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pKSS – A second-generation general purpose cloning vector for efficient positive selection of recombinant clones

(Altered substrate specificity; ampicillin resistance; direct selection plasmid; *Escherichia coli* host strain independence; pBluescript; *p*-chloro-phenylalanine sensitivity; phenylalanyl-tRNA synthetase)

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SUMMARY

A new small plasmid vector (pKSS) for the direct selection of insert-containing plasmid clones is presented. The selection strategy is based on the acquired sensitivity of *Escherichia coli* cells to *p*-chloro-phenylalanine (*p*-Cl-Phe) if they carry a *pheS* allele encoding a phenylalanyl-tRNA synthetase α subunit with relaxed substrate specificity. This *pheS* allele is present on pKSS. Insertion into, or replacement of, the plasmidial *pheS* gene by a cloned fragment enables transformed *pheS* wild-type cells to survive on agar plates containing *p*-Cl-Phe plus ampicillin. This host strain-independent positive selection of recombinant clones proved to be highly efficient (>99%) and did not require purification of the vector fragment prior to cloning. The high-copy-number vector pKSS offers a multitude of restriction sites and all of the features for analysis of cloned fragments that stem from the cloning vector pBluescript (Stratagene, La Jolla, CA, USA). Thus, pKSS represents a valuable alternative to previously reported positive-selection vectors; it should prove particularly useful for cloning when expecting a high fraction of cells transformed with non-recombinant vector, and for construction of DNA libraries.

INTRODUCTION

Modern recombinant DNA technology has provided researchers with a large variety of *Escherichia coli* cloning vectors. A sub-collection of these cloning vehicles, termed 'positive-selection vectors' (Dean, 1981; Stephenson and Kuhn, 1988; Altenbuchner et al., 1992), allows the direct selection of clones carrying inserted DNA fragments. This is accomplished by establishing conditions that do not permit survival or growth of cells transformed with undigested or religated vector DNA. Although the use of positive-selection vectors represents the most efficient and straightforward strategy to eliminate the background of non-recombinant clones, their application for general cloning has remained rare. A survey of currently available positive-selection vectors (Balbás et al., 1986; Stephenson and Kuhn, 1988, and references therein; Hill et al., 1989; Weilguny et al., 1991; Altenbuchner et al., 1992; Christensen and Boye, 1992; Pierce et al., 1992) shows that the vast majority exhibit severe limitations that impair their general utilization. These include inherent features of the applied selection mechanism, such as

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Abbreviations: A_{650}, absorbance (1 cm) at 650 nm; aa, amino acid(s); Ap, ampicillin; βGal, β-galactosidase; bp, base pair(s); IPTG, isopropyl-β-D-thiogalactopyranoside; *lacZpo*, *lac* promoter-operator; LB, Luria-Bertani (medium); MCS, multiple cloning site(s); nt, nucleotide(s); *ori*, origin of DNA replication; *p*-, *para*-; pBLS, pBluescript I KS (+); *p*-Cl-Phe, *p*-chloro-phenylalanine; *p*-F-Phe, *p*-fluoro-phenylalanine; PheRS, phenylalanyl-tRNA synthetase; *pheS*, gene encoding the α subunit of PheRS; [R], resistance/resistant; [S], sensitivity/sensitive; SD, Shine-Dalgarno (sequence); ss, single strand(ed); wt, wild type; XGal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; YEG medium, 0.5% yeast extract/1% NaCl/0.4% glucose; YEG-Cl medium, YEG medium with 10 mM *p*-Cl-Phe; [], denotes plasmid-carrier state; ′ (prime), denotes a truncated gene at the indicated side.

