Background-minimized Cassette Mutagenesis by PCR Using Cassette-specific Selection Markers: A Useful General Approach for Studying Structure–Function Relationships of Multisubstrate Enzymes

Kumud Majumder,¹ Farkad A. Fattah,² Angamuthu Selvapandiyan, and Raj K. Bhatnagar

Plant Molecular Biology Laboratory, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110 067, India

An efficient protocol, termed background-minimized cassette mutagenesis (BMCM) by PCR, has been developed for multiple mutagenesis of DNA. This method uses suitable extension primers for incorporating various mutation(s) and is not limited by either the nature of the mutation or the size and spatial location of mutational loci. Minimization of the wild type background clone and mutant selection at very high frequency were easily achieved through a two-step process. First, a deletion of a unique restriction site within the cassette was introduced through additional silent mutation(s). Then, the recombinant clones were digested with the corresponding enzyme followed by transformation when selective linearization of wild-type clone led to its near total removal leaving the mutant clones as the only practicable transformants. Because it is generally possible to design several such cassette-specific unique background minimization markers for any gene, for multiple mutagenesis involving distally located portions of the gene the present protocol is superior to other currently available methods. The efficiency of BMCM–PCR has been demonstrated here by using the multisubstrate

enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPs) of *Bacillus subtilis* as a model system. Three different sets of cassettes of varying sizes were generated to encompass the three putative active/binding regions in the beginning, middle, and the end of the gene encoding EPSPs. Very high efficiency of mutation incorporation and selection were obtained in all cases. Furthermore, by taking advantage of the unique cassette-specific background elimination markers, it was possible to generate a nested set of double and/or triple mutants. The mutant enzymes were overexpressed in *Escherichia coli* and purified to near homogeneity. Comparison of the kinetic data of these mutants with the wild-type enzyme provided important insight into the mechanism of the binding of EPSPs to its multiple substrates. Significantly, the present concept of background minimization can be easily extended to most of the other methods of site-directed mutagenesis, thereby improving the performances of these methods.

Understanding the complex structure–function relationship between multisubstrate enzyme and its various substrates often requires simultaneous mutagenesis at putative binding sites even if these are present at distal loci on

the structural gene. This ideally necessitates the construction of multiple mutant cassettes, each bearing its own unique selection marker. The availability of such cassettes permits one to create a library of mutants needed to generate the information on the cross-correlation between the different binding sites. However, in the course of our investigation on the multisubstrate enzyme 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPs),⁽¹⁾ we realized that none of the currently available and popular mutagenesis approaches, like mutant selection through phenotypic or genotypic markers,⁽²⁾ the ampicillin-resistant marker,⁽³⁾ the “ung” method,⁽⁴⁾ the phosphorothioate method,⁽⁵⁾ or the restriction site elimination approach,⁽⁶⁾ can be used to specifically remove/reduce the wild-type, or the nonmutant, background clones. Even the PCR-based efficient multiple mutagenesis methods like splicing by overlap extension (SOE) by PCR,⁽⁷⁾ stepwise elongation of sequence (SES) by PCR,^(8,9) site-directed mono- and multiple mutagenesis of double-stranded DNA by PCR,⁽¹⁰⁾ or simultaneous mutagenesis of multiple sites by ligation chain reaction⁽¹¹⁾ do not provide scope for generating this type of specific markers. Such lacuna in the available protocols imposed a serious limitation on our ability to create the desired combinatorial library of mutants as removal of the nonmutant background became increasingly difficult while mixing and matching different cassettes.

Present addresses: ¹Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030; ²Department of Agriculture, University of Baghdad, Iraq.

This prompted us to develop an efficient multiple mutagenesis protocol that allows the creation of selection markers that are unique to specific cassettes. In our method, the subject of this report, the markers are created through selective deletion of unique restriction sites via silent mutations. The absence of the deleted site in mutants is then exploited to remove the background nonmutants by a digestion–transformation step. This approach, termed background-minimized cassette mutagenesis by PCR (BMCM–PCR), provides a nearly 100% efficiency of mutation incorporation irrespective of the number, nature, location, or size of the targeted mutations.

