APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1997, p. 1917–1924 Vol. 63, No. 5
0099-2240/97/$04.00+0
Copyright © 1997, American Society for Microbiology

Combining the *hok/sok*, *parDE*, and *pnd* Postsegregational Killer Loci To Enhance Plasmid Stability

DOUG C. PECOTA,[1] CRAIG S. KIM,[1] KUOWEI WU,[2] KENN GERDES,[3] AND THOMAS K. WOOD[1]*

Department of Chemical and Biochemical Engineering, University of California, Irvine, Irvine, California 92697-2575[1]; GeneMedicine, Inc., Woodlands, Texas 77381[2]; and Department of Molecular Biology, Odense University, DK-5230 Odense M, Denmark[3]

Received 30 October 1996/Accepted 2 March 1997

To enhance plasmid segregational stability in bacterial cells, two pairs of independent postsegregational killing loci (genes which induce host killing upon plasmid loss) isolated from plasmids R1, R483, or RP4 (*hok*$^+$/*sok*$^+$ *pnd*$^+$ or *hok*$^+$/*sok*$^+$ *parDE*$^+$) were cloned into a common site of the β-galactosidase expression vector pMJR1750 (*ptac*::*lacZ*$^+$) to form a series of plasmids in which the effect of one or two stability loci on segregational plasmid stability could be discerned. Adding two antisense killer loci (*hok*$^+$/*sok*$^+$ *pnd*$^+$) decreased the specific growth rate by 50% though they were more effective at reducing segregational instability than *hok*$^+$/*sok*$^+$ alone. With the *ptac* promoter induced fully (2.0 mM isopropyl-β-D-thiogalactopyranoside) and no antibiotic selection pressure, the combination of a proteic killer locus (*parDE*$^+$) with antisense killer loci (*hok*$^+$/*sok*$^+$) had a negligible impact on specific growth rate, maintained high β-galactosidase expression, and led to a 30 and 190% increase in segregational stability (based on stable generations) as compared to plasmids containing either *hok*$^+$/*sok*$^+$ or *parDE*$^+$ alone, respectively. Use of *hok*$^+$/*sok*$^+$ or *parDE*$^+$ alone with high cloned-gene expression led to ninefold and fourfold increases in the number of stable generations, respectively. Two convenient cloning cassettes have been constructed to facilitate cloning the dual *hok*$^+$/*sok*$^+$ *parDE*$^+$ and *hok*$^+$/*sok*$^+$ *pnd*$^+$ killer systems.

Plasmid instability is a significant concern in the industrial utilization of recombinant microorganisms (7). As a vector becomes more effective in directing protein expression, it becomes an increasing burden on cellular metabolism. Betenbaugh et al. have shown that the growth rate of the plasmid-bearing cell is reduced significantly relative to that of the plasmid-free host, as cloned-gene expression is increased through either plasmid amplification or promoter induction (3). Hence, if plasmid instability occurs, faster-growing, plasmid-free segregants can rapidly outnumber the plasmid-bearing population and greatly reduce the yield of recombinant protein from the culture (7).

This segregational instability (complete loss of the plasmid) may occur even if antibiotic selection pressure is used (31). Plasmid-bearing strains can secrete the protein which degrades the antibiotic (e.g., β-lactamase) or become resistant by mutation to an extent that the plasmid-free cells may thrive, even when the antibiotic is added continually (23, 31). Hence antibiotics, besides being expensive and causing unwanted separation and regulation problems (7), may not be completely effective in overcoming plasmid instability.

In order to improve plasmid stability, some scientists have tried addressing the situation mechanically by using two reactors in series (22). In the first reactor, the cell density is increased, whereas in the second reactor, the cloned gene is expressed by using the appropriate inducer (thermal or chemical). This technique requires additional reactors, more sophisticated control equipment, increased maintenance, and complex optimization strategies, resulting in greater likelihood of contamination and higher overall cost.

The problem of plasmid stability has been approached genetically, and some success has been achieved by deleting an essential function from the chromosome and complementing this deficiency by placing the missing gene on the plasmid. One example of this technique that has worked well is complementation of the *Escherichia coli* single-stranded-DNA-binding protein (encoded by *ssb*); *ssb*-deficient hosts which lack the *ssb*-containing plasmid then are unable to grow (23). Cultures were grown for 150 h and remained 100% plasmid bearing (23). The main disadvantage of these systems is that they are not general; for every host/plasmid system, a new essential gene must be both deleted from the chromosome of the host and cloned into a plasmid. Furthermore, these kinds of techniques are usually limited to the bacterial species from which they were derived; for example, for each bacterial species used as an expression system, the *ssb* for that species must be located, excised, and manipulated.

In contrast to systems which require genetic changes for each host, plasmid stability can be enhanced by using the more general technique of killing plasmid-free daughter cells that arise after segregational plasmid instability (postsegregational killing). The postsegregational killer family includes both chromosomal and plasmid genes (at least 12 loci in *E. coli* [9, 15, 20], and members are characterized by a protein toxin and a gene which prevents the toxin from being expressed (15). Cell death is regulated either by controlling translation of the killer protein by feedback inhibition and unstable antisense RNA (e.g., *hok*$^+$/*sok*$^+$, also referred to as *parB* [10, 30] from the *E. coli* R1 plasmid and *pndAB*$^+$ [9] from *E. coli* plasmid R483) or controlling the activity of the proteic killer by a labile antitoxin (e.g., *parDE*$^+$ [16, 17] from the *E. coli* RP4 [5] plasmid).

Plasmid stabilization by a postsegregational killer locus is a general stabilization technique in that the best-characterized member, *hok*$^+$/*sok*$^+$, has been shown to improve stability in a broad spectrum of gram-negative species including *E. coli*, *Pseudomonas putida*, and *Serratia marcescens* (8). To utilize this locus, only the plasmid has to be altered; no changes must be made to the host's chromosome. Hence, once a broad-host

* Corresponding author. Phone: (714) 824-3147. Fax: (714) 824-2541. E-mail: tkwood@uci.edu.

plasmid has been modified, its stability should be enhanced in practically any gram-negative host, as was shown for a pMMB277 derivative which was stabilized in *Pseudomonas* and *Rhizobium* strains by using *hok*$^+$/*sok*$^+$ (13). Other advantages of killer loci include their small size (about 0.6 kb) and their ability to work with different media and reactor configurations. Killer loci have also been used to prevent horizontal transfer (14) of plasmids (a change in environmental conditions induces the killer gene) and for low-background cloning vectors (2) (the killer gene [*ccdB*] is inactivated by insertion of DNA so that the religated vector without the inserted target gene cannot grow).

