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## Accelerated Publications

# Mutants Generated by the Insertion of Random Oligonucleotides into the Active Site of the β-Lactamase Gene†

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ABSTRACT: We have remodeled the gene coding for β-lactamase by replacing DNA at the active site with random nucleotide sequences. The oligonucleotide replacement ($Phe^{66}XXXSer^{70}XXLys^{73}$) preserves the codon for the active serine-70 but also contains 15 base pairs of chemically synthesized random sequences that code for $2.5 \times 10^6$ amino acid substitutions. From a population of *Escherichia coli* infected with plasmids containing these random inserts, we have selected seven new active-site mutants that render *E. coli* resistant to carbenicillin and a series of related analogues. Each of the new mutants contains multiple nucleotide substitutions that code for different amino acids surrounding serine-70. Each of the mutants exhibits a temperature-sensitive β-lactamase activity. This technique offers the possibility of constructing alternative active sites in enzymes on the basis of biological selection for functional variants.

New methods to rearrange nucleotide sequences in DNA have enriched our understanding of how protein structure governs function. By monitoring the effects of particular substitutions on structure and activity, one can explore putative catalytic mechanisms. The most direct approach is to select the region of a gene that codes for the active site of an enzyme and to systematically substitute nucleotides on the basis of a knowledge of the amino acid groups, the mechanism for catalysis, and the three-dimensional structure. This approach has been applied to diverse enzymes including trypsin (Craik et al., 1985), lysozyme (Perry & Wetzel, 1984), and β-lactamase (Dalbadie-McFarland et al., 1986) with considerable success. We demonstrate here an alternative strategy. Instead of making precise substitution on the basis of detailed knowledge of structure and function, one can insert into genes stretches of nucleotides containing random sequences and use biological selection to obtain active enzyme harboring a spectrum of substitutions.

In our initial studies on the selection of nucleotide sequences from random populations, we examined the −35 region of the promotor of the gene for tetracycline resistance (Horwitz & Loeb, 1986, 1988a,b). Bacterial promotors contain short stretches of nucleotides that are biologically active and tolerate nucleotide substitutions at exceptionally high frequency (Horwitz & Loeb, 1989; Oliphant & Struhl, 1988; Schneider & Stormo, 1989). We obtained 85 new active promotors, many of which bore little resemblance to the promotor consensus sequence and some of which were more active than the consensus sequence or the wild-type tetracycline promotor. For studies on the active sites of enzymes we chose to insert random nucleotide sequences into the gene for RTEM-1 β-lactamase (EC 3.5.2.6) that is present in the plasmid pBR322 (Mathew & Heges, 1976). Bacterial β-lactamases hydrolyze the β-lactam ring of penicillin or cephalosphorin transforming them into reactive metabolites. The mechanism of catalysis by the class A enzymes involves a transient acylation of the serine residue at position 70 (Ambler, 1980). The nucleotide sequence and three-dimensional structure of several class A β-lactamases have been determined (Herzberg & Moult, 1987) and indicate a high level of conservation of amino acids surrounding the active Ser-70. Schultz and Richards (1986) used site-saturation mutagenesis to show that even though Thr-71 is conserved, it can be replaced by 14 of the 20 amino acids substituted. Thus, despite the evolutionary conservation of one amino acid within the active site there could be a high degree of tolerance for substitutions. β-Lactams and cephalosporins are among the most frequently prescribed class of pharmaceuticals worldwide, and the rapid evolution of β-

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lactamases in pathogenic bacteria continues to defeat the best efforts of chemists to create new resistant analogues (Bush, 1988).

We have replaced a portion of the active site of β-lactamase in the plasmid pBR322 with an oligonucleotide that retains the codon for the active Ser-70 but also contains two flanking sequences of six and nine random nucleotides. In these experiments we have screened $2 \times 10^5$ tet$^r$ colonies of *Escherichia coli* infected with plasmids containing random inserts and obtained seven new carbenicillin-resistant mutants. DNA sequence analysis of the mutants indicates that multiple amino acid substitutions within the active site can be tolerated and are compatible with enzymatic activity. Furthermore, nucleotide substitutions involving evolutionarily conserved amino acids alter substrate specificity and temperature stability.

