the constructed strains are to be used for the selection of clones encoding DNA methyltransferase genes, it is necessary to be able to recover plasmid DNA from colonies incubated at 30°C long enough to induce the SOS response. The isolated plasmid DNA must also carry a functional DNA methyltransferase gene. The results presented in the previous section have shown that the SOS response can be detected when the colonies formed at 42°C are shifted to 30°C and allowed to grow for 18 h. Some additional experiments have shown that the same level of SOS response can be detected when the colonies are shifted to 30°C for only 3 h and then allowed to grow at 42°C for an additional 8 h. This short incubation time at 30°C allows for the recovery of viable cells.

E. coli AP1-200 and AP1-200-9 were transformed at 42°C with pM.HaeIII and pM.HpaII plasmids respectively and plated on LBA medium supplemented with ampicillin. After 18 h of incubation at 42°C, single colonies were used to inoculate prewarmed LB medium and incubated at 42°C until late logarithmic phase of growth. The cultures were shifted to 30°C and incubated for 1, 2 and 3 h. Half of each sample was grown at 42°C for 18 h, while the other half was chilled on ice for 18 h

1. An overnight culture of AP1-200 or AP1-200-9, grown at 42°C is diluted 1:100 into LB broth (prewarmed to 42°C). Incubation at 42°C with shaking (200 rpm) is continued until the cells reach an A_{600} of 0.25–0.3.
2. The cells are centrifuged (20 min, 4000 rpm) and suspended in 10 ml ice-cold 50 mM CaCl_2 , 10 mM Tris-HCl (pH 8.0). After 15 min the cells are centrifuged again and suspended in 2 ml of ice-cold CaCl_2 solution and kept on ice for 5 h.
3. For transformation, a 0.2 ml aliquot of cells is pipetted into a cold polypropylene tube containing 1 μl to 5 μl DNA (10–100 ng of the appropriate ligation mixture).
4. The cell/DNA mixture is incubated for 30 min at 4°C followed by a 2 min heat shock at 42°C.
5. LB broth (1.8 ml, prewarmed to 42°C) is added, and the cells are incubated at 42°C for 1 hr to allow expression of the antibiotic resistance gene.
6. Aliquots of cells (0.1 ml) are added to 3 ml of soft LB agar (45°C) containing X-gal (35 $\mu\text{g}/\text{ml}$) and the appropriate antibiotic. The cells are mixed with this medium and poured on 10 ml of LB agar plates prewarmed to 42°C containing X-gal (35 $\mu\text{g}/\text{ml}$) and the appropriate antibiotic. After the soft agar has solidified the plates are immediately transferred into a 42°C incubator.
7. After 18 hr of growth at 42°C, the plates are transferred to 30°C and incubated for 3 hr. The cells are reincubated at 42°C for 6–8 hr.
8. Blue colonies (the intensity of a blue color can be different depending on the cloned DNA methyltransferase gene and its expression) are identified and used to inoculate 10 ml LB broth (prewarmed (42°C) containing the appropriate antibiotic and incubated with shaking at 42°C for 18 hr.
9. Plasmid DNA is isolated from 8 ml of culture and the remaining 2 ml is frozen at -70°C , after addition of glycerol to 20%. Cells must always be inoculated into media that has been prewarmed to 42°C and grown at this temperature.
10. The isolated plasmid DNA is used to transform *E. coli* strains deficient in *mcrA*, *mcrB* or *mrr* systems, depending on the cloned DNA methyltransferase gene.

Figure 1. Procedure for Cloning of DNA methyltransferase genes

before the plasmid DNAs were isolated from all samples. The results from this experiment indicated that: (a) in all cases, the total yield of plasmid DNA was the same, (b) the plasmid DNA was resistant to cleavage by the appropriate restriction enzyme and (c) the plasmid DNA carried an active DNA methyltransferase gene as measured by its restriction during transformation of MM294, a strain carrying wild type *mcrA* and *mcrB* gene (data not shown).

Cloning of DNA methyltransferase genes using *E. coli* AP1-200 and AP1-200-9

Based on the results presented above, a procedure for selection of DNA methyltransferase genes from the appropriate bacterial gene banks was developed as summarized in figure 1. Using this protocol, we were able to identify a variety of DNA methylase clones (see table 2). The purity of each clone was checked after the isolation of plasmid DNA by electrophoresis. If the plasmid DNA isolated was from a single transformation event, all the transformants should give the positive SOS response. The purified plasmid DNA was used to retransform the strain originally used for the selection with the standard protocol. When necessary the blue colonies formed after this second transformation were further analyzed. All transformants isolated were able to induce the SOS response.