Earlier studies indicated that the *hok*$^+$/*sok*$^+$ killer locus dramatically increased the stability of pMJR1750 during expression of β-galactosidase (a 17- to 30-fold improvement with β-galactosidase expressed at 7 to 15% of total cell protein [31, 33, 34]), although plasmid loss was still encountered after 35 generations in complex medium and 63 generations in minimal medium, and segregational stability decreased as plasmid-borne, cloned-gene protein expression increased. Based on the success when a single killer locus (*hok*$^+$/*sok*$^+$) was used, the potential of combining two stability loci was investigated to further increase plasmid stability and directly compare *hok*$^+$/*sok*$^+$ with *parDE*$^+$. Therefore, the stability of plasmid pMJR1750 with *hok*$^+$/*sok*$^+$, *parDE*$^+$, *hok*$^+$/*sok*$^+$ *pnd*$^+$, or *hok*$^+$/*sok*$^+$ *parDE*$^+$ was assessed by using sequential shake-flask experiments in which the plasmid-borne cloned gene (β-galactosidase) was expressed fully.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* XL1-Blue (4) was used for plasmid construction, and *E. coli* BK6 [Δ(*lacIPOZ*) *C29 lacY*$^+$ *hsdR galU galK* StrA^r *leuB6 trpC9830* Δ(*srl-recA*)*306*::Tn*10*] (31) was used as the host for all the stability experiments. BK6 contains *lacY*$^+$ in the chromosome but lacks *lacZ*$^+$ for easy detection of *lacZ*$^+$ plasmids and is also a *recA* mutant to reduce structural instability.

The maps of all the plasmids used for segregational stability analysis are shown in Fig. 1. Each plasmid is identical except for a gene(s) inserted at the *Sca*I site. Plasmid pMJR1750 [7.5 kb, *bla*$^+$ (Ap^r) *lacI*^{q} *ptac*::*lacZ*$^+$] (Fig. 1a) (29) was chosen as the parent since it has been found to be unstable, has previously been used for studying plasmid stability (31, 33, 34), has the identification marker β-galactosidase (the product of *lacZ*$^+$) for distinguishing plasmid-containing colonies, has tight control of expression since it contains the *ptac* promoter regulator *lacI*^{q} on the plasmid, and has been used to make pMJR1750-aphA-hok (pTKW106, 9.1 kb, Δ*bla aphA*$^+$ *hok*$^+$/*sok*$^+$ *lacI*^{q} *ptac*::*lacZ*$^+$) (Fig. 1d) (31) which already contains a stability locus. This plasmid contains the pUC origin of replication (29) and has a copy number of 80 to 100 per cell (32).

pMJR1750-aphA (8.7 kb, Δ*bla aphA*$^+$ *lacI*^{q} *ptac*::*lacZ*$^+$) (Fig. 1b) was constructed by cloning the *Bam*HI-*aphA*$^+$-*Eco*RI (1.2 kb) fragment from pKG1022 [4.7 kb, *aphA*$^+$ (Km^r) *bla*$^+$ *hok*$^+$/*sok*$^+$] (8) into the blunt *Sca*I (New England Biolabs, Beverly, Mass.) site of pMJR1750, using *Bam*HI-*Sma*I, *Eco*RI-*Sma*I, and *Sma*I linker adapters (New England Biolabs) to create blunt ends. Plasmid pKG1022 contains *hok*$^+$/*sok*$^+$ adjacent to *aphA*$^+$ (Km^r) and is flanked by multiple cloning sites. This allowed kanamycin resistance to be used for the detection of all clones.

pMJR1750-aphA-parDE (Fig. 1c) was constructed in two steps by cloning simultaneously the *Eco*RI-*parDE*$^+$-*Bam*HI (0.72 kb) fragment from pOU82-parDE (12.7 kb, *bla*$^+$ *parDE*$^+$ λ*CI857 copA copB repA deoC-lacZYA*) (16) together with the *Bam*HI-*aphA*$^+$-*Kpn*I (1.2 kb) fragment from pKG1022 into the multiple-cloning site of pUC18 Not (2.7 kb, *bla*$^+$ *lacI*$^+$ *lacZ*^{α}) (11) cleaved with *Eco*RI and *Kpn*I to make pUC18 Not-aphA-parDE (4.6 kb, *bla*$^+$ *aphA*$^+$ *parDE*$^+$ *lacI*$^+$ Δ*lacZ*^{α}). The *aphA*$^+$-*parDE*$^+$ (1.8 kb) fragment was excised from pUC18 Not-aphA-parDE by using restriction enzymes *Hin*cII (New England Biolabs) and *Eco*RI (Promega, Madison, Wis.) made blunt using *Eco*RI-*Sma*I and *Sma*I linker adapters and ligated into *Sca*I-cut pMJR1750 to make pMJR1750-aphA-parDE (9.4 kb, Δ*bla aphA*$^+$ *parDE*$^+$ *lacI*^{q} *ptac*::*lacZ*$^+$).

pMJR1750-aphA-hok-parDE (Fig. 1e) was constructed by assembling the *aphA*$^+$, *hok*$^+$, and *parDE*$^+$ genes in the multiple cloning site of pUC18 Not and then cloning this fragment into pBluescript II (KS−) to create better flanking sites to facilitate ligation into pMJR1750. The *Bam*HI-*aphA*$^+$-*hok*$^+$-*Bam*HI fragment (1.8 kb) from pKG1022 was cloned into the *Bam*HI (New England Biolabs) site of pUC18 Not to make pUC18 Not-aphA-hok (4.5 kb, *bla*$^+$ *aphA*$^+$ *hok*$^+$/*sok*$^+$ *lacI*$^+$ Δ*lacZ*^{α}). The *aphA*$^+$ gene was removed from pUC18 Not-aphA-hok by digestion with *Eco*RI followed by dilution (to prevent reinsertion of *aphA*$^+$ at the multiple cloning site) and self-ligation to make plasmid pUC18 Not-hok (3.3 kb, *bla*$^+$ *hok*$^+$/*sok*$^+$ *lacI*$^+$ Δ*lacZ*^{α}). The *Eco*RI-*parDE*$^+$-*aphA*$^+$-*Xba*I fragment from pUC18 Not-aphA-parDE was then inserted into the *Xba*I (New England Biolabs) and *Hin*cII sites of pUC Not-hok by using *Eco*RI-*Sma*I and *Sma*I linker adapters to make pUC18 Not-aphA-hok-parDE (5.2 kb, *bla*$^+$ *aphA*$^+$ *hok*$^+$/*sok*$^+$ *parDE*$^+$ *lacI*$^+$ Δ*lacZ*^{α}). The *hok*$^+$-*aphA*$^+$-*parDE*$^+$ fragment was excised with *Eco*RI and ligated into the *Eco*RI site of pBluescript II (KS−) (3.0 kb, *bla*$^+$ *lacZ*^{α}) (Stratagene, La Jolla, Calif.) to make the cloning cassette pBS-aphA-hok-parDE (5.4 kb, *bla*$^+$ *aphA*$^+$ *hok*$^+$/*sok*$^+$ *parDE*$^+$ Δ*lacZ*^{α}) shown in Fig. 2a. The *hok*$^+$-*aphA*$^+$-*parDE*$^+$ fragment was excised from pBS-aphA-hok-parDE with the blunt-end cutters *Eco*I CRI (New England Biolabs) and *Hin*cII. This fragment was then ligated into the *Sca*I site of pMJR1750, giving pMJR1750-aphA-hok-parDE (10.1 kb, Δ*bla aphA*$^+$ *hok*$^+$/*sok*$^+$ *parDE*$^+$ *ptac*::*lacZ*$^+$).