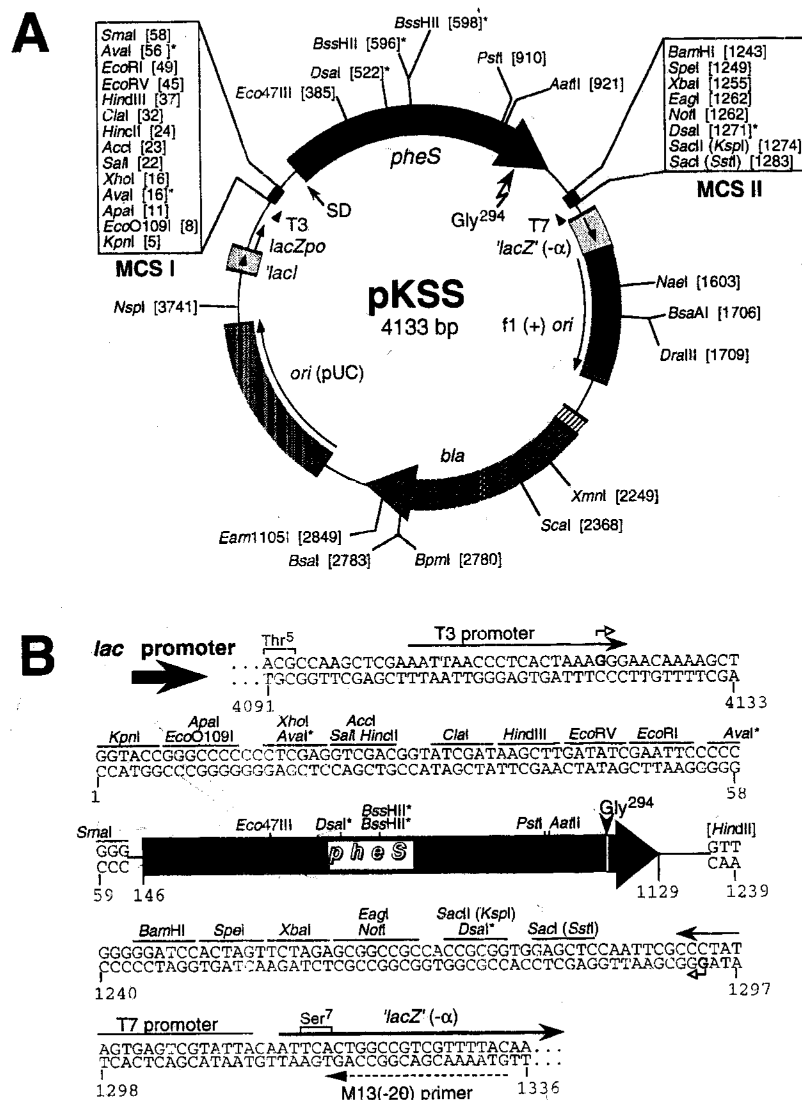

Fig. 1. Structure of pKSS. Numbering starts with the first bp of MCS I. Restriction sites with an asterisk occur twice in pKSS, but are nevertheless useful for positive-selection cloning since they yield incompatible ends (*Ava*I, *Dsa*I) or are so close that they overlap each other (*Bss*HII). All other sites indicated are unique (according to the sequence). The blunt-end site *Eco*47III should be used with caution, since deletion of extra nt could destroy the *pheS* reading frame and yield colonies with religated vectors. **(A)** Circular map of pKSS drawn to scale with the genetic elements {encoded products}; *pheS* {Gly[294] PheRS α subunit for *p*-Cl-Phe[S]} with its SD sequence. a 5′-truncated fragment of *lacZ′* {α-fragment of βGal}, *ori* from filamentous phage f1 for ss DNA synthesis, *bla* {pre-β-lactamase, for Ap[R]} and *ori* (pUC) from plasmid vector pUC19 (Yanisch-Perron et al., 1985), which is a high-copy-number mutant (Lin-Chao et al., 1992) of the pMB1-derived (Balbás et al., 1986) *ori* from pBR322 (Bolivar et al., 1977). Other features include a non-functional 3′-terminal fragment of *lacI* {*lac* repressor}, the *lac* promoter-operator system (*lacZpo*) for controlled in vivo expression of cloned target genes and the T3 and T7 promoters from the universal cloning vector pBluescript I KS (+) (pBLS; Short et al., 1988; Alting-Mees et al., 1992). Restriction sites are provided with the coordinate for the 3′-nt after cleavage. **(B)** Relevant nt sequences flanking the *pheS* gene. T3 and T7 promoters, location, direction and the first nt in the respective transcripts (open arrowhead) are indicated. The universal M13 sequencing primer (dashed arrow) anneals to the *lacZ* coding strand, which is obtained from pKSS as ss DNA upon helper phage superinfection (Alting-Mees et al., 1992). Thr[5] and Ser[7] denote the codons for the 5th and 7th aa in the original βGal sequence. The *Sma*I and the destroyed *Hin*dII site were used for original insertion of the *pheS* fragment (Kast and Hennecke, 1991). The GenBank database accession No. for the complete nt sequence of pKSS is U01668. **Methods:** pKSS is a derivative of plasmid pKSB1-M4G whose construction was previously detailed (Kast and Hennecke, 1991). Briefly, pKSB1-M4G contains a 1181-bp *Sma*I-*Hin*dII fragment carrying the mutated *pheS* allele encoding the Gly[294] PheRS α subunit, inserted into *Sma*I-digested pBLS in-phase with the *lac* promoter. To be able to use the *Pst*I site within *pheS* for cloning foreign DNA fragments, the