## Experimental Procedures

*Materials.* Oligonucleotides were synthesized by using phosphoroamide chemistry by Operon Technologies (San Pablo, CA). Two oligonucleotides each of 47 nucleotides in length

(i) 5′-CGCCCCGAGGAACGTNNNNNNNNNNN-NNNNNNNNNNNNAGTACTGCT-3′

(ii) 5′-CGCCCCGAGGAACGTTTTNNNNNNNNN-AGCNNNNNNAAAGTACTGCT-3′

were used as templates for the construction of the random inserts. The stretches of random nucleotides within these inserts designated by the N's were synthesized by using equimolar mixtures of nucleoside phosphoramidite derivatives. Restriction endonucleases were obtained commercially and were used according to the suppliers' instructions. Standard molecular cloning methods were employed (Maniatis et al., 1982).

*Plasmid Construction.* The vector for the insertion of random sequences, pBDL, is a modification of pBR322 containing within the ampicillin gene two unique restriction sites located on either side of the nucleotides coding for Ser-70. pBDL was assembled by ligating together two modified segments of pBR322 (Figure 1). The first segment was obtained from the plasmid pBR322-R, a generous gift of Dr. J. H. Richards (California Institute of Technology, Pasadena, CA). It is a modification of pBR322 that contains an *Ava*I and a *Sca*I site centered at positions 3972 and 3937, respectively, as well as an additional *Ava*I site at position 1425 (Schultz & Richards, 1986). Digestion with *Pst*I and *Sph*I yielded a fragment of 1315 bp that contained a portion of the ampicillin gene and lacks the second *Ava*I site.

The second segment was obtained by the following steps: (1) digestion of wild-type pBR322 with *Ava*I and *Pvu*II; (2) purification of the large fragment by electrophoresis and electroelution; (3) filling in the *Ava*I termini with *E. coli Pol*I and blunt-end ligation with $T_4$ DNA ligase. The resultant 3725-bp plasmid was also digested with *Sph*I and *Pst*I, and the 2410-bp fragment was purified by electrophoresis and ligated onto the first segment.

*Synthesis of Random Oligonucleotides.* The double-stranded oligonucleotide used in the construction of the nonproducer strain pBNP was synthesized by hybridizing 200 ng of 9-mer primer 5′-AGCAGTACT-3′ to 1 μg of the single-stranded oligonucleotide template 5′-CGCCCCGAG-GAACGT$(N)_{23}$AGTACTGCT-3′ in 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM dithiothreitol at 65 °C for 10 min. This template-primer was extended with the large fragment of *E. coli Pol*I, digested with *Ava*I and *Sca*I, and purified by polyacylamide gel electrophoresis. The double-stranded oligonucleotide for the construction of the plasmid used in selecting new mutants was synthesized by a similar protocol: 200 ng of 9-mer primer 5′-AGCAGTACT-3′ was hybridized to 1 μg of the template oligonucleotide

5′-CGCCCCGAGGAACGTTTT$(N)_9$AGC$(N)_6$AAAGT-ACTGCT-3′

The template-primer was extended with *E. coli Pol*I, digested with *Ava*I and *Sca*I, and used as a replacement for the insert in the nonproducer strain.

*Other Methods.* The preparation of competent DH5α and DH5 *E. coli* and subsequent transformation with plasmid DNA were carried out according to the protocol of Hanahan (1983). Transformants were grown in "SOC" medium for 1 h prior to antibiotic selection. We scored for carbenicillin resistance by incubating the transfected *E. coli* in petri dishes in agar containing 12.5 μg/mL tetracycline and 50 μg/mL carbenicillin at 30 °C for 48 h. Plasmid DNA was purified by the alkaline lysis method (Maniatis et al., 1982), and sequencing of both strands was carried out on double-stranded DNA purified by isopicnic density centrifugation using dideoxy chain termination (Sanger et al., 1977). β-Lactamase activity was scored by using the chromogenic cephalosporin pyridinium-2-azo-*p*-dimethylaniline chromophore (PADAC) (Kobayashi et al., 1988). An overnight culture of each test mutant was diluted 1000-fold with the fresh broth, and a 5-μL inoculum ($10^{-4}$ CFU per spot) of each sample was applied onto agar plates containing 50 μM PADAC and 12.5 μg/mL tetracycline. After 18–20 h of incubation at 30 °C the diameter of the PADAC hydrolysis zone formed around the colony was determined. The highest antibiotic concentration permissible for growth of *E. coli* was determined by using antibiotic concentration gradients generated in L-agar plates (Schultz, 1987).