pMJR1750-aphA-hok-pnd (Fig. 1f) was constructed by cloning the *Eco*RI-*pnd*$^+$-*Sal*I R483 fragment (0.85 kb) from pAN1 (5.2 kb, *bla*$^+$ Δ*Tet*^{r} *pndA*$^+$/*pndB*$^+$) (21) into the multiple cloning site of pUC19 (2.7 kb, *bla*$^+$ *lacI*$^+$ *lacZ*^{α}) (35) to make pDD1024 (3.6 kb, *bla*$^+$ *pndA*$^+$/*pndB*$^+$ *lacI*$^+$ Δ*lacZ*^{α}). pDD1024 was digested with *Eco*RI and *Kpn*I (Promega) giving a 0.9-kb *pnd*$^+$ fragment which was cloned into the *Eco*RI and *Kpn*I sites in between the *aphA*$^+$ and *hok*$^+$ genes on pKG1022 giving the cloning cassette pDD1025 (5.7 kb, *bla*$^+$ *aphA*$^+$ *hok*$^+$/*sok*$^+$ *pndA*$^+$/*pndB*$^+$) shown in Fig. 2b. The *hok*$^+$-*pnd*$^+$-*aphA*$^+$ fragment (2,700 bp) was excised from pDD1025 by using *Sph*I (New England Biolabs), and the 3′ overhangs were made blunt with T4 DNA polymerase (New England Biolabs, 20 min at 11 to 12°C, 1 to 5 U/g of DNA, 10 mM deoxynucleoside triphosphates), followed by blunt-end ligation into the *Sca*I site of pMJR1750, giving pMJR1750-aphA-hok-pnd (10.3 kb, Δ*bla aphA*$^+$ *hok*$^+$/*sok*$^+$ *pndA*$^+$/*pndB*$^+$ *ptac*::*lacZ*$^+$).

The general cloning procedures of Rodriguez and Tait were used (24). All intermediate fragments were cut by using restriction enzymes and purified by using low-melting-point agarose electrophoresis (25) followed by β-agarase treatment (Boehringer Mannheim, Indianapolis, Ind.). Plasmids were purified by a miniprep procedure (24) or a Qiagen DNA Midi kit (Qiagen, Chatsworth, Calif.). The sizes and orientations of all plasmid constructs were confirmed by the restriction enzyme digest pattern of the plasmid DNA on horizontal agarose electrophoresis (a minimum of five different restriction enzymes was used for each plasmid). As an additional check, the presence of each of the *aphA*$^+$, *hok*$^+$/*sok*$^+$, and *parDE*$^+$ gene fragments from pMJR1750-aphA, pMJR1750-aphA-hok, pMJR1750-aphA-parDE, and pMJR1750-aphA-hok-parDE was also verified by using vertical polyacrylamide gel electrophoresis (25). The antibiotic and β-galactosidase phenotypes were confirmed by growth on the appropriate media.

Plasmid constructs were electroporated into host BK6 or XL1-Blue following the procedure of Smith and Iglewski (27) by using a Bio-Rad (Hercules, Calif.) gene pulser and pulse controller (11 to 15 kV/cm, 25 μF, 200 Ω, and τ = 4.2 to 4.8 s). Transformed cells were selected by plating on MacConkey agar (Difco, Detroit, Mich.) with the appropriate antibiotic.

Media. Luria-Bertani medium (LB) (25) was used for all cultures, and 2 mM dioxane-free isopropyl-β-D-thiogalactopyranoside (IPTG) (Fisher, Pittsburgh, Pa.) was added when indicated to induce β-galactosidase. Tetracycline (15 μg/ml; United States Biochemical Corp., Cleveland, Ohio) was added to XL1-Blue cells during cloning to ensure that the F′ plasmid [which contains the part of *lacZ* needed for α-complementation of pBluescript II (KS−)] would not be lost, allowing identification of *lacZ*$^+$-containing cells. MacConkey agar plates with antibiotics (100 to 400 μg of ampicillin per ml or 50 to 100 μg of kanamycin per ml; Fisher) were used to identify plasmid-bearing strains (colonies expressing β-galactosidase are easily distinguished by their red color).

Sequential-dilution plasmid loss experiments. The procedure used for sequential-dilution plasmid loss experiments was based on that of Wu and Wood (34). All cells were grown in 20 ml of LB at 37°C in 250-ml Erlenmeyer flasks shaken at 250 rpm (New Brunswick Scientific, series 25 shaker). Cells for inoculation were grown from −84°C glycerol (15%) stocks by streaking on a MacConkey plate with the appropriate antibiotics. After 24 to 48 h, a single red colony was picked and used to inoculate 20 ml of LB supplemented with 2 mM IPTG to begin the stability experiment or the colony was used to inoculate LB-containing antibiotics from which 10 μl at an optical density at 600 nm (OD_{600}) of 0.1 to 0.3 was taken to inoculate 20 ml of LB with 2 mM IPTG to start the stability experiment. The inoculum was diluted and plated on MacConkey agar (19) (no antibiotics) to ensure it was 100% plasmid bearing at the beginning of the experiment.

After 12 h, the flasks were inspected and if they were turbid, 10 μl was transferred to a new flask of LB–2 mM IPTG; otherwise, the cells were allowed to grow several hours longer and then transferred to a new flask. For every successive 12 h, 10 μl of sample was transferred to a new flask and the OD_{600} was measured. The cells were then diluted and plated on MacConkey agar (targeting 100 to 400 colonies of cells/plate), and a 0.7- to 5-ml sample was saved for a plasmid miniprep (2 OD_{600} units of GM33/pBR322 was added as an internal control to make sure errors were not made during the plasmid isolation) to determine the mechanism of plasmid loss (structural or segregational) (34). The MacConkey plates were examined 20 to 36 h after plating, and the numbers of red and white colonies were counted. Serial dilutions were performed until the plasmid-bearing population was less than 10%.