second *Pst*I site present in the MCS of pKSB1-M4G was eliminated by partial digestion of pKSB1-M4G with *Pst*I, purification of the linearized fragment, removal of the 3′-protruding ends by T4 DNA polymerase, and subsequent blunt-end religation. A plasmid clone lacking the *Pst*I site in MCS I was designated pKSS. Sequence analysis of the manipulated region revealed that an extra GC bp from the former *Pst*I site was also deleted (between nt 55 and 56; accounted for in Fig. 1B); this deletion, however, has no impact on the function of pKSS. All DNA manipulations were carried out according to standard procedures (Sambrook et al., 1989). Restriction and DNA-modifying enzymes were from Boehringer (Mannheim, Germany). Double-strand DNA sequencing (Sanger et al., 1977; Sambrook et al., 1989) of pKSS was performed with the M13 reverse primer (−24) (#1201, New England Biolabs, Beverly, MA, USA) and the T3 primer (Stratagene, La Jolla, CA, USA) which anneal to pKSS nt 4072–4087 and 4105–4123, respectively, using Sequenase™ (United States Biochemical, Cleveland, OH, USA). Standard transformations were done using *E. coli* HB101 (see Table I). Bacteria were always grown at 37°C.

limited host range, relatively high background, the need to use highly mutagenic agents or complex media and procedures for selection, maintenance, propagation or purification of the vectors. Other drawbacks associated with many of the vectors themselves include a relatively large size and complex structure, relatively few restriction sites suitable for cloning, and low copy number. Finally, even the most useful and efficient positive-selection vectors reported lack the versatility of today's most popular general cloning vehicles (see e.g., Brosius, 1992).

The novel positive-selection vector pKSS presented in this work combines the universal features of the general cloning vector pBluescript (Short et al., 1988; Alting-Mees et al., 1992) with a newly designed and highly efficient selection mechanism. This selection strategy is based on previous work on determinants of phenylalanine (Phe) specificity of phenylalanyl-tRNA synthetase (PheRS) from *E. coli* (Kast and Hennecke, 1991). As predicted from a model for substrate binding, replacement of the Ala^{294} of the PheRS α subunit with a smaller Gly residue yields an enzyme with relaxed substrate specificity. The Gly^{294} PheRS accepts Phe derivatives with bulky *p*-substituents, such as *p*-Cl-Phe and *p*-bromo-phenylalanine (Kast and Hennecke, 1991), in addition to the substrates of wt PheRS, Phe and *p*-F-Phe.