## Results

In order to substitute random DNA sequences for designated nucleotides at the active site of the β-lactamase gene, we first constructed a derivative of plasmid pBR322 that contains two unique restriction sites flanking the targeted sequence (see Experimental Procedures). The new DNA vector contains both the tetracycline and ampicillin resistance genes. Within the ampicillin resistance gene is an *Ava*I and a *Sca*I site centered at positions 3329 and 3294, respectively. Since it is likely that only a small fraction of random nucleotide sequences at the active site of β-lactamase code for viable amino acid substitutions, and since it is difficult to completely cleave restriction sites when present at the ends of double-stranded DNA (Horwitz & Loeb, 1986), we designed a two-step strategy to minimize contamination with wild-type sequences. We first constructed a nonproducer plasmid, pBNP, that contains an inactive nucleotide replacement, obtained DNA from isolated clones, and then exchanged the inactive sequence with a random nucleotide sequence to be used for mutant selection.

The nonproducer was obtained by digesting the modified pBR322 (Figure 1) with *Ava*I and *Sca*I and inserting a double-stranded 47 oligomer that contains a 23-base random nucleotide sequence (Figure 2A). Plasmid-infected *E. coli* were selected on the basis of resistance to tetracycline and then screened for sensitivity to low concentrations of carbenicillin (10 μg/mL). A tet$^r$ clone was selected that was sensitive to all of the analogues tested and exhibited no detectable β-lactamase activity (Figure 3). DNA sequence analysis demonstrated that this clone contains a 23 base pair insert between Arg-66 and Val-74 (the *Ava*I and *Sca*I sites) and codes for

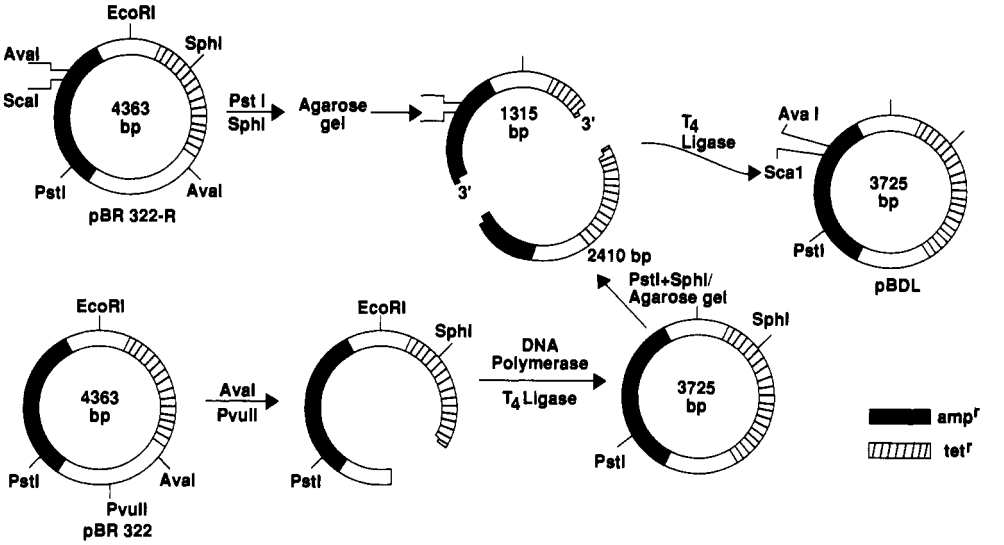


FIGURE 1: Construction of pBDL. pBDL was constructed as detailed under Experimental Procedures.