The time of exponential growth was computed by using 9 h for each 12-h

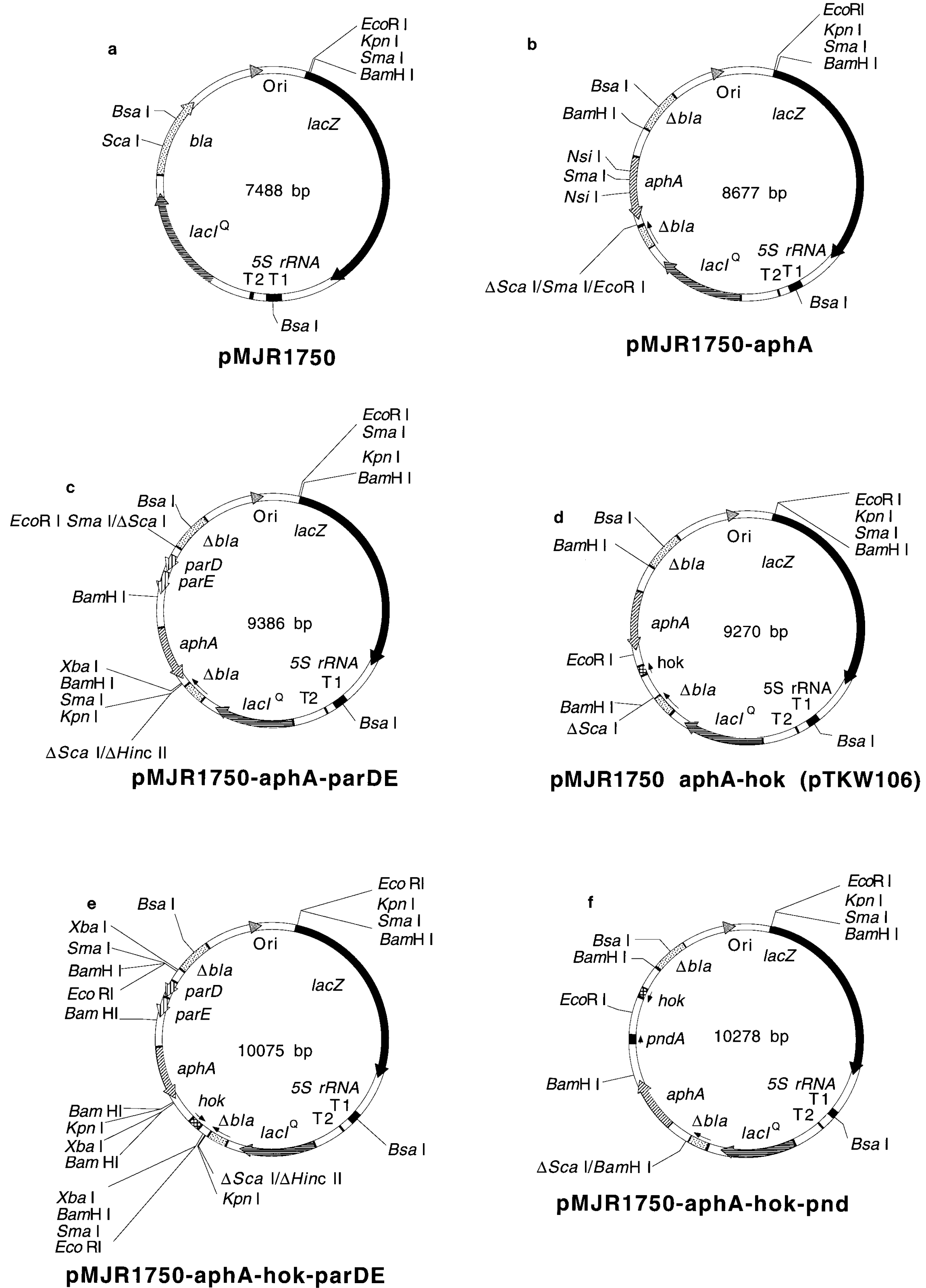

FIG. 1. Plasmid restriction maps. ori, origin from pBR322; *lacZ*, wild-type β-galactosidase gene from *E. coli*; T1/T2, the transcription terminators of *rrnB*; *5S RNA*, the gene for the 5S RNA of *E. coli*; *lacI*^{q}, lac repressor mutant; *bla*, β-lactamase from Tn*3* which confers ampicillin resistance; *hok*, killer gene of *hok*$^+$/*sok*$^+$ antisense killer locus; *pndA*, killer gene of *pnd*$^+$ killer locus; *parE*, killer gene of the *parDE*$^+$ proteic killer locus of RP4; *parD*, antitoxin gene of *parDE*$^+$; *aphA*, aminoglycoside 3′-phosphotransferase A gene from Tn*903* which confers kanamycin resistance.

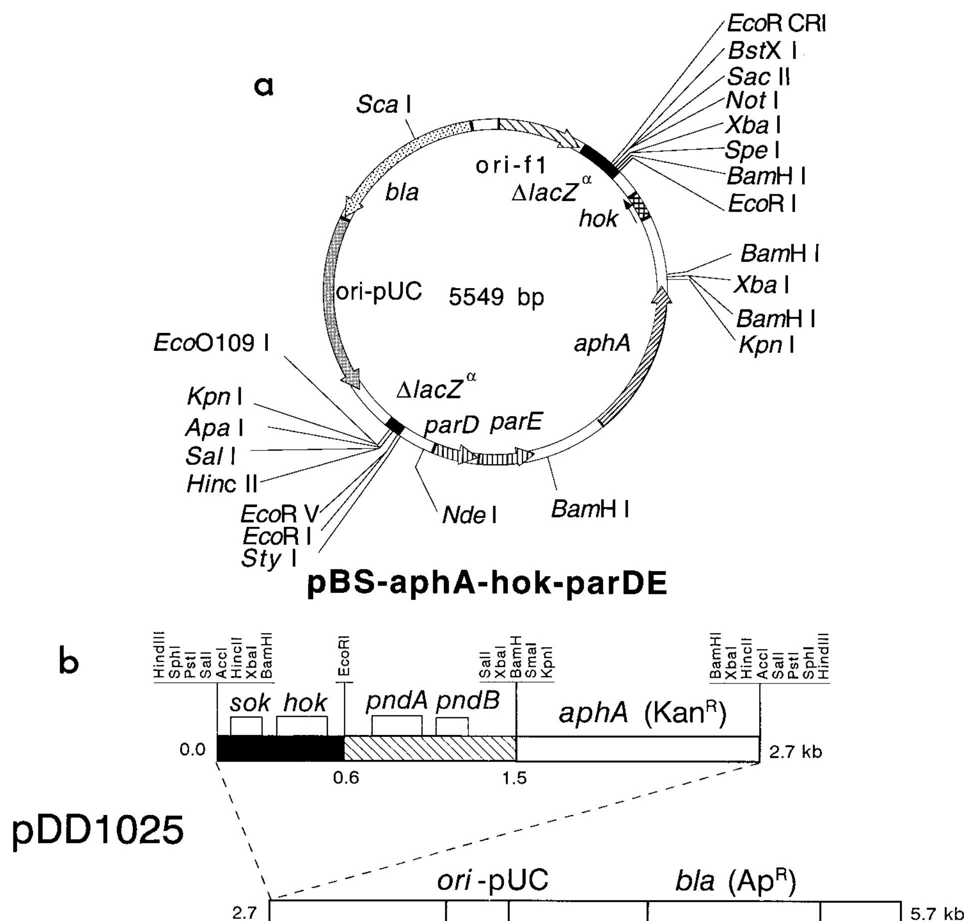

FIG. 2. Plasmid restriction maps. (a) $aphA^+$-hok^+/sok^+-$parDE^+$ cloning cassette plasmid pBS-aphA-hok-parDE; (b) $aphA^+$-hok^+/sok^+-pnd^+ cloning cassette plasmid pDD1025. Abbreviations are as defined in the legend for Fig. 1 except for the following: ori-f1, f1 filamentous phage origin of replication; $\Delta lacZ^\alpha$, α-complementation fragments of *lacZ* (the gene was disrupted by the inserted genes); ori-pUC, origin of replication ColE1 from pUC plasmid series.

