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The 'endo-blue method' for direct cloning of restriction endonuclease genes in *E.coli*

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ABSTRACT

A new *E.coli* strain has been constructed that contains the *dinD1*::*LacZ*$^+$ fusion and is deficient in methylation-dependent restriction systems (McrA$^-$, McrBC$^-$, Mrr$^-$). This strain has been used to clone restriction endonuclease genes directly into *E.coli*. When *E.coli* cells are not fully protected by the cognate methylase, the restriction enzyme damages the DNA *in vivo* and induces the SOS response. The SOS-induced cells form blue colonies on indicator plates containing X-gal. Using this method the genes coding for the thermostable restriction enzymes *Taq*I (5'TCGA3') and *Tth*111I (5'GACNNNGTC3') have been successfully cloned in *E.coli*. The new strain will be useful to clone other genes involved in DNA metabolism.

INTRODUCTION

The SOS response in *E.coli* is induced by DNA damaging agents or drugs that inhibit DNA replication (reviewed in refs 1, 2). A set of about 20 genes are part of this regulon. One of these is *dinD1* to which a *lacZ* fusion has been made by insertion of Mu dI phage (Ap^r or Kan^r, LacZ$^+$, refs. 3, 4). In cells carrying this fusion the level of β-galactosidase increases following treatment with DNA damaging agents such as UV radiation or mitomycin treatment, or when DNA breaks/nicks are generated by the *Eco*RI or *Bam*HI restriction endonucleases or by McrA, McrBC, or Mrr (3–11).

McrA, McrBC, and Mrr are methylation-dependent endonucleases of *E.coli* K12 that cleave target sites only when they are methylated (reviewed in refs 12, 13). Plasmids carrying one of many methylase genes have lower transformation efficiency into cells that are McrA$^+$, McrBC$^+$, and Mrr$^+$ (14–16). Expression of methylase genes in wild-type K12 induces expression from the *dinD* locus (5). Temperature sensitive *mcrA*, *mcrBC*, *mrr* strains that carry the *dinD1*::*lacZ* fusion were constructed and used for direct cloning of methylase genes into *E.coli* from other bacterial sources (17, 18). Upon transformation of ligated genomic/vector DNA into such a strain, transformants containing an appropriate methylase gene form white colonies at 42°C and blue colonies at 30°C on X-gal indicator plates as a result of SOS DNA repair induction and β-galactosidase expression. Because most methylase genes are closely linked to the cognate restriction enzyme genes, cloning of a methylase gene on a reasonable size DNA fragment may lead to concomitant cloning of the cognate endonuclease gene.

We are interested in methods for direct cloning of restriction endonuclease genes in *E.coli*. There are two general methods for cloning of restriction endonuclease genes (19–22). The first method uses phages to select clones from libraries that contain endonuclease gene since the presence of restriction-modification (RM) systems in bacteria enables them to partially resist infection by bacteriophages. The phage challenge method requires expression of the endonuclease gene to an appropriate level to allow restriction of incoming phages (19, 20). However, not all endonuclease genes are appropriately expressed in *E.coli* and the selection often does not work. Another cloning approach is to select for an active methylase gene since restriction and modification genes are often closely linked, both genes can often be cloned simultaneously. Although a majority of endonuclease genes have been obtained using the methylase selection method, the method sometimes yields only the methylase genes (21–25).

It has been shown that DNA breaks/nicks introduced by T7.3 endonuclease (phage T7 gene 3 product), *Eco*RI, or *Bam*HI induce the SOS response in *E.coli* (6, 9, 10). We reasoned that when ligated genomic DNA fragments and vector are introduced into a *dinD1*::*LacZ*$^+$ indicator strain deficient in methylation-dependent restriction and transformants plated on X-gal plates, one might find the endonuclease-containing clones directly by picking blue colonies. In order to inflict the *in vivo* DNA damage, the expression of the methylase gene should not fully protect the host chromosome or the methylase gene should be absent. In this paper we demonstrate that indeed the new *dinD1*::*LacZ*$^+$ indicator strain (*hsd*, *mcrA*, *mcrBC*, *mrr*) can be used for direct cloning of restriction endonuclease genes. The strategy described here differs from the method used to clone methylase genes (17) although both methods rely on increases in β-galactosidase expression as an indicator. The method to clone methylase genes in a *dinD1*::*LacZ*$^+$ host is based on the DNA damage elicited by methylation-dependent restriction systems McrA, McrBC or Mrr on methylated DNA. Our method (the 'endo-blue method') depends on the expression of a restriction endonuclease gene in the *dinD1*::*LacZ*$^+$ host and the production of DNA damage on unmethylated DNA.