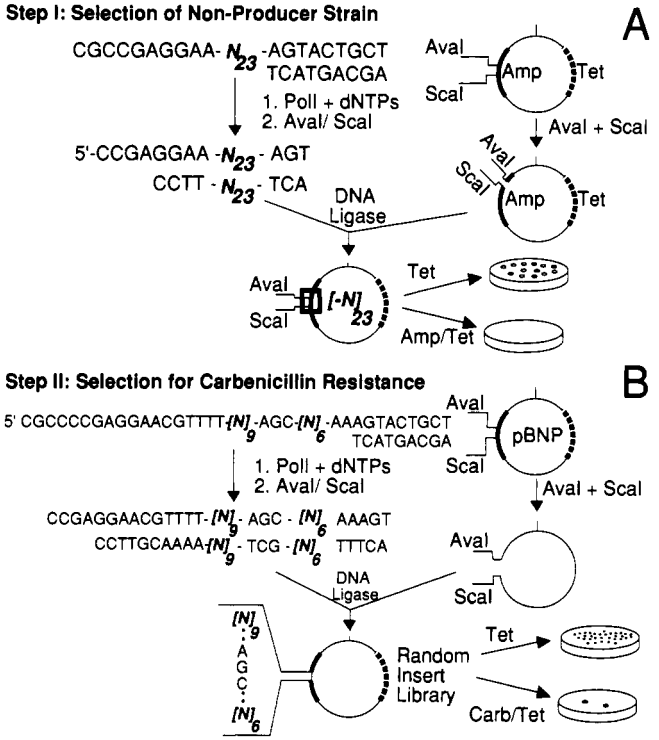


FIGURE 2: Overall scheme for the insertion of oligonucleotides containing random DNA inserts. The construction of the nonproducer strain pBNP is given in step I. Replacement of the oligonucleotide in the nonproducer strain with random DNA sequences is given in step II. Abbreviations: amp, ampicillin; tet, tetracycline; N, unspecified bases.

| | | 70 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Amp (wt) | CGT Arg | TTT Phe | CCA Pro | ATG Met | ATG Met | AGC Ser | ACT Thr | TTC Phe | AAA Lys |
| Non Producer | CGT Arg | ***CAT*** ***His*** | ***TTT*** ***Phe*** | ***CTG*** ***Leu*** | ***GGT*** ***Gly*** | ***GTC*** ***Val*** | ***GTT*** ***Val*** | CAT His | CA |
| Mutant 1 | CGT Arg | TTT Phe | CC***C*** Pro | ***GTC*** ***Val*** | ATG Met | AGC Ser | A***TC*** ***Ile*** | ***ATC*** ***Ile*** | AAA Lys |
| Mutant 2 | CGT Arg | TTT Phe | CC***G*** Pro | ATG Met | ***CTT*** ***Leu*** | AGC Ser | AC***A*** Thr | ***ATA*** ***Ile*** | AAA Lys |
| Mutant 3 | CGT Arg | TTT Phe | ***GCC*** ***Ala*** | ***CTC*** ***Leu*** | A***AT*** ***Asn*** | AGC Ser | AC***A*** Thr | TT***T*** Phe | AAA Lys |
| Mutant 4 | CGT Arg | TTT Phe | CC***T*** Pro | ***GTG*** ***Val*** | ***TGT*** ***Cys*** | AGC Ser | AC***G*** Thr | ***CAT*** ***His*** | AAA Lys |
| Mutant 5 | CGT Arg | TTT Phe | CCA Pro | ***CAA*** ***Gln*** | ***TTG*** ***Leu*** | AGC Ser | AC***C*** Thr | ***CAC*** ***His*** | AAA Lys |
| Mutant 6 | CGT Arg | TTT Phe | CC***C*** Pro | ***CTT*** ***Leu*** | ***TCT*** ***Ser*** | AGC Ser | ***CAC*** ***His*** | ***CGT*** ***Arg*** | AAA Lys |
| Mutant 7 | CGT Arg | TTT Phe | CC***C*** Pro | AT***A*** ***Ile*** | ***CTA*** ***Leu*** | AGC Ser | ***CCA*** ***Pro*** | T***CT*** ***Ser*** | AAA Lys |

FIGURE 3: Active-site sequence substitutions. The nucleotide sequence of each of the carbenicillin-resistant mutants was determined as given under Experimental Procedures, and the amino acid sequence was deduced.

an amino acid sequence having no homology with the wild-type sequence and also containing a single base frameshift at the carboxy terminus of the insert.

For the construction of active-site mutants, DNA from the nonproducer strain was digested with the same two restriction enzymes and purified by agarose gel electrophoresis. Into this DNA was ligated an oligonucleotide containing an AGC codon for serine flanked by stretches of nine and six random nucleotides (Figure 2B). Nonselective growth in the presence of tetracycline in four separate experiments yielded a total of approximately $2 \times 10^5$ colonies. Seven of the tetracycline-resistant colonies were also resistant to carbenicillin (50 $\mu$g/mL) (Figure 3, mutants 1–7). Each of the carbenicillin-resistant clones contained the AGC codon at amino acid position 70. The distribution of nucleotides within the random positions in the mutants is 39% C, 28% T, 23% A, and 10% G. The less than equal representation of G in the coding strand of the insert could be the result of a bias in the incorporation of nucleotides during chemical synthesis or could be indicative of the repertoire of substitutions that yield active molecules. Considering all seven mutants, 53% of the nucleotides in the random positions do not correspond to those in the present strain. Of the 35 codons capable of being substituted, 32 contained nucleotide substitutions; 10 of these were silent and 22 resulted in amino acid changes. Of the five substituted amino acids, the proline at position 67 is the most conserved; however, it is still lacking in one of the new sequences.