shake-flask culture if the final OD_{600} was 2 to 3 (Spectronic 20D spectrophotometer, Milton Roy), by using the full 12 h if the OD_{600} was <0.5, or by subtracting 1 h from 12 h for each 0.5 OD unit less than 2 (only a factor for the first two flasks of some sequential dilutions). The plasmids without killer loci were lost very rapidly, so samples were taken every 2 h. The probability of the plasmid loss (p) was computed by using the model of Imanaka and Aiba (12, 34):

$$F = \frac{(1-\alpha-p)}{(1-\alpha-p2^{(\alpha+p-1)N})}$$

where F is the fraction of plasmid-bearing cells, N is the number of generations of plasmid-bearing cells, and α is the growth rate of plasmid-free cells divided by the growth rate of plasmid-bearing cells. This equation is derived by solving the two simultaneous ordinary differential equations which govern the exponential growth of the plasmid-free and plasmid-containing cells while assuming the probability of random plasmid loss upon division is p. In this work, p was calculated for each stabilized plasmid for any N by determining F after each shake-flask culture (by the proportion of red colonies on MacConkey plates) and by determining α from the measured growth rates.

Specific growth rates and β-galactosidase activity. The specific growth rates (μ) in LB supplemented with 2 mM IPTG were measured by growing the cells in LB with the appropriate antibiotic and then transferring 0.02 to 0.5 absorbance units of exponentially growing cells to LB with 2 mM IPTG. The change in OD_{600} was measured (0.03 to 0.5), but only data obtained after the cells had recovered from IPTG induction shock (at least 2 h) were used for calculations (three or four separate measurements were made for each strain). This ensured that balanced growth was achieved and that the growth rate was reproducible. The cells were checked at the end of each experiment to ensure that they were 100% plasmid containing.

β-Galactosidase activity was measured as described previously (32). Cells were grown to an OD_{600} of 0.5 to 0.7 in LB with appropriate antibiotics, centrifuged to remove the antibiotic, resuspended in LB, and inoculated into 20 ml of LB containing 2 mM IPTG for an OD_{600} of 0.05. After 2 h of induction, the OD was measured, and the cells were sonicated and then preserved by placement on ice and the addition of phenylmethylsulfonylfluoride (Life Technologies Gaithersburg, Md.) to prevent enzyme degradation. The activity was calculated by measuring the conversion rate of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma, St. Louis, Mo.) to *o*-nitrophenol by using a spectrophotometer (Beckman DU 640) at 410 nm. The activity measurements were made specific by using the OD_{600} of the cells.

RESULTS

Construction of plasmids containing killer loci. A series of six plasmids were constructed to see the effect of the stability loci on plasmid segregational stability. Both orientations of gene $aphA^+$ in pMJR1750-aphA were obtained, and since there was no observed difference in the plasmids, a plasmid with the orientation in Fig. 1b was chosen for further study. The only orientation of $aphA^+$-$parDE^+$ in pMJR1750-aphA-parDE obtained is that depicted in Fig. 1c. The orientation of $aphA^+$-hok^+ in pMJR1750-aphA-hok (Fig. 1d) was used previously (although the opposite orientation was also obtained and both had comparable stability [31]). The orientation of hok^+-$aphA^+$-$parDE^+$ in plasmid pMJR1750-aphA-hok-parDE (Fig. 1e) was chosen for further study since out of eight colonies, only one had the opposite orientation (probably due to read-through transcription from the bla^+ gene promoter into the $parDE^+$ fragment which perhaps disrupts regulation of the

TABLE 1. Segregational plasmid stability in shake-flask, serial-dilution experiments with full induction (2 mM IPTG) of the plasmid-borne gene and no antibiotics

Plasmid	Growth rate (h^{-1})	Median time (h) until 90% plasmid bearing	Avg total time (h) until 90% plasmid bearing	Average h of exponential growth to 90% plasmid bearing	Median no. of generations until 90% plasmid bearing	No. of stability experiments	Probability of plasmid loss at division	β-Galactosidase activity ± SD (nmol/min/OD unit)
None	1.13 ± 0.08							
pMJR1750	0.68[a]	4	3.0 ± 1.3	3.0 ± 1.3	3.9	5	1.4×10^{-2}	14,300 ± 1,200
pMJR1750-aphA	0.68[a]	4	3.7 ± 0.7	3.7 ± 0.7	3.9	5	1.4×10^{-2}	12,900 ± 2,500
pMJR1750-aphA-parDE	0.62 ± 0.16	18	29 ± 28	27 ± 24	14.7	11	2.0×10^{-5}	10,700 ± 900
pMJR1750-aphA-hok	0.68 ± 0.02	37	38 ± 4	33 ± 2.3	32.9	10	2.4×10^{-8}	9,300 ± 1,300
pMJR1750-aphA-hok-pnd	0.31	27	28 ± 1.2	28 ± 1.2	12.1	3	6.8×10^{-11}	12,600 ± 800
pMJR1750-aphA-hok-parDE	0.66 ± 0.16	50	43 ± 16	41 ± 15	42.9	9	5.4×10^{-11}	10,600 ± 600

[a] Estimated from BK6/pMJR1750-aphA-hok.

parDE$^+$ operon). The orientation of *hok*$^+$-*pnd*$^+$-*aphA*$^+$ in pMJR1750-aphA-hok-pnd (Fig. 1f) was also found to be important. The configuration with *hok*$^+$ adjacent to the *bla*$^+$ promoter was stable for 2 h, as compared to 27.6 h when *aphA*$^+$ was adjacent to the *bla*$^+$ promoter; hence, the more stable configuration was chosen for further study. This is surprising since pMJR1750-aphA-hok has *hok*$^+$/*sok*$^+$ adjacent to the *bla*$^+$ promoter.

β-Galactosidase activity and specific growth rates. The β-galactosidase activities were determined to ensure that any difference in stability was not due to a difference in *lacZ*$^+$ expression (34). In previous work, induction of BK6/pMJR1750-aphA-hok in LB medium with 2 mM IPTG resulted in an enzyme activity of approximately 9,100 nmol of ONPG/(min OD_{600}) and 15% of the cellular protein (34) was β-galactosidase, which placed a significant amount of metabolic load on the cells. Table 1 shows that all the plasmids produced approximately the same level of β-galactosidase except for pMJR1750, which was ~30% higher. Therefore, differences in stability were not due to different levels of plasmid-borne gene expression.

The growth conditions used to determine plasmid segregational stability represent the worst case for plasmid stability: high cloned-gene expression, intermittent stationary-phase conditions, and no antibiotic. The specific growth rates of host BK6 and BK6 with each plasmid are shown in Table 1. The growth rates of BK6/pMJR1750 and BK6/pMJR1750-aphA were estimated to be the same as that of BK6/pMJR1750-aphA-hok since balanced growth was not achieved with these two unstable plasmids. For BK6/pMJR1750 and BK6/pMJR1750-aphA, it was noted that the viable cell count declined by 10- to 100-fold after induction with IPTG and then recovered; the OD_{600} did not decline but leveled off for a few hours and then continued to increase exponentially (after most cells were plasmid-free). This phenomenon has been seen by others (1, 6) and has been attributed to the shock caused by a sudden shift in metabolism caused by induction.