MATERIALS AND METHODS

The oligomers were synthesized on an automated DNA synthesiser (model 380B, ABI, USA) and processed for use as PCR primers.^(8,9) For the sake of convenience, the *n*th mutant primer at the 5' end is designated as MP*n* (mutant primer, number), and the *n*th mutant primers at the 3' end as MT*n* (mutant terminator, number). The plasmid pEPB5 containing the wild-type gene encoding EPSPs enzyme from *Bacillus subtilis*⁽¹²⁾ was used as the template for the first step of the present BMCM–PCR method. All initial steps of PCR were carried out in a 25- μ l volume using 1–2 units of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, USA) in the manufacturer's supplied reaction buffer. Denaturation was done at 94°C (1 min), annealing at 37–55°C (1 min), and amplification at 72°C (1 min). The primers and templates added were as follows: For the first PCR, 1–10 ng of pEPB5 and 10–15 pmoles of the MP1 and MT1 primers were used and amplified for 20–30 cycles. A 2.5- μ l aliquot of the product of the first PCR was diluted to 250 μ l, and 10 μ l was used as the template for the second PCR using 10–15 pmoles of primer MP1 and MT2, along with other PCR components. The amplification was carried out for 30–40 cycles using the same conditions as above. Nondenaturing PAGE analysis of the amplification products from each step of PCR showed that the major band always corresponded to the molecular size of the targeted product (see Fig. 3, below). The final PCR product was extracted with phenol–chloroform and precipitated with ethanol, and the dried precipitate

was resolubilized in 20 μ l of 10 mM Tris and 1 mM EDTA (pH 8.0), and digested with *Eco*RI and *Hind*III. Cloning of the insert in pBSKII or pKMZB,⁽¹³⁾ identification of recombinant clones by blue–white color selection, and DNA sequencing of the positive clones were carried out by standard methods.⁽¹⁴⁾

For subcloning of Cassette 1 in pEPB5, the sequenced clones were digested with *Nsi*I + *Nhe*I and the minigene cassettes were recovered by PAGE followed by agarose-mediated electroelution. The cassettes were inserted in a *Nsi*I + *Nhe*I digest of pEPB5. After cloning and transformation,⁽¹⁴⁾ the transformed sample was used for plasmid isolation. The DNA was then digested with *Sma*I, as this site has been deleted in the mutant cassette, and retransformed in *Escherichia coli* DH5 α . Alternatively, the ligation mixture was digested with *Sma*I and then used for transformation. The other cassettes were also similarly cloned using a suitable combination of enzymes.

The wild-type and mutant genes cloned on pBSKII were expressed to a 30% level as soluble proteins using the bacteriophage T7 RNA polymerase/promoter system.⁽¹⁵⁾ The expressed protein was purified by ammonium sulfate followed by recovery of the enzyme by dialysis.⁽¹⁶⁾ The dialyzed extract was used as the source of enzyme. Analysis of extract by SDS-PAGE revealed only one major band corresponding to ~90% purity of EPSPs. Identical purification protocol with all mutants resulted in a comparable amount of nearly homogenous preparation of EPSPs. Purified EPSP synthase preparations containing no detectable phosphatase activity were assayed by measuring the rate of appearance of Pi (inorganic phosphate) as described by Lanzetta et al.⁽¹⁷⁾

RESULTS AND DISCUSSION

Figure 1 shows a schematic representation of the principles underlying the BMCM–PCR method. As shown, during the PCR-based synthesis of the mutant cassettes, in addition to the targeted mutations, auxiliary silent mutations leading to the deletion of a unique restriction site (shown as *Sma*I) and possible creation of a different unique restriction marker (shown as *Not*I) were carried out. This resulted in two different permanent cassette-linked markers, namely, the background elimination marker (here

*Sma*I) and a mutant screening marker (here *Not*I). Because the *Sma*I site is present only in the wild-type cassette and not in the mutant ones, selective removal of the wild-type clones can be achieved easily by digesting the ligation mixture with *Sma*I prior to the transformation step. As the transformation efficiency of the linearized plasmid is extremely low as compared with the circularized molecules,⁽¹⁴⁾ under the conditions of complete digestion (by *Sma*I) the bacterial machinery itself nearly eliminates the wild-type clones leaving the mutants as the only transformants. This process of mutant enrichment by selective removal of the wild-type background has been termed as the background-minimized cassette mutagenesis by PCR or BMCM–PCR.