The sensitivity to *p*-F-Phe of *E. coli* cells containing a wt *pheS* gene has been previously exploited to develop a positive-selection system based on the vector pHE3 carrying the wt *pheS* allele and the *p*-F-Phe^R mutant *E. coli* strain RR28 (*pheS*12; Hennecke et al., 1982). Charging $tRNA^{Phe}$ with *p*-F-Phe by wt PheRS leads to deleterious incorporation of the aa analog into cellular protein (Fangman and Neidhardt, 1964; Hennecke et al., 1982; Kast and Hennecke, 1991). In the presence of *p*-F-Phe, the wt *pheS* gene is dominant and lethal, unless it is inactivated by cloning a DNA fragment into the unique *Pst*I site of pHE3. However, this system is limited by the lack of MCS, the need for minimal medium in the selection step and, in particular, the requirement for the specific host strain, RR28. The observation that wt *E. coli* cells become highly sensitive to *p*-Cl-Phe as soon as the *pheS* allele encoding the Gly^{294} PheRS variant α subunit is introduced (Kast and Hennecke, 1991) inspired the elaboration of a versatile, second-generation positive-selection vector by reversing the principles of selection used for the pHE3/RR28 system.

EXPERIMENTAL AND DISCUSSION

(a) Structure of pKSS

Structure and construction of the positive-selection vector pKSS is described in Fig. 1. The nt sequence encompassing the two polylinkers (MCS I and II) is shown in Fig. 1B; it includes a deviation from the published sequence of pBluescript (the deletion of a GC bp 5′ to the *Kpn*I site, between positions 4133 and 1; unpublished observation), which was very recently reported to occur in all pBluescript I KS vectors (Alting-Mees et al., 1992). The complete and corrected sequence of pKSS can be accessed (No. U01668) in the GenBank (Benson et al., 1993).

(b) The pKSS positive-selection system

In a *pheS* wt *E. coli* cell, the high substrate specificity of wt PheRS prevents detrimental incorporation of *p*-Cl-Phe into cellular protein. However, when a plasmid (e.g., pKSS) carrying the *pheS* allele encoding the Gly^{294} PheRS α subunit (conferring relaxed substrate specificity) is introduced into such a cell, it becomes extremely sensitive to this Phe analog (Kast and Hennecke, 1991). Thus, in the presence of *p*-Cl-Phe, the *pheS* allele of pKSS is dominantly lethal over the recessive chromosomal wt *pheS* locus in such merodiploid transformants. The strategy for direct selection of recombinant clones involves inactivating the plasmid-borne *pheS* allele by inserting the target DNA fragment either directly into a restriction site within the *pheS* reading frame, or by replacing part or all of the *pheS* gene via two different, appropriate sites. pKSS provides four different sites for the first option and a myriad of possible combinations for the second (Fig. 1), so that virtually any DNA fragment can be cloned into pKSS using positive selection.

Different selective conditions were evaluated for optimal efficiency and reproducibility. Among the *p*-halogenated Phe derivatives that are accepted as substrates by Gly^{294} PheRS, but not by the wt enzyme, D,L-*p*-Cl-Phe is clearly the best choice for the selection. Besides being the most effective substrate (Kast and Hennecke, 1991), it caused the most dramatic phenotype (no growth) of Gly^{294} PheRS-producing cells, but did not impair plating efficiency of *pheS* wt strains (see section **d**). Furthermore, D,L-*p*-Cl-Phe is sufficiently soluble and readily available. The cost of D,L-*p*-Cl-Phe does not exceed that of either XGal or IPTG, the ingredients needed for blue-white screening of insert-carrying clones in, for example, pUC or pBluescript vectors (Yanisch-Perron et al., 1985; Brosius, 1992). On minimal medium (as detailed by Kast and Hennecke, 1991) containing as little as 1.1 mM racemic *p*-Cl-Phe, no background growth of strains producing Gly^{294} PheRS was observed, contrasting with the residual slow growth seen in the RR28[pHE3] system. This finding inspired the attempt to use rich medium, which accelerates growth and is easier to prepare. Initial experiments with LB agar (Miller, 1992) showed that sensitivity started at D,L-*p*-Cl-Phe concentrations of

TABLE I

Selection efficiency in different *E. coli* host strains after cloning chromosomal DNA fragments into different sites of pKSS[a]