The growth rate of pMJR1750-aphA-hok-pnd (Table 1) was ~30% that of BK6. This low growth rate was unexpected since different plasmids containing *pnd*$^+$ and *hok*$^+$ were found previously to be compatible when each locus was on a separate plasmid (21).

The growth rate of BK6/pMJR1750-aphA-hok-parDE (Table 1) was almost the same as that of the single killer locus strains. Colonies from the freshly streaked −84°C stock of BK6/pMJR1750-aphA-hok-parDE are distinguishable from other colonies by their more intense red color, which suggests greater β-galactosidase expression and enhanced plasmid stability. Similarly, colonies of strains with a single killer locus are more red than those that lack a stability locus. Colonies of BK6/pMJR1750-aphA-hok-parDE were also smaller (about half the size of a typical BK6 colony containing any of the other plasmids) on MacConkey agar but were normal size on LB. After about 25 generations of growth in medium without antibiotics, larger red colonies (about normal size for other plasmid-bearing strains) started to appear on MacConkey plates. These colonies did not appear significantly different when plated on LB, nor did one cell type outgrow the other in liquid LB medium. The two colony sizes persisted on MacConkey plates even after the cells lost the plasmid. This suggests that the large colony size was due to a mutation in the chromosome.

Plasmid segregational stability. The results of the shake-flask segregational stability studies are shown in Table 1. For some experiments, the number of plasmid-bearing cells dropped precipitously upon induction, indicating the inoculations were not 100%; these experiments (approximately 15% of the experiments) were not included in the analysis and are not shown in Table 1. As was expected from previous studies (34), the stability of pMJR1750 in BK6 was very low (<5 h to become 90% plasmid bearing for each experiment). Figure 3 depicts a typical plasmid-loss curve for each plasmid. The addition of *aphA*$^+$ did not alter segregational stability (<5 h to become 90% plasmid bearing for each experiment) in the absence of antibiotic. Almost all experiments with the plasmids without killer loci resulted in segregational, not structural, instability; however, plasmid structural instability was encountered in ~30% of the experiments. The plasmids which resulted from structural instability lacked all of *lacZ*$^+$ and the first part of *lacI*^{q}, as seen previously (34), indicating that the structural instability was inherent to the pMJR1750 vector and not caused by the addition of the killer genes. Experiments with the vectors in which *lacZ*$^+$ was deleted due to structural instability (ascertained by plasmid minipreps conducted for each shake-flask experiment) were not included in the segregational stability analysis (Table 1).

The addition of *parDE*$^+$ to the plasmid increased the median number of generations before plasmid loss by approximately fourfold over that for experiments with pMJR1750-aphA. The variability in the number of generations until segregational stability occurred was very high (8.0, 8.9, 14.3, 14.3, 14.7, 14.7, 17.0, 20.5, 25.9, 50.9, and 79.4 generations until 90% plasmid bearing); the experiment that retained the plasmid longest had a 10-fold-greater generation count than that of the experiment that retained it the shortest, and the median time (17.5 h) to lose the plasmid was 60% that of the average time (29 h). The data tended to be clustered around the me-

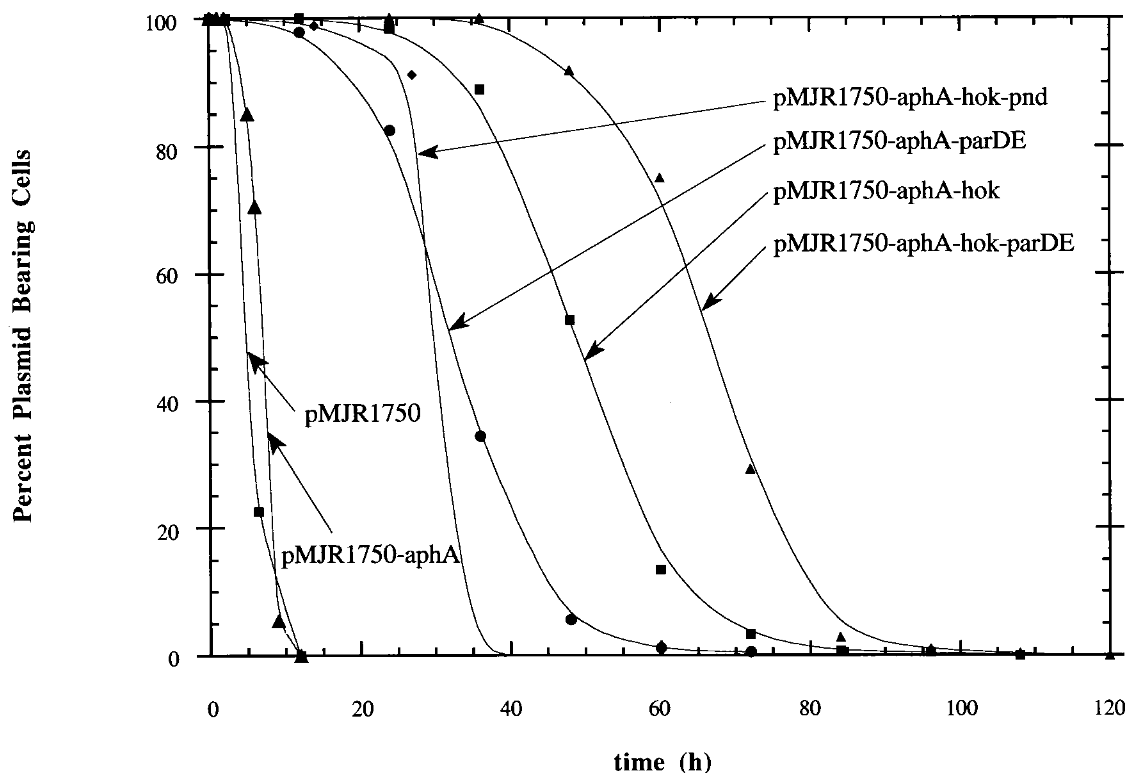

FIG. 3. Representative plasmid segregational stability curves for the shake-flask, serial dilution experiments (37°C, full induction [2 mM IPTG], and no antibiotics). The data shown are the median experimental results.

dian rather than the mean due to extreme values distorting the mean. Therefore the median was chosen as more representative of the data and was used for all comparisons and calculations.

Adding hok^+/sok^+ increased the median time to plasmid loss approximately ninefold over that for pMJR1750-aphA (Table 1). The variation in the number of generations measured in each experiment was much smaller than for the strains containing the other killer locus plasmids (standard deviation of 5%). These results are in agreement with previous work in this laboratory in which hok^+/sok^+ increased segregational stability 17- to 30-fold (34). In contrast to these results, Jensen et al. (16) found $parDE^+$ to be more efficient at stabilization than hok^+/sok^+; however, in those experiments different growth conditions were used, the media contained glucose, the temperature was lower (35°C), no cloned gene was induced, and cells did not reach stationary phase.