The resistance of *E. coli* infected with each of the mutant DNAs was quantified by observing the extent of bacterial growth on agar plates containing concentration gradients of carbenicillin, ampicillin, or benzylpenicillin. At 30 °C, the control strain, pBDL, was resistant to each of the antibiotics at >500 $\mu$g/mL, while the nonproducer strain, pBNP, failed to grow at the lowest concentration in the gradiant. The extent of resistance was confirmed by using agar plates containing defined concentrations of each of the antibiotics. At 30 °C, mutants 2, 3, 4, and 6 rendered *E. coli* resistant to the highest concentration of antibiotic tested. However, at the elevated temperatures, all of the mutants was more sensitive to the antibiotics than was the control, pBDL. Mutant 7 was the

Table I: Maximum Level of Resistance and PADAC Hydrolysis Zone of Strains with Mutations at β-Lactamase[a]

| | max antibiotic concn (mg/L) permitting bacterial growth | | | | | | | | | PADAC hydrolysis zone diam (mm), 30 °C |
|---|---|---|---|---|---|---|---|---|---|---|
| | carbenicillin | | | ampicillin | | | benzylpenicillin | | | |
| strain/mutant | 30 °C | 37 °C | 42 °C | 30 °C | 37 °C | 42 °C | 30 °C | 37 °C | 42 °C | |
| wild type | >500 | >500 | >500 | >500 | >500 | >500 | >500 | >500 | >500 | 20 |
| nonproducer | NG | NG | NG | NG | NG | NG | NG | NG | NG | <4 |
| mutant 1 | >500 | 75 | NG | >500 | 100 | NG | 250 | NG | NG | <4 |
| mutant 1R | >500 | 75 | NG | >500 | 100 | NG | 250 | NG | NG | <4 |
| mutant 2 | >500 | >500 | 200 | >500 | >500 | 80 | >500 | >500 | 50 | 14 |
| mutant 3 | >500 | >500 | 70 | >500 | 160 | NG | >500 | NG | NG | 10 |
| mutant 4 | >500 | >500 | 220 | >500 | 330 | 75 | >500 | 40 | NG | 6 |
| mutant 5 | 320 | 83 | NG | 400 | 55 | NG | 250 | NG | NG | <4 |
| mutant 6 | >500 | >500 | 75 | >500 | 420 | NG | >500 | 170 | NG | 6 |
| mutant 7 | 110 | NG | NG | 310 | 100 | NG | 190 | NG | NG | <4 |

[a] Antibiotic resistance was determined by concentration gradients, as described under Experimental Procedures; NG, no growth observed. PADAC hydrolysis of less than 4 mm in diameter could not be visualized due to growth of bacteria. One of the mutant sequences was duplicated into a second plasmid to rule out mutations outside the site of random sequence insertion. The insert of mutant 1 was reconstructed in plasmid pBNP by ligating into it a double-stranded oligonucleotide: 47-mer 5′-CGCCCCGAGGAACGTTTTCCCGTCATGAGCATCATCAAAGTACTGCT-3′ and its complement. The double-stranded oligonucleotide was constructed as described under Experimental Procedures and then digested with *Ava*I and *Sca*I before ligation.

most sensitive to each of the antibiotics. All the other mutants are more resistant toward carbenicillin than ampicillin or benzylpenicillin, particularly at higher temperatures. A chromogenic cephalosporin, PADAC, was used to measure β-lactamase activity of each of the mutant-infected *E. coli*. In general, the resistance of the mutants to carbenicillin and the other β-lactam antibiotics parallels the production by β-lactamase as measured by the hydrolysis of PADAC. However, differences in the relative resistance of the mutants to the different analogues suggest that some of the β-lactamase mutants exhibit altered substrate specificity.

To unambiguously establish that the replacement at the active site was responsible for the carbenicillin resistance in plasmid-infected *E. coli*, we reassembled one of the new mutants. We chemically synthesized a double-stranded oligonucleotide identical with the sequence of nucleotides in mutant 1 (Figure 3). This oligonucleotide was used to replace the insert in the nonproducer strain. A comparison of the drug resistance of *E. coli* infected with mutant 1 containing the biologically selected random sequence and that bearing the chemically synthesized insert (mutant 1R) is included in Table I. The pattern of resistance is identical. Thus, antibiotic resistance is conferred by the substitution at the active site and not by some other mutation within the plasmid or within the *E. coli* chromosome.