E. coli strain[b]	Cloning sites used	Analysis of clones isolated from YEG-Cl plates[c]		
		Analyzed transformants[d]	pKSS with insert	Plasmid of size pKSS
HB101	*Aat*II	12	12	0
	*Pst*I	11	11	0
	*Bam*HI + *Hin*dIII	14	14	0
	*Eco*RI + *Xba*I	13	12	1
DH5α	*Aat*II	8	8	0
	*Pst*I	13	13	0
	*Bam*HI + *Hin*dIII	11	11	0
	*Eco*RI + *Xba*I	10	10	0
JM109	*Pst*I	2	2	0
	*Bam*HI + *Hin*dIII	11	11	0
	*Eco*RI + *Xba*I	2	2	0
Total:		107	106	1

[a]pKSS was digested with the indicated restriction endonuclease(s) and heat-treated for 30 min at 85°C. Chromosomal DNA from HB101 was fractionated on a 0.8% low-melting agarose gel. The size range of fragments excised for direct ligation to the vector allowed subsequent easy identification of plasmid clones with an insert. For procedures and materials see Fig. 1.

[b]The three *E. coli* strains possess a wt *pheS* gene. The genotypes of HB101 (Boyer and Roulland-Dussoix, 1969) and JM109 (Yanisch-Perron et al., 1985) were listed earlier (Kast and Hennecke, 1991). DH5α™ {(F^- (ϕ80d*lacZ*ΔM15) Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17 (r_k^-, m_k^+), *supE*44, *thi*-1, λ^-, *gyrA*96, *relA*1} was from Gibco-BRL Life Technologies (Gaithersburg, MD, USA).

[c]Selective YEG-Cl agar plates were made with 5 g yeast extract (Oxoid, Basingstoke, UK)/10 g NaCl/2 g D,L-*p*-Cl-Phe (No. 13,071-0, Aldrich, Milwaukee, WI, USA)/15 g agar per liter of deionized H_2O. After thorough mixing (to ensure dissolution of most *p*-Cl-Phe after subsequent autoclaving), the medium was steam sterilized. Then, sterile solutions of glucose and Ap were added to 0.4% (w/v) and 200 µg/ml final concentrations, respectively. No IPTG induction of *pheS* expression was necessary.

[d]No colonies without plasmids were detected on transformation plates.

4–10 mM; however, some background growth persisted even up to 60 mM, presumably due to the abundance of competing Phe in LB. Increasing the Ap concentration from 150 to 750 µg/ml did not sufficiently reduce the number of colonies that have lost the Gly[294] mutant *pheS*-carrying vector. This background problem was, however, completely solved by omitting tryptone from LB to give 'YEG-Cl' agar (0.5% yeast extract/1% NaCl/0.4% glucose/1.5% agar/10 mM D,L-*p*-Cl-Phe/200 µg Ap per ml; for preparation see Table I).

(c) Analysis of chromosomal DNA cloning into pKSS: variation of restriction sites and *E. coli* host strains

The utility of pKSS for cloning foreign DNA fragments with positive selection was assessed by cloning experiments using four different insertion sites in pKSS (Table I). It must be emphasized here that the vector DNA was not purified after digestion, but only heat-treated to inactivate the restriction enzyme(s). To demonstrate the power of this system, digested and size-selected chromosomal DNA of *E. coli* HB101 was ligated to the vector fragment, and three different commonly used *E. coli* host strains were transformed with the ligation mixtures. Analysis of 107 randomly picked clones from the selective YEG-Cl plates showed that 106 transformants carried plasmids with an insert, corresponding to the cloning efficiency of over 99% (Table I).