The addition of both the hok^+/sok^+ and $parDE^+$ genes increased the median generations until plasmid loss approximately 12-fold compared to pMJR1750-aphA, 2.9-fold compared to pMJR1750-aphA-parDE, and 1.3-fold compared to the addition of hok^+/sok^+ alone (pMJR1750-aphA-hok). The variation in the number of generations in each experiment was larger than that for the addition of hok^+/sok^+ alone but less than that for the addition of $parDE^+$ alone (the standard deviation was approximately 40%).

The addition of both the hok^+/sok^+ and pnd^+ genes increased the median generations until plasmid loss approximately threefold compared to pMJR1750-aphA. This stability enhancement was not as large as that upon the addition of hok^+/sok^+ alone (ninefold) based on the number of generations; however, the probability of plasmid loss (p) was improved by almost the same amount (3 orders of magnitude) as the addition of hok^+/sok^+ and $parDE^+$ (Table 1). Comparing p is a better indicator of changes in segregational stability for strains with very different growth rates since the formulation of Imanaka and Aiba takes into account both the growth rate and the number of stable generations. It was also found that fresh transformants containing hok^+/sok^+ and pnd^+ grew very slowly (growth rate not measured) whereas colonies formed by restreaking or by retransforming grew faster (0.3/h) (Table 1), indicating a change had occurred on the plasmid. The plasmid stability in both cases was comparable.

The cloning cassettes pBS-aphA-hok-parDE and pDD1025 (Fig. 2) were constructed so that the double killer loci could be readily cloned along with the kanamycin resistance gene into any plasmid which needs to be stabilized. Each set of double killer genes can be readily cut for blunt-end cloning (e.g., pBS-aphA-hok-parDE cut with *Eco*RV and *Eco*RCRI and pDD1025 cut with *Hin*cII) or for sticky-end cloning with a number of commercially available restriction enzymes.

DISCUSSION

Killer proteins are attractive for improving the production of recombinant proteins by enhancing plasmid segregational stability. However, the hok^+/sok^+ killer system is not capable of completely stabilizing plasmids (instability ensues after approximately 50 generations in minimal media in continuous cultures with recombinant-protein production at 15% of total cell protein [33]), and both the pnd^+ and $parDE^+$ systems have been shown to stabilize plasmids about as well as hok^+/sok^+ (16, 21). This level of stability enhancement is suitable for batch and fed-batch fermentations; however, it is not adequate

for continuous operations. It is not surprising that a few cells escape the postsegregational killing mechanism, since it has been shown that even severe overproduction of Hok killer protein does not lead to complete sterilization of a growing bacterial culture (10).

Although there are many possibilities why a killer locus may not be completely effective, placing two killer loci on a single vector to improve segregational stability is straightforward and has many advantages. This approach will decrease the possibility of plasmid-free cells occurring due to a point mutation inside the stability locus. The mutation rate is approximately 10^{-6}/gene/generation for chromosomal genes (18), and if one assumes this applies to the killer genes on the plasmid, then the possibility of mutations occurring inside two distinct killer genes will be reduced to roughly 10^{-12}. Hence, the possibility of a mutation that inactivates both killer loci or creates resistant host cells will be reduced dramatically. In addition, by having two sources of killer protein, there is an increased likelihood that plasmid-free cells will be killed.

The first candidate chosen as the second killer locus to enhance plasmid stability was the *pnd*$^+$ locus of plasmid R483. The *pnd*$^+$ locus kills plasmid-free cells in a manner similar to that of *hok*$^+$/*sok*$^+$, and it is 40% dissimilar to *hok*$^+$/*sok*$^+$ in structure (21). Furthermore, both *hok*$^+$/*sok*$^+$ and *pnd*$^+$ have been shown to enhance segregational stability independently in many gram-negative hosts, and both loci have been shown to be compatible on separate plasmids (21). In addition, natural analogs of such a system exist since some stable plasmids such as F have two antisense killer loci (e.g., *flm*$^+$ and *srnB*$^+$) (8). However, with our construct, a 50% reduction in growth rate was seen. This reduction in growth rate may be an artifact of the close proximity of the two killer genes; read-through from the *pndA*$^+$ or *hok*$^+$ promoters may terminate transcription prematurely, disrupt fold-back inhibition, and allow some toxic protein to be expressed.

Since our construct, pMJR1750-aphA-pnd-hok, caused the host to grow too slowly for practical use (though it did enhance plasmid stability significantly as shown by the relative loss rate p), another killer locus, *parDE*$^+$, was chosen that does not depend on antisense control and is not related to *hok*$^+$/*sok*$^+$. This second candidate, *parDE*$^+$ of RP4, is a proteic killer that kills by an unknown mechanism. Cells killed by *parDE*$^+$ are enlarged (16) and filamented (28), whereas cells killed by *hok*$^+$/*sok*$^+$ are condensed at the poles with a clear center which yields a ghostlike appearance (10); this indicates the mechanisms of killing are different. The *parABCDE*$^+$ region of RP4 which contains *parDE*$^+$ has been found to help stabilize plasmids in *Alcaligenes eutrophus*, *Alcaligenes latus*, *Azotobacter chroococcum*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, and *E. coli* (5, 26). Much of the stabilization has been attributed to *parDE*$^+$, especially in strains with low copy number (28) so it is likely that it functions in a broad range of hosts. The addition of *parDE*$^+$ should complement *hok*$^+$/*sok*$^+$ since *parDE*$^+$ kills cells more slowly (16) and more effectively under some growth conditions (28).

The use of *hok*$^+$/*sok*$^+$ with *parDE*$^+$ was more effective than either of the single-killer systems but still resulted in formation of plasmid-free cells. The improvement in segregational stability upon addition of a postsegregational killer locus is due to killing of the plasmid-free cells. Therefore the effectiveness of a killer locus depends on the fraction of cells that survive killing. The fraction of cells that survive killing can be estimated by assuming the rate of formation of plasmid-free cells is unchanged by the addition of the killer locus (killer loci do not discriminate how the plasmid is lost). Dividing the loss frequency (p) for a cell with a stability locus by the loss frequency without a killer locus yields the fraction of cells that survive killing (8). The fraction of cells that survive killing with *parDE*$^+$ is 1.46×10^{-3}, and that survive killing with *hok*$^+$/*sok*$^+$ is 1.71×10^{-6} (Table 1). If these two killer loci kill independently, the fraction of cells that survive killing should be the product of the fraction that survive killing with each independent killer locus. Therefore, for the plasmid with *hok*$^+$/*sok*$^+$ and *parDE*$^+$, one expects the fraction of cells that survive killing to be 2.5×10^{-9} [$(1.46 \times 10^{-3}) \cdot (1.71 \times 10^{-6})$], which is close to the value observed, 3.8×10^{-9} (5.4×10^{-11}/0.014). This result is consistent with this notion that *hok*$^+$/*sok*$^+$ and *parDE*$^+$ kill independently. Since the growth rate with this dual killer system was not significantly lower than that with a single killer locus and since plasmid stability was enhanced, the addition of a second killer locus to a high-copy-number *E. coli* plasmid is an attractive way to improve fermentation productivity.

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation under grant BES-9224864 and the Plasmid Foundation of Denmark (award 17066).

