Positive Selection of Recombinant DNA by CcdB

Philippe Bernard
Institut de Biologie, Montpellier, France

BioTechniques 21:320-323 (August 1996)

Cloning or subcloning a DNA fragment into a plasmid vector is a routine procedure in modern recombinant DNA technology. After restriction or modification with appropriate enzyme to generate compatible termini, the insert DNA and the linearized plasmid molecule are joined by ligase. Plasmid recircularization can occur with or without integration of the insert DNA. Only a fraction of subsequently transformed bacterial cells acquires a plasmid recombined with the insert DNA. We are faced with the problem of separating bacteria carrying the insert DNA from the majority of nonrecombinants. The blue/white screening allows a color discrimination of recombinants from nonrecombinants (16) but has no incidence on the overall cloning efficiency. To reduce the number of colonies to be screened for recombinant plasmids, alkaline phosphatase can be used to remove 5′ terminal phosphate from vector DNA to limit self-ligation. However, the extent of dephosphorylation is variable, and significant background may subsist. To overcome those limitations, an innovative technology allowing direct positive selection of inserts by means of disruption of the lethal *ccdB* gene has been developed (5). As only recombinant DNA allow formation of viable colonies, CcdB selection revolutionizes the cloning procedures by elimination of vector dephosphorylation and blue/white color screening.

The F plasmid contains two genes, *ccdA* and *ccdB*, which contribute to the plasmid stability by killing newborn daughter cells that have not inherited a plasmid copy at cell division (14,18). The *ccdB* gene product is a potent cytotoxin of 101 amino acid residues whose activity is negatively regulated by the CcdA protein (17). Because half-life of the CcdA antidote is shorter than that of the CcdB poison, persistance of the CcdB protein leads to the death of F^- bacterial segregants (19). To investigate the mechanism by which the CcdB protein kills cells, an *E. coli gyrA*462 resistant mutant was isolated (3). Tetrameric A_2B_2 gyrase, the bacterial topoisomerase II, is an essential enzyme that catalyzes the ATP-dependent negative supercoiling of DNA. Gyrase alters DNA topology by effecting a transient double-strand break in the DNA backbone, passing the double helix through the gate and resealing the gaps. The CcdB poison acts by trapping DNA gyrase in a cleaved complex with the gyrase A subunit covalently closed to the cleaved DNA (6). The CcdB three C-terminal residues play an important role in the killer function (1), and the poison was observed to be active towards *Pseudomonas aeruginosa* gyrase (A. Gaballa and P. Cornelis, personal communication) but inactive on mammalian topoisomerase II.

By handling a potent killer gene and a *gyrA*462 resistant mutant, which allows propagation of vectors expressing the lethal gene, I got the idea of using *ccdB* for the selection of recombinant DNA. A multiple cloning site (MCS) with 13 endonuclease recognition sequences was fused to the promoter-proximal end of the *ccdB* gene (5). The fused-CcdB protein, although a very less potent killer than the shorter wild-type product, has been proven to be an efficient positive-selection effector. The CcdB-selection system was used to clone, with no trace of background, the seven pBR322 *Taq*I fragments whose sizes range from 141 to 1444 bp. Introduction of +1 or +2 frame shifts at level of the MCS was observed to prevent the inhibition of growth normally mediated by the *ccdB* gene (2). Cloning experiments performed at Invitrogen laboratories (San Diego, CA, USA) with the Zero Background™ pZErO™-1 vector have shown that *ccdB* gene is inactivated by in-frame insertions as small as 75 bp (12,13).

The CcdB selection simplifies cloning procedures to the

utmost: linearization of the vector (even partial) and inactivation of the restriction endonuclease, ligation of the insert, transformation and picking of colonies. The ratio of nonrecombinant to recombinant is very low but is influenced by the quantity and the quality of the insert DNA. Major potential contributions to background derive only from *ccdB* disruption by nucleases or spontaneous mutagenesis. Since most restriction endonuclease preparations are contaminated with traces of exonucleases, it is preferable not to over-digest the vector to avoid degradation of the vector termini and disruption of the *ccdB* frame. Background related to partial digestion or perfect recircularization of the vector is very low because the frequency of *ccdB* mutations resulting in viability of a selective host approximates 10^{-5} when transformed with a closed circular pKIL or pZErO vector (5,13). Deletions and rearrangements that can be observed in cloning experiments can also be due to in vivo recyclization of linear molecules present in ligation reaction mixtures (8).