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MATERIALS AND METHODS

Bacterial strains and constructions

Thermus aquaticus YT-1 (ATCC #25104), *Thermus thermophilus* 111 and *E.coli*H709c were from the New England Biolabs' collection. *E.coli* strains JH140 *dinD1*::Mu dI1734 (Kan^r, LacZ$^+$) (4); RR1 (F$^-$ l$^-$ *leu* Δ*(gpt-proA)62 supE44 ara-14 galK2* Δ*(mcrC-mrr) rpsL20 xyl-5 mtl-1 thi-1 lacY*) (26, 27); ER1458 (F$^-$ λ$^-$ Δ*(argF-lac)U169 lon-100 hsdR2 adaD139 rpsL* (Str^r) *supF58 mcrA zjj202*::Tn*10* (Tet^r) *mcrB1*) (28); and ER1821 (F$^-$ λ$^-$*supE44 e14*$^-$ *rfbD1? relA1? endA1 spoT1? thi-1* Δ*(mcrC-mrr)114::IS10*) were previously described (29). ER1578 is ER1458 transformed with pMC9 (Ap^r *LacI*$^+$). ER1991 is described below. ER2267 is ER1991 *recA1 F' proA*$^+$*B*$^+$ *lacI*^{q} Δ*(lacZ)M15 zzf*::miniTn*10* (Kan^r) and was constructed by standard methods (30).

ER1992 (F$^-$ λ$^-$ Δ*(argF-lac)U169 supE44 e14*$^-$ *dinD1*::Mu dI1734 (Kan^r, LacZ$^+$) *rfbD1? relA1? endA1 spoT1? thi-1* Δ*(mcrC-mrr)114::IS10*) was constructed in three steps: (i) A Lac$^-$ derivative of ER1821 was obtained by transduction with a *proC*::Tn*5* linked to Δ*(argF-lac)U169* from NK6993, selecting for Kan^r and screening for Lac$^-$ Pro$^-$ to yield ER1984; (ii) this derivative was made Pro$^+$ Kan^s by transduction from ER1578, yielding ER1991; (iii) *dinD1*::Mu dI1734(Kan^r, LacZ$^+$) was introduced by transduction from JH140, selecting KanR and screening for nalidixic acid-inducible expression of β-galactosidase mediated by the *dinD* fusion. This was tested on X-gal plates with a central well containing this DNA-damaging agent. Purified transductants were streaked radially from the well. One that yielded a gradient of dark blue color was designated ER1992. This strain showed light blue color on X-gal in the absence of any DNA damage.

Media and reagents

Luria-Bertani (LB) medium and LB agar were prepared as described previously (31). When cells contained plasmids coding for ampicillin resistance (Ap^r), media were supplemented with 50 μg/ml ampicillin. 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal) was added to media at a final concentration of 80 μg/ml. All restriction enzymes, DNA modifying enzymes, vector DNA, and DNA size markers were from New England Biolabs.

Plasmid and genomic DNA preparation and transformation

Plasmid DNA was prepared on a small scale (1.5 ml cell culture) by the boiling method with minor modifications (32) and on a large scale (250−500 ml cell culture) by Qiagen column purification according to the manufacturer's instruction (Qiagen Inc., Chatsworth, CA). After the boiling step and centrifugation, the supernatant was extracted once with phenol-$CHCl_3$ and once with $CHCl_3$ and plasmid DNA precipitated with ethanol. *Thermus aquaticus YT1* and *Thermus thermophilus 111* genomic DNA were prepared as described (31). *E.coli* cells were made competent by growing cells in SOB medium followed by $CaCl_2$ treatment (31). Ligated genomic and vector DNA were then introduced using standard procedures (31).

Library construction

Genomic DNA from *T.aquaticus*, *T.thermophilus*, or *E.coli* 709c was partially digested with *Sau*3AI and 10−30 μg of the cleaved genomic DNA was ligated with 1−3 μg of *Bam*HI-digested and dephosphorylated pBR322. The ligation mixtures (approximately 100 ng) were introduced into ER1992 (for the endo-blue method) or ER2267 (for the methylase selection method) by transformation. In the methylase selection method, the *T.thermophilus* and *E.coli*709c libraries were processed according to previously described (23).