Figure 2A shows the schematic representation of the physical location of the three targeted cassettes in the *aroE* gene encoding EPSPs. Figure 2B shows the general strategy used for the assembly of Cassette 1 (M1 and M2) and Cassette 3 (M6). The general steps used in the synthesis of Cassette 2 (M3, M4, and M5) are shown in Figure 2C. The specific base changes introduced in the three cassettes are shown in Table 1. Figure 2D shows the specific details of the construction of Cassette 1. Here, panels i–iv show the sequences of the primers used for PCR and v shows the wild-type (WT) and mutant cassettes (M1 and M2). For all of the cassettes, all mutations, including the silent ones, were incorporated by using only the degenerate codons biased for the *Bacillus* genome and specifically for its *aroE* gene coding for EPSPs. Such a codon replacement strategy was chosen to avoid any codon-induced variation in the yield of the recombinant enzyme during its expression in *E. coli*.

As the BMCM–PCR protocol depends critically on the availability of unique restriction sites within the cassette, the size of the cassette might be driven at times by this factor rather than the size of the mutation and/or nearest cloning sites at the two ends of the cassette. This was the case for mutations M3, P105S; M4, R104K; and M5, R104Q (Cassette 2 of Table 1). Accordingly, it became necessary to synthesize cassettes of 411 bp. Because, here, the targeted mutation is located at the center and the silent mutation at the beginning of the cassette (sequence not shown), assembly of Cassette 2 was achieved by a combination of

FIGURE 1 A graphic presentation of the principles of the BMC-PCR method. During the PCR-based mutagenesis in addition to the targeted mutations (solid rectangles), supplementary silent mutations (shaded rectangles) leading to the deletion of a unique restriction site (here *Sma*I) and possible creation of a unique restriction site (here *Not*I) were carried out using mutagenic primers. After digestion of the PCR amplification product with suitable enzymes (here *Nsi*I and *Nhe*I), it was ligated to a compatibly digested vector. Because the *Sma*I site is present only in the wild type and not in the mutants, digestion of the ligation mixture with *Sma*I (or ligation in the presence of *Sma*I) would linearize only the wild-type clones. Consequently, transformation of the ligation mixtures produces (practically) the mutants as the only transformants.

the approach in Figure 2B and the splicing by overlap extension or SOE-PCR method.⁽⁷⁾ The resulting protocol is shown in Figure 2C. For synthesizing larger cassettes with multiple mutations, one can extrapolate the approach of Figure 2C to link more than two minicassettes.

All of the PCR products thus obtained exhibited the presence of DNA bands of the correct size (Fig. 3). The desired product was recovered from the polyacrylamide gel and cloned into the pBS or pKMZB⁽¹³⁾ vector. Several randomly se-

lected clones were restriction mapped, and at least two of each type were sequenced for both the strands. Correct sequences containing only the desired mutations were observed in these clones. Extensive sequence analysis of a large number of clones showed the presence of an average of one random point mutation for 20–25 clones. This amounted to a fidelity error of 1 in $\sim 4 \times 10^4$, which agrees well with literature data on *Taq* polymerase,⁽¹⁸⁾ thereby suggesting that for cassettes of kilobase length, a certain degree of caution should be exercised in

choosing the number of steps of PCR. Proper regimen of conditions will entail maximum benefit from this method while keeping the number of random undesired mutations at a minimum. Presently, the mutant genes have been successfully used for the expression of mutant EPSPs.⁽¹⁵⁾

To generate a double mutant involving M1 and M6, the M1 cassette bearing the first mutation was cloned as a *Nsi*I–*Nhe*I fragment in the M6-containing recombinant clone pMEPB6. After cloning and transformation, the new recombinant plasmid pMEPB16 was isolated and digested with *Sma*I (see Table 1) to selectively linearize the background pMEPB6. The clones of pMEPB16 obtained after retransformation are necessarily the double mutants because in these cases the *Sma*I site has been replaced by a *Not*I site (Table 1). Thus, double mutants were easily identified without having to perform laborious hybridization experiments. The synthesis of the triple mutants are easily achieved by cloning the desired third mutant cassette (e.g., M3) in a plasmid containing the other two mutant cassettes (e.g., pKMEPB16). Because the wild-type background deletion markers are unique to each cassette, mutant selection is done as above by digestion with *Nhe*I (see Table 1). The expression of the double mutant and triple mutant proteins are currently under study.