The final step in this procedure is to transform the plasmid DNA isolated from the selected clones into *E. coli* strains that are defective in the *mcrA*, *mcrB* or *mrr* genes, depending on the type of the cloned DNA methyltransferase gene. The introduction of the cloned DNA methyltransferase into a Mcr or Mrr nuclease deficient background assures that there will be no selective pressure against the loss of the plasmid or an accumulation of the clones carrying mutated DNA methyltransferase genes.

To test the developed procedure, gene banks were prepared from the following bacteria: *Moraxella* sp., *Neisseria gonorrhoeae*, *Haemophilus influenzae* Rd, *Pseudomonas aeruginosa*, *Xanthomonas oryzae*, *Saccharopolyspora erythraea* and *Treponema hyodentariae*. These gene banks were used to transform both AP1-200 and AP1-200-9 according to the procedure outlined in figure 1. After selection of the presumptive clones carrying cloned DNA methyltransferase genes, the plasmid

DNAs were isolated and used to transform ERI562 or DH5MCR. Each clone was tested for the presence of a cloned DNA methyltransferase gene by: (a) isolation of the plasmid DNA (b) if possible, by testing the resistance of plasmid DNA to digestion with an appropriate restriction enzyme, and (c) determination of *in vitro* DNA methyltransferase activity in the crude extracts obtained from appropriate subclones.

The results presented in Table 2 show that the constructed strains and the new strategy for cloning of DNA methyltransferase genes allows for very fast and efficient cloning of such genes. Of the 143 clones described in Table 2, all encoded a DNA methyltransferase, as detected by either imparting resistance to cleavage with a known restriction enzyme or by the presence of DNA methylating activity in crude extracts. In contrast to the existing strategies for cloning of DNA methyltransferase genes (1) it allowed us not only to clone the predicted DNA methyltransferase genes, as in the case of *mspIM* from *Moraxella* sp., but also a great variety of DNA methyltransferase genes whose existence was not known (For example, *thylM* (an isoschizomer of *PstI*) from *T. hyodentariae* or *serIM* from *S. erythraea*). The use of gene banks prepared after cleavage of chromosomal DNA with different restriction enzymes can substantially increase the variety of cloned DNA methyltransferase genes isolated, as was observed in the case of *N. gonorrhoeae*. At present, we do not know the limitations of this cloning method. One would assume that only those DNA methyltransferase genes whose activity makes the methylated DNA sensitive to the action of the McrA, McrB or Mrr nucleases could be cloned. The determination of the recognition sequences for all of the DNA methylases that we have cloned will help provide a final answer.

Additional Applications

Since most DNA methyltransferases are linked to genes encoding the corresponding restriction endonucleases, this method should allow for the rapid identification of clones encoding these enzymes as well. This is demonstrated by our cloning the DNA encoding the restriction endonuclease *MspI* concomitant with the cloning of *M.MspI*. It is also possible to clone restriction enzymes in the absence of DNA methylases, if the level of expression of

Table 2. Methyltransferase clones selected by AP1-200 and AP1-200-9

Bacterial specificity ^d species	number of ^a transformants	number of ^b clones	system ^c	
<i>Moraxella</i> sp.	800	12	S.MspI	CCGG
<i>Neisseria gonorrhoeae</i> WR302	750	2	ND ^e	ND
<i>N. gonorrhoeae</i>	30000	42	S.NgoI	(A/G)GCGC(T/C)
			S.NgoV	GGNNCC
			S.NgoVI	GATC
			S.NgoVII	GC(C/G)GC
			S.NgoX	ND
			S.NgoXI	ND
<i>Haemophilus influenzae</i> RD	900	2	S.HincII	GT(T/C)(A/G)AC
<i>Treponema hyodentariae</i>	1500	70	S.ThyI	CTGCAG
<i>Xanthomonas oryzae</i>	800	7	ND	ND
<i>Pseudomonas aeruginosa</i>	900	6	ND	ND
<i>Saccharopolyspora erythraea</i>	1400	2	ND	ND

^aThe number of transformants is the number of recombinant plasmids screened.

^bThe number of clones is the number of colonies that were able to induce the SOS response.

^cThe system is the DNA methyltransferase system cloned.

^dSpecificity is the recognition sequence of the cloned system.

^eND = not determined.

the enzyme is low, as these clones will induce the SOS response at both 30°C and 42°C.

Several research groups are currently attempting to clone DNA methyltransferase enzymes from a variety of eucaryotic organisms. The system that we have constructed lends itself to these types of applications, where one would need only to screen cDNA libraries for colonies that give a positive signal. These clones would most likely encode DNA methyltransferase genes.

There are many research groups that would like to extend the recognition sequence of previously cloned DNA methyltransferases. However, in the absence of restriction endonucleases with novel specificities, the detection of these clones will be tedious. The system that we have constructed has the ability to identify DNA methylation activity, and should allow for the detection of novel DNA methylation activities.

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