REFERENCES

1. **Bentley, W. E., R. H. Davis, and D. S. Kompala.** 1991. Dynamics of induced CAT expression in *E. coli*. Biotechnol. Bioeng. **38:**749–760.
2. **Bernard, P.** 1996. Positive selection of recombinant DNA by CcdB. BioTechniques **21:**320–323.
3. **Betenbaugh, M. J., C. Beaty, and P. Dhurjati.** 1989. Effects of plasmid amplification and recombinant gene expression on the growth kinetics of recombinant *E. coli*. Biotechnol. Bioeng. **33:**1425–1436.
4. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. BioTechniques **5:**376–379.
5. **Burkhardt, H., G. Riess, and A. Pühler.** 1979. Relationship of group P1 plasmids revealed by heteroduplex experiments: RP1, RP4, R68 and RK2 are identical. J. Gen. Microbiol. **114:**341–348.
6. **Donovan, R. S., C. W. Robinson, and B. R. Glick.** 1996. Review: optimizing inducer and culture conditions for expression of foreign proteins under the control of the *lac* promoter. J. Ind. Microbiol. **16:**145–154.
7. **Ensley, B. D.** 1985. Stability of recombinant plasmids in industrial microorganisms. Crit. Rev. Biotechnol. **4:**263–277.
8. **Gerdes, K.** 1988. The *parB* (*hok*/*sok*) locus of plasmid R1: a general purpose plasmid stabilization system. Bio/Technology **6:**1402–1405.
9. **Gerdes, K., L. K. Poulsen, T. Thisted, A. K. Nielsen, J. Martinussen, and P. H. Andreasen.** 1990. The *hok* killer gene family in gram-negative bacteria. New Biol. **2:**946–956.
10. **Gerdes, K., P. B. Rasmussen, and S. Molin.** 1986. Unique type of plasmid maintenance function: postsegregational killing of plasmid-free cell. Proc. Natl. Acad. Sci. USA **83:**3116–3120.
11. **Herrero, M., V. de Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. **172:**6557–6567.
12. **Imanaka, T., and S. Aiba.** 1981. A perspective on the application of genetic engineering: stability of recombinant plasmid. Ann. N. Y. Acad. Sci. **369:**1–14.
13. **Jahng, D., C. S. Kim, R. S. Hanson, and T. K. Wood.** 1996. Optimization of trichloroethylene degradation using soluble methane monooxygenase of *Methlosinus trichosporium* OB3b expressed in recombinant bacteria. Biotechnol. Bioeng. **51:**349–359.
14. **Jensen, L. B., J. L. Ramos, Z. Kaneva, and S. A. Molin.** 1993. A substrate-dependent biological containment system for *Pseudomonas putida* based on the *Escherichia coli gef* gene. Appl. Environ. Microbiol. **59:**3713–3717.
15. **Jensen, R. B., and K. Gerdes.** 1995. Programmed cell death in bacteria: proteic plasmid stabilization systems. Mol. Microbiol. **17:**205–210.
16. **Jensen, R. B., E. Grohmann, H. Schwab, R. Diaz-Orejas, and K. Gerdes.** 1995. Comparison of *ccd* of F, *par*DE of RP4, and *parD* of R1 using a novel conditional replication control system of plasmid R1. Mol. Microbiol. **17:**211–220.
17. **Johnson, E. P., A. R. Ström, and D. R. Helinski.** 1996. Plasmid RK2 toxin protein ParE: purification and interaction with the ParD antitoxin protein. J. Bacteriol. **178:**1420–1429.
18. **Knudsen, S. M., and O. H. Karlström.** 1991. Development of efficient suicide mechanisms for biological containment of bacteria. Appl. Environ. Microbiol. **57:**85–92.

19. **MacConkey, A. T.** 1908. Bile salt media and their advantages in some bacteriological examinations. J. Hyg. **8:**322–334.
20. **Masuda, Y., K. Miyakawa, Y. Nishimura, and E. Ohtsubo.** 1993. *chpA* and *chpB*, *Escherichia coli* chromosomal homologs of the *pem* locus responsible for stable maintenance of plasmid R100. J. Bacteriol. **175:**6850–6856.
21. **Nielsen, A. K., P. Thorsted, T. Thisted, E. G. H. Wagner, and K. Gerdes.** 1991. The rifampicin-inducible genes *srnB* from F and *pnd* from R483 are regulated by antisense RNAs and mediate plasmid maintenance by killing of plasmid-free segregants. Mol. Microbiol. **5:**1961–1973.
22. **Park, S., D. D. Y. Ryu, and J. Y. Kim.** 1990. Effect of cell growth rate on the performance of a two-stage continuous culture system in a recombinant *Escherichia coli* fermentation. Biotechnol. Bioeng. **36:**493–505.
23. **Porter, R. D., S. Black, S. Pannuri, and A. Carlson.** 1990. Use of the *Escherichia coli ssb* gene to prevent bioreactor takeover by plasmidless cells. Bio/Technology **8:**47–51.
24. **Rodriguez, R. L., and R. C. Tait.** 1983. Recombinant DNA techniques: an introduction. Benjamin/Cummings Publishing, Menlo Park, Calif.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning, a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
26. **Saurugger, P. N., O. Hrabak, H. Schwab, and R. M. Lafferty.** 1986. Mapping and cloning of the *par*-region of broad-host-range plasmid RP4. J. Biotechnol. **4:**333–343.
27. **Smith, A. W., and B. H. Iglewski.** 1989. Transformation of *Pseudomonas aeruginosa* by electroporation. Nucleic Acids Res. **17:**10509.
28. **Sobecky, P. A., C. L. Easter, P. D. Bear, and D. R. Helinski.** 1996. Characterization of the stable maintenance properties of the *par* region of broad-host-range plasmid RK2. J. Bacteriol. **178:**2086–2093.
29. **Stark, M. J. R.** 1987. Multicopy expression vectors carrying the *lac* repressor gene for regulated high-level expression of genes in *Escherichia coli*. Gene **51:**255–267.
30. **Thisted, T., N. S. Sørensen, and K. Gerdes.** 1995. Mechanism of post-segregational killing: secondary structure analysis of the entire *hok* mRNA from plasmid R1 suggests a fold-back structure that prevents translation and antisense RNA binding. J. Mol. Biol. **247:**859–873.
31. **Wood, T. K., R. H. Kuhn, and S. W. Peretti.** 1990. Enhanced plasmid stability through post-segregational killing of plasmid-free cells. Biotechnol. Tech. **4:**36–41.
32. **Wood, T. K., and S. W. Peretti.** 1991. Effect of chemically-induced, cloned-gene expression on protein synthesis in *E. Coli*. Biotechnol. Bioeng. **38:**397–412.
33. **Wu, K., D. Jahng, and T. K. Wood.** 1994. Temperature and growth rate effects on the *hok/sok* killer locus for enhanced plasmid stability. Biotechnol. Prog. **10:**621–629.
34. **Wu, K., and T. K. Wood.** 1994. Evaluation of the *hok/sok* killer locus for enhanced plasmid stability. Biotechnol. Bioeng. **44:**912–921.
35. **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.