EPSPs (E.C. 2.5.1.9) is a shikimate pathway enzyme that catalyzes the transfer of the enol pyruvyl (carboxy vinyl) group from phosphoenol pyruvate (PEP) to the 5-hydroxy group of shikimate-3-phosphate (S3P) to yield 5-enol pyruvyl shikimate-3-phosphate (EPSP) and Pi. EPSPs is an agronomically important enzyme as it is the target for the popular and extensively used postemergence and broad-spectrum herbicide glyphosate, the active ingredient of commercial herbicide Roundup.⁽¹⁾ Although considerable variation exists in the nucleotide and deduced amino acid sequences for EPSPs obtained from several bacteria, fungi, and plants, the reaction mechanism and enzyme structure have been expected to show strong conservation.⁽¹⁾ On the basis of random mutagenic analysis and recognition of the charged nature of the substrates, a few conserved cationic residues located in three different segments of the enzyme along with the conserved uncharged residue P105 have been sug-

FIGURE 2 (A) Schematic view of the three cassettes, 1, 2, and 3, of the wild-type *aroE* gene as present in pEPB5. The location and size of each cassette have also been indicated. (B) The flowchart for background-minimized multiple mutagenesis of gene-encoding EPSPs using cassette-linked unique markers. Both the mutant cassettes 1 (i.e., M1 and M2) and 3 (i.e., M6) were synthesized following this generalized scheme. The first two steps indicate introduction of various mutations. A multistep approach was followed to avoid the necessity of synthesizing very long oligomers. The last step indicates the incorporation of the mutant cassettes either in a cloning vector or a cassette exchange with the wild-type ones present in the wild-type gene-bearing plasmid. (C) The flowchart for the BCM-PCR approach for mutation of bases located away from unique restriction sites. The mutant Cassette 2 (i.e., M3, M4, and M5) was synthesized using this scheme. The first step involves the PCR-mediated synthesis of two mutant/nonmutant minicassettes using the primer sets MP11+MT11 and MP21+MT21. The second step consists of PCR-mediated linking of the two minicassettes through a bridging primer (MBP). The last step is similar to that shown in B. (D) Generation of mutants Lys¹⁹ → Glu (M1) and Arg²⁴ → Asp (M2). (i) Primer MP1, used for deleting the unique *Sma*I site (CCCGGG) of wild-type and replacing it by a *Not*I site (GCGGCCGC). To effect this, the sequence GC-CCGGGC was mutated to GCGGCCGC in the noncoding portion of the gene. (ii) Terminator MT2; (iii) terminator MT1, used for converting Lys¹⁹ → Glu (M1) by mutating AAA → GAG; (iv) terminator MT1, used for changing Arg²⁴ → Asp (M2) by mutating CGC → GAT. (v) The protein and DNA sequence of the wild-type and mutant cassettes M1 and M2. All sequences are shown in the 5' → 3' direction, with their lengths indicated at the right end of each line. Overlapping areas between MT1 and MT2 are underlined. Double underlines on i, iii, and iv indicate the sites for priming the wild-type pEPB5. For all the cassettes, the 5'-terminal primers used in the last step contained an *Eco*RI site and, similarly, the 3'-terminal primers contained a *Hind*III site. An additional 3–4 bases were introduced at the end of the terminal primers to facilitate proper restriction digestion of the PCR products.⁽⁹⁾ Cloning of the PCR products onto pBSKII (Stratagene Inc., USA) or pKMZB⁽¹³⁾ vector was carried out as *Eco*RI–*Hind*III fragments. Conditions of PCR amplification are given in the text.

TABLE 1 Background-minimized Multiple Mutagenesis of Gene Encoding EPSPs Using Cassette-linked Unique Markers

Cassette no.	mutant no.	Mutational substitution carried out		Silent mutations introduced			Restriction enzymes used for background minimization ^c
		amino acid changes ^a	nucleotide changes	nucleotide changes	restriction site deleted ^b	restriction site created ^b	
1	M1	K → E	AAA → GAG	CCGG → GGCC	<i>Sma</i> I	<i>Not</i> I	<i>Sma</i> I
	M2	R → D	CGC → GAT				
2	M3	P → S	CCT → AGC	A → G and G → C	<i>Nhe</i> I	<i>Eco</i> 52I and <i>Nae</i> I	<i>Nhe</i> I
	M4	R → K	CGT → AAA				
	M5	R → Q	CGT → CAG				
3	M6	H → K	CAT → AAG	T → G	<i>Hind</i> III and <i>Bst</i> BI	—	<i>Bst</i> BI

^aThe amino acids are indicated by single-letter IUB/IUPAB codes.