Preparation of cell extract and restriction enzyme activity assay

Individual blue colonies were picked and inoculated into 10 ml of LB plus Ap and incubated overnight in a shaker at 37°C. Cells were harvested by centrifugation and resuspended in 1 ml of sonication buffer (10 mM Tris−HCl, pH 7.8, 2 mM EDTA, pH 8.0, 10 mM β-mercaptoethanol) plus lysozyme (10 mg/ml). Cell lysis was completed by sonication. Prior to assaying thermalstable restriction endonuclease activity, *E.coli* proteins were heat-denatured by incubation of the lysate at 65°C for 30 min. Insoluble components were removed by centrifugation and the supernatant was used for endonuclease activity assay. DNA substrates λ and pBR322 DNA were incubated with varying amount of cell extract at 65°C for one hour to overnight. DNA fragments were resolved in 0.8% agarose gels and detected by ethidium bromide staining.

RESULTS

Cloning of *taqIR* and *tth111IR* genes in *E.coli*

The *Sau*3AI partially digested genomic DNA from *Thermus aquaticus* YT-1 (ATCC 25104) was ligated with *Bam*HI-cleaved and CIP-treated pBR322 DNA. A total of 4,000 transformants of ER1992 were obtained from one transformation experiment. Ten blue colonies were found. Each was inoculated into 10 ml of LB plus Ap and incubated overnight at 37°C in a shaker. When cell extracts were examined for endonuclease activity on pBR322 DNA substrate, two isolates yielded *Taq*I endonuclease activity. The *Taq*I activity assay of one isolate is shown in Figure 1 (lane 2). Plasmid DNA was extracted from these two isolates and subjected to *Taq*I endonuclease digestion. One plasmid was partially resistant to *Taq*I digestion and the other was completely digested (data not shown). We inferred from the above result that one clone contains the *Taq*I methylase gene and the second clone may not. To estimate *Taq*I endonuclease yield, a one liter cell culture was made and the cell extract assayed for activity. Both isolates yielded 5×10^4 units of *Taq*I per gram of wet cells (data not shown).

The above procedure was repeated using genomic DNA prepared from *Thermus thermophilus* 111 which produces *Tth*111I and *Tth*111II. Forty blue colonies were found among 8,000 transformants. Fourteen out of forty blue isolates yielded *Tth*111I endonuclease activity. The *Tth*111I activity from one isolate is shown in Figure 1 (lane 6). To examine methylation of *Tth*111I site by the cognate methylase, plasmid DNAs prepared from these isolates were digested with *Tth*111I endonuclease. Three isolates were partially resistant to *Tth*111I digestion (data not shown), suggesting that the methylase gene is also present in the cloned DNA fragment. The *E.coli* strains carrying *tth111IR* gene produce approximately $1-4\times10^4$ units of *Tth*111I endonuclease/gram of wet cells.

No *Tth*111II [5'CAARCA3' (11/9)] activity was detected among 40 blue isolates, which may be due to low specific activity of *Tth*111II (Robinson, D.P. personal communication).

In order to compare this method with the methylase selection method, the same *Sau*3AI partial plasmid library was digested

with *Tth*111I restriction endonuclease for two hours at 65°C and the resulting DNA was used to transform ER2267 competent cells. The transformation results were summerized in Table 1 (lines 3 and 4). Plasmid DNA was prepared from cell cultures of 36 transformants and digested with *Tth*111I endonuclease. Six plasmid DNAs are resistant to *Tth*111I digestion. Among the six resistant clones two plasmids suffered large deletions (the *Tth*111I site in pBR322 vector was lost in the two deletion clones, data not shown). When overnight cell cultures containing the remaining four resistant clones were examined for *Tth*111I activity, two isolates produced *Tth*111I endonuclease (4×10^4 units and 4×10^3 units/gram of wet cells, respectively). Therefore, 2 out of 36 isolates produce *Tth*111I endonuclease activity using the methylase seletion (6% R^+), while 14 out of 40 blue isolates produce *Tth*111I with the 'endo-blue' method (35% R^+).

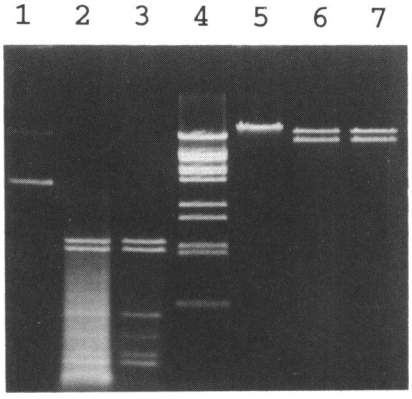

Figure 1. Assay of *Taq*I and *Tth*111I endonuclease activity in cell extracts. Lane 1, uncut pBR322 DNA; lane 2, pBR322 cleaved with cell extract containing *Taq*I endonuclease; lane 3, pBR322 cleaved with purified *Taq*I; lane 4, *Bst*EII-cleaved λ DNA size standard; lane 5, uncut λ DNA; lane 6, λ DNA cleaved with cell extract containing *Tth*111I endonuclease; lane 7, λ DNA cleaved with purified *Tth*111I endonuclease. *Taq*I and *Tth*111I restriction digestions were performed at 65°C for one hour in buffers as described in New England Biolabs catalog 1993/94.