CcdB selection improves the efficiency of shotgun library construction and should facilitate the cloning aspects of genome sequencing projects. Large-scale DNA sequencing strategies depend upon random fragmentation of the target molecule into small pieces, subcloning into a vector and enzymatic sequencing analysis. CcdB selection alleviates the need for DNA sample analysis on agarose gel, while the effort of sequencing self-religated vector is reduced to zero. In addition, by treating DNA to be inserted with alkaline phosphatase, CcdB selection allows the construction of low background shotgun libraries with a reduced number of chimeric clones. Thus, the assembling of sequence data for overlapping is facilitated. Note that in case of blunt-ended fragments, dephosphorylation of insert DNA can lead to an appreciable

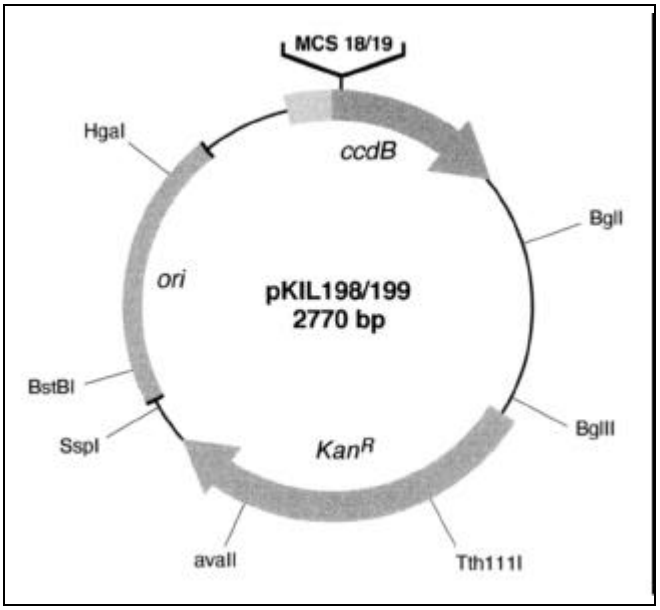

Figure 1. Structure of plasmids pKIL198/199. The location of unique restriction sites, the kanamycin resistance gene, the p15a origin of replication (*ori*) and the *ccdB* gene with the preceding *lacP* promoter are represented. These positive-selection vectors have an intermediate-low copy number replicon derived from pACYC184. They were constructed by cloning the *Eco*RI-*Bgl*I fragments from pKIL128/129 into the *Eco*RI + *Bgl*I-digested pK184/pK194 (15). Their complete nucleotide sequences appear in the EMBL, GenBank® and DDBJ Nucleotide Sequence Databases under the Accession Nos. L47836 and L47837. Two *Xba*I sites are present in pKIL198/199.

drop of ligation efficiency. The "Turbo cloning procedure" (7) combined with *ccdB* positive selection could allow forced cloning of blunt-ended DNA with an efficiency approaching that obtainable in cohesive-end cloning.

Most of the blue/white screening vectors should be converted into powerful positive-selection cloning tools by replacing the *lacZa*-complementation gene with *ccdB.* A pair of positive-selection vectors with a p15a origin of replication and a kanamycin selectable marker has been developed (Figure 1). These intermediate-copy-number pKIL198/199 vectors are compatible with ColEI replicon and are thus able to coexist in the same bacteria with plasmids like pBR or pUC. They can be used in experiments that require the presence of more than one recombinant plasmid per cell and find application in genetic complementation tests where adverse effects due to elevated gene dosage will be minimized.