(d) Background analysis

No colonies without pKSS were detected upon transformation with ligation mixtures. Further analysis of the only clone with a plasmid of the size of pKSS (see section **c** and Table I) showed that although the plasmid appeared to possess a pKSS-typical restriction pattern, the corresponding transformant strain was fully *p*-Cl-Phe resistant, suggesting that this plasmid had picked up a yet uncharacterized mutation. Transformations with religated, unpurified vector alone gave a background of less than 0.5% colonies on YEG-Cl plates as compared to YEG plates. A stationary phase culture of HB101 harboring pKSS was titrated on YEG-Cl(Ap) and YEG(Ap) plates to assess the combined background due to host strain mutations, plasmid loss and pKSS mutants subsequently dominating the plasmid pool within a cell. A control culture of HB101[pBLS] did not show decreased plating efficiency on YEG-Cl, whereas background

TABLE II

Characterization of a randomly chosen clone obtained by positive-selection cloning

Strain[plasmid][a]	Growth[b] YEG	YEG-Cl	YEG-F	% of PheRS activity in the presence of competitor[c]		
				p-Cl-Phe	*p*-F-Phe	Phe
HB101	– –	– –	– –	99.5	32.9	7.3
HB101[pBLS]	+	+	–	102.9	35.8	9.5
HB101[pKSS]	+	– –	– –	76.2	23.8	6.5
HB101[pKSS-AAT]	+	+	–	100.6	32.2	5.8

[a] HB101[pKSS-AAT] was picked from a YEG-Cl agar plate of the cloning experiment from section **c**. It contains an insertion in the *Aat*II site of pKSS. pBLS and pKSS transformants served as negative (no plasmidial *pheS*) and positive (Gly[294] *pheS*) controls, respectively.

[b] The growth of single colonies streaked on YEG, YEG-Cl and YEG-F agar plates (200 μg Ap/ml) was scored from no growth at all (– –), marginal growth (–) to good growth (+). YEG-F is YEG medium with 10 mM D,L-*p*-F-Phe (for more details see Table I, footnote c).

[c] Crude extracts were prepared from cultures grown to stationary phase. The amount of cells corresponding to one A_{650} unit were spun down and resuspended in 400 μl of 20 mM Tris·HCl pH 7.5/0.2 mM EDTA/6 mM β-mercaptoethanol/30 mM NH_4Cl/10 mM $MgCl_2$/1 μg per ml of DNase I. The cells were disrupted by ultrasonication (Branson sonifier cell disrupter 200, micro tip, 5 × 15 pulses, output level 6.0, duty cycle 10%, at 0°C). After centrifugation at 12 000 × *g*, the supernatant was assayed for PheRS activity. PheRS competition assays were carried out for 5 min at 28°C as described previously (Kast and Hennecke, 1991) with a 50-fold molar excess of the competing compound (L-enantiomer). The value in the absence of a competitor (+ H_2O) was defined as 100%. Each data point is the mean of two experiments with a deviation of less than 5%.

growth of individual colonies with HB101[pKSS] was only observed at cell concentrations of and above 10^6 per plate. Re-streaking of such background colonies on Ap-containing agar as well as attempted plasmid isolation showed that they had eliminated pKSS (data not shown). Thus the apparently extremely low background with HB101[pKSS] is predominantly due to cell density effects.

(e) Insertion of foreign DNA into the plasmidial *pheS* mutant gene inactivates the relaxed specificity Gly[294] PheRS variant

A randomly chosen colony from cloning experiments of bacterial chromosomal DNA in pKSS (section **c**) was tested for in vivo and in vitro activity of the plasmid-encoded PheRS α subunit. The plasmid harbored by HB101 had approximately 1500 bp inserted in the *Aat*II site of pKSS and was designated pKSS-AAT. As shown in Table II, the insert abolished sensitivity to *p*-Cl-Phe seen with pKSS-carrying cells, whereas the less prominent sensitivity to *p*-F-Phe of wt *pheS* became apparent again. In vitro PheRS aminoacylation assays (Kast and Hennecke, 1991) with radio-labeled Phe in the presence of competing Phe derivatives confirmed the difference in substrate utilization expected for extracts of cells with pKSS (relaxed) or pKSS-AAT (wt) (Table II). Thus, this experiment verified the link between survival on YEG-Cl agar plates and disabling Gly[294] PheRS activity.