Cloning of the *ecoO109IR* gene by a combination of methylase selection method and blue colony screening

We also tested the possibility of combining two methods, the methylase selection and blue colony screening. Genomic DNA was prepared from *E.coli* H709c cells (the *Eco*O109I producing strain) and partially digested with *Sau*3AI and ligated to pBR322 (*Bam*HI-cleaved and CIP-treated). The ligation DNA mixture was transformed into RR1 competent cells. About 10^5 transformants were pooled from plates and inoculated into one liter of LB medium. Plasmid DNA was prepared from an overnight cell culture and digested with *Eco*O109I. The digested plasmid DNA was used to transform ER1992 competent cells and transformants plated on Ap, X-gal plates. Fourteen blue colonies were found among 120 transformants. Ten ml of cell culture was made from all 14 blue isolates and cell extracts assayed for *Eco*O109I endonuclease activity. Apparent *Eco*O109I endonuclease activities were detected in eight isolates (57% R^+). By combining the methylase selection method and the blue colony screening method one could eliminate those clones that carry only the methylase gene or vector mutants that have lost cleavage sites after challenge, identifying those that carry the endonuclease gene alone or together with a less-expressed methylase gene.

In the methylase selection method, the *Eco*O109I-digested plasmid library was also used to transform ER2267 and transformants were screened for resistant to *Eco*O109I digestion. Three partial resistant clones were found among 24 screened. Two out of three partial resistant clones produced *Eco*O109I endonuclease activity. Therefore, 2 out of 24 survivors are R^+ (8%).

Table I. Summary of results with different cloning methods

Source of DNA	1st test	# of transformants (tf)	# tf pass test	# tested further	R^+	# tested further that were M^+	$M^\pm$	M^-	Freq of R^+ clones
a	b	c	d	e	f	g	h	i	j
1. *T.aquaticus*	endo-blue	4×10^3	10	10	2	0	1	1	5×10^{-4}
2. *T thermophilus*	endo-blue	8×10^3	40	40	14	0	3	11	2×10^{-3}
3.	*Tth*111I 40 u	$>10^3$	30	18	2	3	0	15*	$\leq3\times10^{-3}$
4.	*Tth*111I 5 u	$>10^3$	100	18	0	1	0	17	$<3\times10^{-3}$
5. *E.coli*H709c	*Eco*O109I	$\sim10^5$	120	24	2	0	3	21	1×10^{-4}
6.	*Eco*O109I+ endo-blue	$\sim10^5$	14	14	8	NT	NT	NT	8×10

Data on clone recovery using the endo-blue method (lines 1–2), the methylase selection method (lines 3–5) or a combination (line 6). Columns show:
a: Organism.
b: Criterion used for further evaluation was SOS-induction ('endo-blue') or plasmid survival of digestion by the indicated amount of restriction endonuclease.
c: Number of transformants evaluated. For lines 1–2, those resulting from the ligation mix; for lines 3–6 those resulting from retransformation of the unselected plasmid pool.
d: Number of blue colonies on the original plates (lines 1–2) or number obtained after retransformation of the selected plasmid pool (lines 3–5) or blue colonies formed from the selected plasmid pool.
e. Number in column (d) carried further, tested next either for endonuclease production or for plasmid resistance to cleavage.
f–i: Production of endonuclease detectable in crude extract (R^+), or production of methylase *in vivo* sufficient to protect completely (M^+), partially ($M^\pm$), or not at all (M^-) from digestion by the relevant endonuclease *in vitro*. In lines 1, 2, 6, all isolates of column (e) were tested for R^+ and (lines 1–2) positives were tested for M; in lines 3–5, the order was reversed. M^- clones from lines 3–5 were not tested for endonuclease production.
j: Frequency of R^+ clones in the library was calculated as: [fraction of R^+ clones among those rescreened (=f/e)]×[fraction of library that passed the first selection/screen (=d/c)]. Note that in column c, lines 1–2 and lines 3–6 do not measure the same quantity. See text and Fig. 1.
*Two plasmids in this group were resistant to digestion by *Tth*111I but also carried vector deletions spanning the *Tth*111I site.
NT= not tested.