To investigate the ability of small in-frame insertions to inactivate the *ccdB* gene, the 141-bp *Taq*I DNA fragment of pBR322 was ligated with *Acc*I-linearized pKIL199 plasmid. TOP10F′ transformants were plated on LB medium containing 50 µg/mL kanamycin and 1 mM isopropylthio-β-D-galactoside (IPTG). After an overnight incubation at 37°C, two types of colonies distinguishable by their sizes were present in equivalent ratio. A dozen clones from each type were cultivated in liquid LB without IPTG to prevent transcription from the *lacp*, and the plasmid DNA was extracted. Restriction endonucleases analysis showed all the clones were recombinants. The orientation of the 141-bp DNA insert was tightly related to the size phenotype of original clones. In faster growing clones, the DNA fragment was inserted in an antisense orientation with respect to *ccdB*, and translation, according to the DNA sequence of pBR322, is disrupted by stop codons. In smaller clones, the insert had a sense orientation with no in-phase translational stop signals, and a residual activity of the *ccdB* gene product may be responsible for a longer generation time. In any case, the 141-bp DNA fragment was recovered in both orientations with a similar positive-selection cloning efficiency. A remarkable feature of the CcdB selection system is that the killer activity is regulated at two distinct levels when a *lac*I^q F′ strain is used as a selective host. When positive recombinants are grown without IPTG to restrain effects of possible partial *ccdB* disruption, the CcdA antidote encoded by the episome is liable to prevent any residual activity of the lethal gene.

A variety of other positive-selection systems have been reported. Most of these vectors are not well adapted for general use because of their large size, the limited range of useful cloning sites, high background, the need of a special selective host strain or complex culture medium. A positive-selection vector based on insertional inactivation of a modified lysis gene *E* of bacteriophage ϕX174 has been recently developed (11). Ten unique restriction sites were introduced within the native *E* gene without changing the original amino acid sequence of the lysis protein, which remains fully active and is able to prevent formation of false-positive colonies when overproduced from a *lac-tac* double promoter. However, the unique cloning sites are not clustered in a MCS but are dispersed through the *E* gene. Therefore, different sets of primers located at a relatively short distance from any of the cloning sites must be designed and used for sequencing according to the insertion point. An assay system selecting positively for colonies harboring LacZ$^-$ plasmids has been elaborated (9). A galactose-sensitive β-galactosidase-deficient *E. coli* strain must be used as selective host. Another disadvantage is cells have to be plated on a complex selective minimal agar medium supplemented with phenylgalactoside; bacterial growth rate is slowed down, and the cost of phenylgalactoside is rather high. A positive-selection strategy for shotgun cloning and sequencing in M13 was accomplished by insertional inactivation of the gene *X* protein, which is a specific inhibitor of phage M13 DNA synthesis when overproduced (10). Only one unique restriction site, placed between the Shine-Dalgarno sequence and the gene *X* start codon, is suitable for cloning, and the use of the strategy is restricted to cloning into M13 bacteriophages. Although positive selection represents the most efficient process to eliminate background of parental clones, its application for general use had not yet been widely adopted because reported positive-selection systems lacked the versatility of the well-known blue/white screening assay. The small size of the CcdB selection effector, its broad host range, the easy procedure for selection, the high number of useful cloning sites, the low background of nonrecombinants and the facility to propagate the cytotoxic vectors make pKIL or pZErO competitively stronger than all other reported positive-selection vectors.

The β-galactosidase (β-gal) inactivation assay, in which recombinant and parental vector molecules are visually distinguished as white and blue colonies, can prove subjectively difficult to interpret. A high expense is associated with the chromogenic compound 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-gal), which turns blue when cleaved by β-gal, and plates cannot be stored for long. Another disadvantage is a high cloning efficiency cannot be guaranteed without dephosphorylation of the vector. In addition, the formation of blue colonies by nonrecombinant DNA molecules is the result of α-complementation of β-gal activity by the *lacZa* gene product, and a special LacZ$^-$ host encoding for an α-acceptor protein is necessary. The α-acceptor protein is usually encoded by a *lac*ZΔM15 F′ episome, and the presence of F$^-$ bacteria in the pool of competent cells may increase the background of false recombinants. With *ccdB* positive-selection cloning, dephosphorylation of the vector is not required and neither a special selective host strain nor expensive X-gal is needed.

Positive selection relying on insertional inactivation of *ccdB* gene is filed for patent protection (4) and licensed to Invitrogen Corporation.

ACKNOWLEDGMENTS

Thanks are due to Ahmed Gaballa and Pierre Cornelis for communication of data. The author is very grateful to Albertina De Sario and Gérard Roizès for critical review of the manuscript and Solange Heerinckx, Bertrand Marçais, Alphonse Calenda, Jacques Puechberty and Patrick N. Higgins for useful comments. I also thank Yves Bernard and Magic Media for the http://www.magic.be/pbernard Web pages on the Internet. This work was supported by the CNRS.