(f) Conclusions

(1) The new versatile cloning vector pKSS was shown to function efficiently for direct selection of clones with recombinant plasmids. Because cells transformed with religated or uncut pKSS do not grow under selective conditions, pKSS should greatly facilitate any cloning experiment which benefits from very high ratios of recombinant to non-recombinant clones. The selection system employs the inactivation of a plasmid-borne mutant *pheS* gene, and eliminates all of the drawbacks of the previous positive-selection system with pHE3 carrying wt *pheS* (Hennecke et al., 1982). The switch from minimal to the much simpler YEG-Cl rich medium expedites cloning (one day instead of two for appearance of colonies). Furthermore, the large number of cloning sites and the host strain independence increase tremendously the applicability of the pKSS system.

(2) pKSS offers all of the advantages of the multipurpose cloning vector pBluescript, including a high copy number, single-stranded DNA production, IPTG-inducible gene expression, gene fusion with the α-complementing fragment of *lacZ*, convenient MCS, unidirectional deletions of predictable length into inserted DNA, and RNA transcription into the cloned fragment from the T3 and T7 promoters (Fig. 1; Short et al., 1988; Alting-Mees et al., 1992).

(3) Due to the low background inherent to the pKSS selection system, no dephosphorylation and/or vector purification is required, thus greatly accelerating the cloning process.

(4) The enumerated properties of pKSS make this new vector strongly competitive with the best positive-selection cloning systems currently available (Nilsson et al., 1983; Nikolnikov et al., 1984; Hashimoto-Gotoh et al., 1986; Henrich and Plapp, 1986; Kuhn et al., 1986; Altenbuchner et al., 1992). Specialized applications of pKSS or appropriate derivatives may include gene library construction (Pierce and Sternberg, 1992), trapping of rare transposition events (Simon et al., 1991) and select-

ing allelic exchange of chromosomal markers in any desired *E. coli* strain (Blomfield et al., 1991; Donnenberg and Kaper, 1991; Quandt and Hynes, 1993).

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REFERENCES

Altenbuchner, J., Viell, P. and Pelletier, I.: Positive selection vectors based on palindromic DNA sequences. Methods Enzymol. 216 (1992) 457–466.

Alting-Mees, M.A., Sorge, J.A. and Short, J.M.: pBluescript II: multifunctional cloning and mapping vectors. Methods Enzymol. 216 (1992) 483–495.

Balbás, P., Soberón, X., Merino, E., Zurita, M., Lomeli, H., Valle, F., Flores, N. and Bolivar, F.: Plasmid vector pBR322 and its special-purpose derivatives – a review. Gene 50 (1986) 3–40.

Benson, D., Lipman, D.J. and Ostell, J.: GenBank. Nucleic Acids Res. 21 (1993) 2963–2965.

Blomfield, I.C., Vaughn, V., Rest, R.F. and Eisenstein, B.I.: Allelic exchange in *Escherichia coli* using the *Bacillus subtilis sacB* gene and a temperature-sensitive pSC101 replicon. Mol. Microbiol. 5 (1991) 1447–1457.

Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L., Boyer, H.W., Crosa, J.H. and Falkow, S.: Construction and characterization of new cloning vehicles, II. A multipurpose cloning system. Gene 2 (1977) 95–113.

Boyer, H.W. and Roulland-Dussoix, D.: A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. 41 (1969) 459–472.

Brosius, J.: Compilation of superlinker vectors. Methods Enzymol. 216 (1992) 469–483.

Christensen, L.S. and Boye, M.: Establishment of a genomic bank of bovine herpesvirus 1 using a novel positive selection plasmid vector. J. Virol. Methods 36 (1992) 277–282.

Dean, D.: A plasmid cloning vector for the direct selection of strains carrying recombinant plasmids. Gene 15 (1981) 99–102.

Donnenberg, M.S. and Kaper, J.B.: Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infect. Immunol. 59 (1991) 4310–4317.