Fraction of RM systems identifiable by the combination method

In an effort to assess how frequently the endo-blue method should be applicable, we surveyed primary clones that had been obtained using the methylase selection method for ability to induce SOS response in ER1992. Systems were classified according to whether they formed dark blue, medium blue, or light blue colonies (the strain forms light blue colonies with no plasmid or with the vector). Dark blue colonies were formed by *Aat*IIRM, *Ava*IIRM, *Bbv*IRM, *Hpa*IIRM, and *Msc*IRM. Transformants of *Nla*IIIRM, *Sap*IRM, and *Xmn*IRM form medium blue colonies. The *Nco*IRM, *Sfi*IRM, *Sma*IRM, and *Xcm*IRM transformants, however, form light blue colonies. (These strains are from the New England Biolabs' collection, unpublished data).

DISCUSSION

The screening method for isolation of restriction endonuclease clones described here makes use of DNA-damage-inducible expression of β-galactosidase from the *dinD1*::*LacZ*$^+$ fusion originally described by Kenyon and Walker (3) and subsequently modified by Heitman and Model (4). Its use here is based on the expectation that newly created restriction endonuclease clones will frequently be imperfectly adapted to the new host and sequence context. The methylase gene, required to protect endogenous DNA from the action of the endonuclease, may be poorly expressed or absent altogether. The action of restriction enzymes on endogenous DNA *in vivo* is known to induce the SOS response (4−11). Clones can be identified by blue color on X-gal plates, as shown for *Taq*I, *Tth*111I, and *Eco*O109I (Figure 1 and Table 1).

The endo-blue method is simpler than the methylase selection method. The latter includes pooling all transformants in the primary library, making plasmid DNA pool, selective digestion, retransformation, plasmid DNA preparation from individual survivors, digestion, identification of resistant clones and assaying endonuclease activity in the cell extract of the resistant clones. The endo-blue method identifies blue colonies directly in the primary library and assays endonuclease activity among the blue isolates. In the endo-blue method, however, isolation of viable clones with active endonuclease does require a reparable level of damage. What this level is will depend on whether the methylase gene is present but inadequately expressed, or is absent altogether. For two reasons, our method is likely to be most useful for cloning genes encoding thermalstable restriction enzymes, as with *Taq*I and *Tth*111I. First, native nucleases and other proteins can be heat-denatured to enhance the sensitivity of crude extract assays. Second, the endonuclease will be partially inactive during cell growth but fully active during testing. Most endonucleases from thermophilic organisms have temperature optima well above the *E.coli* growth range. Thus, a level of expression high enough to detect *in vitro* at the endonuclease optimum temperature may still be low enough *in vivo* at *E.coli* growth temperature to spare the cell from lethal damage. This appears to be the case for many *Tth*111I clones.

A second major use is in screening survivors of methylase selection (the combination method), as was done here with *Eco*O109I (Table 1, line 6). This will reduce the amount of work needed to identify an R$^+$ clone. A large fraction (6/14) of initial clones obtained by selecting first for methylase activity nevertheless are still capable of inducing SOS response by this assay. These six may be unstable clones. When a blue colony was inoculated into 10 ml LB and cultured overnight, the unstable clones may delete out insert DNA, leading to non-detectable endonuclease activity in the cell extract. It is also possible to clone a *lacZ* gene from the original endonuclease-producing strain.

Data in Table 1 allow us to estimate the enrichment for the methylase gene achieved by the methylase selection: from line 2, a minimum of 3/8000 clones carried the M gene in the absence of selection, while 4/36 carried the M gene with selection (lines 3 and 4), for an enrichment of 296-fold. However, this enrichment does not yield a population containing solely the desired gene (Table 1, lines 3−5). As seen here with *Eco*O109I, screening of survivors of selection by the endo-blue method used here can identify those clones that carry the endonuclease gene in addition to the methylase gene.

As with the methylase selection method, there is a background with the endo-blue method. Some of these may simply be R$^+$ clones that express too little endonuclease to detect by our assay. This may account for failure to recover *Tth*111II clones. In contrast with *Tth*111I, *Tth*111I is a Type II-S enzyme, recognizing an asymmetric site; such enzymes frequently have a low specific activity. In addition, other kinds of enzymes might induce SOS if overproduced or if specificity were relaxed when expressed in a foreign cytoplasm. For example, transposases are known to do this (33). Single-stranded DNA nucleases also induce SOS response (ref. 9 and A.Fomenkov and S.-y.Xu, unpublished result).

This method differs from one described previously (the 'methylase indicator method'; 17), in that the earlier method indirectly detected expression of the methylase, via the DNA-damaging action of endogenous methylation-specific restriction enzymes. Our method detects the endonuclease only and not the methylase, because the relevant methylation-dependent restriction systems are absent from the host. We have not evaluated the methylase indicator method in this experiment.

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