## Discussion

We have replaced nucleotide sequences within the active site of the β-lactamase gene with random chemically synthesized DNA sequences and selected from a heterogeneous population those sequences that render *E. coli* resistant to carbenicillin. The nucleotide sequence of each of the new mutants has many differences from that of the parental plasmid and from any natural β-lactamases so far reported (Bush, 1988; Brenner, 1988; Kelly et al., 1980; Spratt & Cromie, 1988; Nicholas & Strominger, 1988). This supports the notion that the active sites of enzymes may be more flexible than is generally recognized. As designed, each of the mutants maintained the codon for Ser-70 present within the oligonucleotide insert. Brenner (1988) hypothesized that the Ser-70 in β-lactamase evolved from an ancestral enzyme containing cysteine, and in fact, substitution of Ser-70 with cysteine yields a β-lactamase with 1–2% of the activity of the wild-type parent (Sigal et al., 1982). In six out of the seven mutants we have obtained, the most conserved amino acid is Pro-67 followed by Thr-71 (four of seven). Even though Thr-71 is conserved in class A β-lactamases, it has been reported that 14 out of 19 single amino acid replacements at this site yield active enzyme (Schultz & Richards, 1986). Our finding that four of the seven random mutants contain codons other than Thr confirms the fact that threonine is not essential for catalysis.

The amino acids within the active site that determine the substrate specificity of β-lactamase are unknown. The differences in resistance to β-lactam antibiotics among the mutants we have obtained suggest that some of the mutants have an altered substrate specificity (Table I). The region extending from Ser-70 to Lys-73 may not be involved in substrate recognition; this region is conserved in natural β-lactamases that exhibit differences in substrate specificity (Dale et al., 1985; Spratt & Cromie, 1988; Nicholas & Strominger, 1988). Alternatively, mutations within the region from Pro-66 to Ser-70 may be those responsible for alterations in substrate specificity. However, this sequence from Ser-70 to Lys-73 may be required for enzyme stability. In the studies of Schultz and Richards, most—but not all—substitutions for Thr-71 resulted in decreased activity at elevated temperatures, and evidence was presented that this resulted from increased proteolysis (Schultz & Richards, 1986). We observed a similar thermal lability of mutants selected from the active-site inserts that contained random nucleotide sequences in this region. Further studies will be required to determine which substitutions reduce the stability of the β-lactam-resistant phenotype and if this thermal inactivation results from enzyme denaturation or increased susceptibility to proteolysis. Most of the mutants exhibited a greater resistance toward carbenicillin than to the other analogues particularly at 40 °C (Table I), and this could be the result of selection by carbenicillin. Alternatively, carbenicillin could protect against thermal denaturation by preferentially binding to β-lactamases; the $K_m$ values for carbenicillin, ampicillin, and benzylpenicillin have been reported to be 10, 31, and 21 μM, respectively (Labia et al., 1979).

This use of random DNA for the generation of new mutants is based on the hypothesis that multiple amino acid substitutions can be tolerated within the active sites of enzymes and that many of these substitutions could yield enzymes with altered or even new catalytic activities. Consider a chronology of selected prebiotic evolutionary events that might offer advantages to sequences with the best fit. Assume that an average gene was initially coded by 2000 nucleotides and thus was selected for from a reservoir of $4^{2000}$ possible permutations. Early steps in selection could involve DNA and RNA struc-

ture, replication, and transcription (Orgel, 1986; Eigen & Schuster, 1977). Selections based on protein structure and specificity of catalysis are likely to be relatively late events and might be limited by the interdependence of metabolic pathways and by the stringencies of protein–protein interactions in multicomponent systems. A smaller number of possible permutations would be obtained if genes were assembled in units on the basis of structural domains (Schultz et al., 1987; Savageau, 1986). In either case, as a consequence of progressive selective processes, a large number of potential nucleotide arrangements may have been eliminated early in evolution, yet may code for active enzymes.

The overall frequency of multiple amino acid codon substitutions in the random collection of β-lactamase mutants is much higher than that found in nature. By selecting active genes from random DNA inserts, it might be possible to circumvent the sequential selective pressures that have occurred during evolution. Using a series of small random oligonucleotide inserts, we should be able to identify most substitutions that yield reactive molecules and thus define the topology of the active site on enzymes. Small changes in the structural configuration at the active site may have profound influences on the rates and specificities of enzymatic reactions and/or thermo- and proteolytic stability. New active sequences selected from random DNA inserts might be able to catalyze reactions at a rate greater than that of the native enzymes or might utilize unusual substrates and thus be of practical importance.

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