Fangman, W.L. and Neidhardt, F.C.: Protein and ribonucleic acid synthesis in a mutant of *Escherichia coli* with an altered aminoacyl ribonucleic acid synthetase. J. Biol. Chem. 239 (1964) 1844–1847.

Hashimoto-Gotoh, T., Kume, A., Masahashi, W., Takeshita, S. and Fukuda, A.: Improved vector, pHSG664, for direct streptomycin-resistance selection: cDNA cloning with G:C-tailing procedure and subcloning of double-digest DNA fragments. Gene 41 (1986) 125–128.

Hennecke, H., Günther, I. and Binder, F.: A novel cloning vector for the direct selection of recombinant DNA in *E. coli*. Gene 19 (1982) 231–234.

Henrich, B. and Plapp, R.: Use of the lysis gene of bacteriophage φX174 for the construction of a positive selection vector. Gene 42 (1986) 345–349.

Hill, R.T., Illing, N., Kirby, R. and Woods, D.R.: Development of pLR591, a *Streptomyces-Escherichia coli* positive selection shuttle vector. FEMS Microbiol. Lett. 57 (1989) 223–226.

Kast, P. and Hennecke, H.: Amino acid substrate specificity of *Escherichia coli* phenylalanyl-tRNA synthetase altered by distinct mutations. J. Mol. Biol. 222 (1991) 99–124.

Kuhn, I., Stephenson, F.H., Boyer, H.W. and Greene, P.J.: Positive-selection vectors utilizing lethality of the *Eco*RI endonuclease. Gene 44 (1986) 253–263.

Lin-Chao, S., Chen, W.-T. and Wong, T.-T.: High copy number of the pUC plasmid results from a Rom/Rop-suppressible point mutation in RNA II. Mol. Microbiol. 6 (1992) 3385–3393.

Miller, J.H.: A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1992.

Nikolnikov, S., Pósfai, G. and Sain, B.: The construction of a versatile plasmid vector that allows direct selection of fragments cloned into six unique sites of the *c*I gene of coliphage 434. Gene 30 (1984) 261–265.

Nilsson, B., Uhlén, M., Josephson, S., Gatenbeck, S. and Philipson, L.: An improved positive selection plasmid vector constructed by oligonucleotide mediated mutagenesis. Nucleic Acids Res. 11 (1983) 8019–8030.

Pierce, J.C. and Sternberg, N.L.: Using bacteriophage P1 system to clone high molecular weight genomic DNA. Methods Enzymol. 216 (1992) 549–574.

Pierce, J.C., Sauer, B. and Sternberg, N.: A positive selection vector for cloning high molecular weight DNA by the bacteriophage P1 system: improved cloning efficacy. Proc. Natl. Acad. Sci. USA 89 (1992) 2056–2060.

Quandt, J. and Hynes, M.F.: Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria. Gene 127 (1993) 15–21.

Sambrook, J., Fritsch, E.F. and Maniatis, T.: Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

Sanger, F., Nicklen, S. and Coulson, A.R.: DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74 (1977) 5463–5467.

Short, J.M., Fernandez, J.M., Sorge, J.A. and Huse, W.D.: λ ZAP: a bacteriophage λ expression vector with in vivo excision properties. Nucleic Acids Res. 16 (1988) 7583–7600.

Simon, R., Hötte, B., Klauke, B. and Kosier, B.: Isolation and characterization of insertion sequence elements from Gram-negative bacteria by using new broad-host-range, positive selection vectors. J. Bacteriol. 173 (1991) 1502–1508.

Stephenson, F.H. and Kuhn, I.: Plasmid positive selection vectors. In: Rodriguez, R.L. and Denhardt, D.T. (Eds.), Vectors: A Survey of Molecular Cloning Vectors and Their Uses. Butterworth Publishers, Stoneham, MA, 1988, pp. 131–152.

Weilguny, D., Prætorius, M., Carr, A., Egel, R. and Nielsen, O.: New vectors in fission yeast: application for cloning the *his2* gene. Gene 99 (1991) 47–54.

Yanisch-Perron, C., Vieira, J. and Messing, J.: Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33 (1985) 103–119.