^bExcept for *Nae*I (in the second cassette) and *Hind*III (in the third cassette), all other restriction enzyme sites are unique for both the gene and plasmid. The codon bias of the *aroE* gene of *B. subtilis* was taken into account for incorporating all of the mutations. In the second cassette the silent mutation of A → G resulted in the deletion of the unique *Nhe*I site, and the G → C mutation (silent) led to the creation of the nonunique *Nae*I site for M3, M4, and M5 plus a unique *Eco*52I site only for M3.

^cThe enzymes used here were chosen based on the unique site that has been deleted through silent mutation. These enzymes can be used in conjunction with each other if multiple mutants involving different cassettes are desired.

gested to be involved in the enzyme–substrate interaction.^(1,12) Currently, we have carried out a total of six mutations in these three regions (Table 1), and the

FIGURE 3 Nondenaturing (6%) PAGE analysis of the amplification products obtained from various steps of BMCM–PCR. Lanes B–C correspond to mutant M1, and lanes E–F are for the mutant M2. Amplification products obtained after the first step, B; second step, C and F; and third step, i.e., after digestion with *Eco*RI and *Hind*III followed by phenol–chloroform extraction and precipitation, E. (Lanes A, D) 92-bp DNA as size marker. (Lane M) *Hae*III digest of pBR322 as DNA molecular size marker. Band from lane E was used for DNA extraction⁽¹⁴⁾ and then cloning in pBSKII or pKMZB vector as an *Eco*RI–*Hind*III fragment.

results of kinetic analysis of R24D, P105S, H285K, R104K, and R104Q are presented here. Comparison of the kinetic features like the Michaelis–Menten constant and Hill coefficients of the first three mutants, R24D, P105S, H385K, with the nonmutant enzyme showed that there are two sites for PEP binding—a catalytic and a noncatalytic site. Glyphosate competes for binding at the catalytic site and does not interact at the secondary site. Notably, the presence of the second binding site has been hitherto unknown, and only a single binding site is reported in earlier literature.⁽¹⁹⁾ We find that the native enzyme from H385K exists as a dimer and acquires a tetrameric conformation in the presence of glyphosate. The presence of the dimers and tetramers has been confirmed by native PAGE analysis using activity staining of the enzyme.⁽¹²⁾ The R104Q mutation reduces the sensitivity of the interaction with glyphosate by approximately threefold. The binding constant of PEP increased by the same margin in both R104Q and R104K, suggesting preferential interaction of R104 with PEP. Unlike the dissociation constants of PEP, the Michaelis constant, K_m (S3P), varied diversely in the two mutants. Whereas R104K resulted in a marginal drop in the K_m (S3P), mutation R104Q reduced affinity of S3P with the enzyme. Of the three interacting molecules (S3P, PEP, and glyphosate), significant alteration in the binding of glyphosate is observed for mutants R104Q and R104K. R104K mu-

tation is designed to maintain the positive charge at position 104, and the R104Q mutation replaces the charge by a polar uncharged amino acid; from these two mutations we conclude that the guanidinium side chain of the arginine residue is involved in the glyphosate binding. However, to obtain a clearer picture of how the local conformation at the active sites influences the interaction of the three substrates with EPSPs, the kinetic behavior of various single, double, or triple mutations involving the above conserved sites needs to be studied. And in this regard, the BMCM–PCR method is rather advantageous because it allows easy generation of multiple mutants from the single mutants.

In the past, several techniques like the phenotypic or genotypic selection,⁽²⁾ ung method,⁽⁴⁾ phosphorothioate method,⁽⁵⁾ ampicillin resistance marker,⁽³⁾ and restriction site elimination approach⁽⁶⁾ have been developed to facilitate differentiation of mutants from the wild-type DNA. Although the last two approaches can in principle be used to generate cassettes comprising of portions of the insert and the vector, creation of selection markers without involving the vector sequence is, however, not possible. This limits the usefulness of these protocols for creating multiple cassettes usually needed for the extensive mutagenesis of large DNA. An additional drawback of most of the existing methods of multiple mutagenesis is the necessity of time-consuming southern hybrid-

ization or sequencing of a large number of clones for identifying the mutants.⁽¹⁴⁾