REFERENCES

1. **Bahassi, E.M., M.A. Salmon, L. Van Melderen, P. Bernard and M. Couturier.** 1995. *ccdB* gene mutants coding for non-cytotoxic proteins which retain their regulatory functions. Mol. Microbiol. *15*:1031-1037.
2. **Bernard, P.** 1995. New *ccdB* positive-selection cloning vectors with

kanamycin or chloramphenicol selectable markers. Gene *162*:159-160.
3. **Bernard, P. and M. Couturier.** 1992. Cell killing by the F plasmid CcdB protein involves poisoning of DNA-topoisomerase II complexes. J. Mol. Biol. *226*:735-745.
4. **Bernard, P. and P. Gabant,** inventors; Université Libre de Bruxelles, assignee; Cloning and/or sequencing vector. PCT patent PCT/BE93/00051 and Belgian patent 09200696.
5. **Bernard, P., P. Gabant, E.M. Bahassi and M. Couturier.** 1994. Positive-selection vectors using the F plasmid *ccdB* killer gene. Gene *148*:71-74.
6. **Bernard, P., K. Kézdy, L. Van Melderen, J. Steyaert, L. Wyns, M.L. Pato, N.P. Higgins and M. Couturier.** 1993. The F plasmid CcdB protein induces efficient ATP-dependent DNA cleavage by gyrase. J. Mol. Biol. *234*:534-541.
7. **Boyd, A.C.** 1993. Turbo cloning: a fast, efficient method for cloning PCR products and other blunt-ended DNA fragments into plasmids. Nucleic Acids Res. *21*:817-821.
8. **Conley, E.C., V.A. Saunders and J.R. Saunders.** 1986. Deletion and rearrangement of plasmid DNA during transformation of *E. coli* with linear plasmid molecules. Nucleic Acids Res. *14*:8905-8932.
9. **Gossen, J.A., A.C. Molijn, G.R. Douglas and J. Vijg.** 1992. Application of galactose-sensitive *E. coli* strains as selective hosts for LacZ$^-$ plasmids. Nucleic Acids Res. *20*:3254.
10. **Guilfoyle, R.A. and L.M. Smith.** 1994. A direct selection strategy for shotgun cloning and sequencing in the bacteriophage M13. Nucleic Acids Res. *22*:100-107.
11. **Henrich, B. and B. Schmidtberger.** 1995. Positive-selection vector with enhanced lytic potential based on a variant of ϕX174 phage gene E. Gene *154*:51-54.
12. **Invitrogen Corporation.** 1995. Faster and easier cloning with the Zero Background™ Cloning Kit. Expressions *2.1*:1-3. San Diego, CA.
13. **Invitrogen Corporation.** Zero Background™ Cloning Kit, Instruction Manual, Version A. San Diego, CA.
14. **Jaffé, A., T. Ogura and S. Hiraga.** 1985. Effects of the *ccd* function of the F plasmid on bacterial growth. J. Bacteriol. *163*:841-849.
15. **Jobling, M.G. and R.K. Holmes.** 1990. Construction of vectors with the p15a replicon, kanamycin resistance, inducible *lacZa* and pUC18 or pUC19 multiple cloning sites. Nucleic Acids Res. *18*:5315-5316.
16. **Messing, J., B. Gronenborn, B. Müller-Hill and P.H. Hofschneider.** 1977. Filamentous coliphage M13 as a cloning vehicle: insertion of a *Hin*dII fragment of the *lac* regulatory region in M13 replicative form *in vitro*. Proc. Natl. Acad. Sci. USA *74*:3642-3646.
17. **Miki, T., K. Yoshioka and T. Horiuchi.** 1984. Control of cell division by sex factor F in *E. coli*, I. The 42.84-43.6 F segment couples cell division of the host bacteria with replication of plasmid DNA. J. Mol. Biol. *174*:605-625.
18. **Ogura, T. and S. Hiraga.** 1984. Mini-F plasmid gene that couple host cell division to plasmid proliferation. Proc. Natl. Acad. Sci. USA *80*:4784-4788.
19. **Van Melderen, L., P. Bernard and M. Couturier.** 1994. Lon-dependent proteolysis of CcdA is the key control for activation of CcdB in plasmid-free segregant bacteria. Mol. Microbiol. *11*:1151-1157.

Address correspondence to Philippe Bernard, BioRix, 13 Avenue Françoise, 1330 Rixensart, Belgium. Internet: pbernard@dbm.ulb.ac.be