The present BMCM-PCR approach overcomes these limitations of the currently existing methods. This approach of single or multiple mutant recovery, unaided by Southern hybridization, is simple and efficient. Although we have resorted to the creation as well as deletion of unique restriction sites, the deletion of sites within each cassette is strategically more important. Creation of new sites can merely help in the screening of recombinants by restriction mapping, whereas deletion of unique restriction sites allows linearization of nonrecombinants through selective digestion prior to transformation. The extremely inefficient transformation of linear plasmid⁽¹⁴⁾ virtually eliminates the wild-type background. As the cassette-specific deletion of unique restriction sites is performed through silent mutation, it becomes inherent to the cassette. Thus, unlike the other available methods, the present PCR approach is endowed with a permanent provision to destroy specifically the undesired wild-type sequence in a cassette-specific manner. This background-minimization concept is capable of providing nearly 100% efficiency for both mutagenesis and mutant recovery irrespective of the number and loci of mutations in a DNA sequence.

Having established that BMCM-PCR is the only approach that provides unique cassette-linked permanent markers for wild-type background removal/mutant selection, the question we need to address at this juncture is how does an end user benefit by using BMCM-PCR in lieu of other methods like ung, phosphorothioate or phenotypic selection. Understandably, the answer to such a question would depend on the particular mutagenesis at hand. In general, we find that for only one round of mutagenesis or mutation at a single locus, all of the above methods perform nearly equally well, although BMCM-PCR, unlike most of the other methods, does not depend on the use of special vectors, growth media, or enzymes. However, the results of this comparative analysis change considerably in favor of the BMCM-PCR if we take into account the cases of mutagenic analysis of multisubstrate enzymes. For better illustration let us use EPSPs as a representative case of the multisubstrate enzyme. In EPSPs three different regions

have been implicated in enzyme-substrate interaction, and to understand the mechanism of substrate-binding, mutageneses of the amino acids at all these three loci need to be carried out singularly as well as in conjunction with each other. Here, for the sake of logistics, we will consider only the mutations M1-M6 cited in this paper, that is, two from the first region, three from the second region, and one from the third region (see Table 1). This represents 6 single mutants that can be combined to produce 11 double and 6 triple mutants or a total of 23 mutants. Now, if we were to employ the BMCM-PCR method to generate all of these mutants, we would need to carry out only six independent mutagenesis experiments for generating the single mutants. The 17 double and triple mutants can then be easily generated by simple subcloning experiments because each of the single mutants carries independent background elimination markers that allow efficient removal of wild-type/non-mutant clones. In contrast, for phenotypic/genotypic or phosphorothioate, ung, or similar methods, where the use of mutant enrichment (wild-type background removal) criteria are limited to only the first round of mutagenesis, each of the 17 double/triple mutations has to be treated as separate mutagenesis experiments. This in turn would require sequence characterization of the entire mutant cassette/gene for all of the 23 mutants because there is no easy way of determining whether, for multiple mutants, more than one mutant primer actually hybridized to the same template or not. In contrast, for the BMCM-PCR approach, the restriction mapping of the deleted sites is generally sufficient to confirm the presence of multiple mutations. This naturally translates into considerable savings in time, money, and effort when using the BMCM-PCR approach instead of the other existing methods. These advantages of BMCM-PCR make it the method of choice for studying multisubstrate enzymes.

The major limitation of the BMCM-PCR method is that the prior availability of unique restriction sites is a prerequisite for deleting the sites in the target DNA/gene. However, as shown in the case of Cassette 2, this drawback is primarily surmountable through the construction of large cassettes. Interestingly, even though developing a cassette-specific wild-type background elimination

marker via the deletion of a unique restriction site appears to be a natural choice, any reference to such an approach is conspicuously absent in the available literature. It is also possible to extend the present background-minimization paradigm to other existing mutagenesis protocols, thereby improving their efficiency significantly.

ACKNOWLEDGMENTS

This paper is dedicated to Professor Irwin C. Gunsalus as a mark of respect and in appreciation of his continued encouragement to us. Thanks are also due to Professor K.K. Tewari for his support, to Naresh Arora and Ashima Bhardwaj for technical assistance, and to Radha for help in typing the manuscript.

REFERENCES

1. Kishore, G.M. and D.M. Shah. 1988. Amino acid biosynthesis inhibitors as herbicides. *Annu. Rev. Biochem.* **57**: 627-663.
2. Smith, M. 1985. In vitro mutagenesis. *Annu. Rev. Genet.* **19**: 423-462. Rossi, J. and M. Zoller. 1987. Site-specific and regionally directed mutagenesis of protein-encoding sequences. in *Protein engineering* (ed. D.L. Oxender and C.F. Fox), pp. 51-63. Alan R. Liss, Inc., New York.
3. Lewis, M.K. and D.V. Thompson. 1990. Efficient site-directed in vitro mutagenesis using ampicillin selection. *Nucleic Acids Res.* **18**: 3439.
4. Kunkel, T.A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci.* **82**: 488-492.
5. Olsen, D.B. and F. Eckstein. 1990. High-efficiency oligonucleotide-directed plasmid mutagenesis. *Proc. Natl. Acad. Sci.* **87**: 1451-1455.
6. Deng, W.P. and J.A. Nickoloff. 1992. Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. *Anal. Biochem.* **200**: 81-88.
7. Horton, R.M., R.D. Hunt, S.N. Ho, J.K. Pullen, and L.R. Pease. 1989. Engineering hybrid genes without the use of restriction enzymes: Gene splicing by overlap extension. *Gene* **77**: 61-68.
8. Majumder, K. 1992. Ligation-free gene synthesis by PCR: Synthesis and mutagenesis at multiple loci of a chimeric gene encoding OmpA signal peptide and hirudin. *Gene* **110**: 89-94; **115**: 115-116.
9. Majumder, K. 1992. Cost-effective gene synthesis, multiple mutagenesis and gene fusion by polymerase chain reaction. In *Downstream processing in biotechnology*

- (ed. R.N. Mukherjea), pp. 198–215. Tata-McGraw Hill, Delhi, India.
10. Weiner, M.P., K.A. Felts, T.G. Simcox, and J.C. Braman. 1993. A method for the site-directed mono- and multi-mutagenesis of double-stranded DNA. *Gene* **126**: 35–41.
 11. Rouwendel, G.J.A., J.H. Wolbert, L.-H. Zwiers, and J. Springer. 1993. Simultaneous mutagenesis of multiple sites: Application of the ligase chain reaction using PCR products instead of oligonucleotides. *BioTechniques* **15**: 68–76.
 12. Majumder, K., A. Selvapandiyar, F.A. Fattah, N. Arora, S. Ahmad, and R.K. Bhatnagar (in prep.).
 13. Majumder, K., S. Choudhury, and R.K. Bhatnagar. 1994. Recombinant enrichment by exploitation of the restriction sites with interrupted palindromes: Design, synthesis and incorporation of zero background linkers in cloning and expression vectors. *Gene* **151**: 147–151.
 14. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*, pp. 1.21–1.104. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
 15. Tabor, S. and C.C. Richardson. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled expression of specific genes. *Proc. Natl. Acad. Sci.* **82**: 1074–1078.
 16. Selvapandiyar, A., K. Majumder, F.A. Fattah, S. Ahmad, N. Arora, and R.K. Bhatnagar. (in prep.).
 17. Lanzetta, P.A., L.J. Alvarez, P.S. Reinach, and O.A. Candia. 1979. An improved assay for nanomole amounts of inorganic phosphate. *Anal. Biochem.* **100**: 95–97.
 18. Eckert, K.A. and T.A. Kunkel. 1990. High fidelity DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Nucleic Acids Res.* **18**: 3739–3744.
 19. Steinrücken, M.S. and N. Amrhein. 1984. 5-Enolpyruvylshikimate-3-phosphate synthase of *Klebsiella pneumoniae*: 2. Inhibition by glyphosate (N-phosphonomethyl glycine). *Eur. J. Biochem.* **143**: 351–357.

Received August 29, 1994; accepted in revised form December 22, 1994.

Background-minimized cassette mutagenesis by PCR using cassette-specific selection markers: a useful general approach for studying structure-function relationships of multisubstrate enzymes.

K Majumder, F A Fattah, A Selvapandiyan, et al.

Genome Res. 1995 4: 212-218

References

This article cites 14 articles, 3 of which can be accessed free at:
<http://genome.cshlp.org/content/4/4/212.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).

Affordable, Accurate
Sequencing.

To subscribe to *Genome Research* go to:
<https://genome.cshlp.org/subscriptions>