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Oligonucleotide-directed mutagenesis as a general and powerful method for studies of protein function

(β-lactamase/plasmid pBR322/enzyme mechanisms/colony screening/protein structure–function)

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ABSTRACT **We have used oligonucleotide-directed mutagenesis to make a specific change in the β-lactamase (EC 3.5.2.6) (ampicillin resistance) gene of the plasmid pBR322. Evidence suggests that the active site for this enzyme may include a serine-threonine dyad (residues 70 and 71). By priming *in vitro* DNA synthesis with a chemically synthesized 16-base oligodeoxyribonucleotide, we have inverted the Ser-Thr dyad to Thr-Ser and thereby generated a mutant with an ampicillin-sensitive phenotype. This "double-mismatch" method is relatively simple and also very general because detection of mutants is at the level of DNA and involves only colony hybridization. Accordingly, the procedure can be applied to any DNA sequence and does not depend on the phenotype of the mutant.**

Rational study of the influence of amino acid sequence on the three-dimensional structure and function of a protein would benefit greatly from the ability to change specific residues. When the gene for the protein has been cloned and is expressed in an appropriate vector, site-directed mutagenesis (1, 2) provides a method for creating new proteins whose structural and functional characteristics may be of practical value or may offer significant mechanistic insights.

Oligonucleotide-directed mutagenesis, the most specific form of directed mutagenesis, has been used to produce specific base changes in the single-stranded phage φX174 (3, 4). The method (Fig. 1) involves priming *in vitro* DNA synthesis with a chemically synthesized oligodeoxynucleotide that carries at least a single base mismatch with the complementary strand of the "wild-type" DNA. Because DNA polymerases require a double-stranded segment for initiation of DNA replication, the synthetic oligodeoxynucleotide primes DNA synthesis and is, itself, incorporated into the resulting heteroduplex molecule. After molecular cloning, semiconservative *in vivo* replication of this heteroduplex gives rise to homoduplexes whose sequences are either that of the original wild-type DNA or that of the synthetic oligodeoxynucleotide. With single-stranded circular DNAs, priming with oligodeoxynucleotides has been used to cause transitions, transversions, and deletions, in some cases very efficiently (5–11). Wallace *et al.* (12, 13) have extended the technique to double-stranded circular DNAs, and even though the frequency of directed mutagenesis may be lower, screening of colonies with the ^{32}P-labeled synthetic oligodeoxynucleotide allows easy detection of the desired mutant (12, 13).

The particular virtue of oligonucleotide-directed mutagenesis for structure–function studies lies in allowing one to produce mutant proteins with very specific changes in particular residues, which, for example, may be directly involved in catalysis or in determining substrate specificity. In this way, one can test ideas about the roles of particular amino acid side chains. Other procedures are valuable for producing random or less specific changes as, for example, in the creation of a mutant of β-lactamase (EC 3.5.2.6) with enhanced activity toward cephalosporins (14). Interesting mutants of β-lactamase have also been generated by segment-directed mutagenesis (15, 16) or by misrepair of gaps placed at restriction sites (17).

β-Lactamase provides a particularly favorable system for developing the technique of oligonucleotide-directed mutagenesis and for demonstrating the general feasibility of this approach to the study of protein function. The gene for β-lactamase is contained in the common double-stranded cloning vehicle plasmid pBR322, which has a total of only 4,362 base pairs and which contains a second selectable marker for resistance to tetracycline (18). The complete nucleotide sequence of the β-lactamase gene has been determined (19), and when pBR322 is grown in *Escherichia coli*, the enzyme is expressed and secreted into the periplasmic space, from which it can be readily isolated (20).

β-Lactamase catalyzes the hydrolysis of the amide bond of the lactam ring of penicillins and related antibiotics; the catalytic pathway includes an acyl-enzyme intermediate (20). A serine residue probably participates in catalysis (21–26) and is part of a conserved Ser-Thr dyad (26). [These residues have been numbered Ser-70 and Thr-71 (26).] Presumably the hydroxyl group of Ser-70 adds nucleophilically to the carbonyl group of the β-lactam ring in a mechanism somewhat analogous to that of serine proteases. The role of Thr-71 is less clear, but it seems essential for catalytic activity because a mutant, probably with isoleucine at this position, shows no catalytic activity (26).

In this work, whose main focus was on the procedures for generating and identifying site-specific mutants, we used a double-mismatch method to invert the order of the dyad from Ser-Thr to Thr-Ser (i.e., a double mutation: Ser-70 → Thr and Thr-71 → Ser). The mutant bacteria show no β-lactamase activity, which suggests a functional defect in the mutant enzyme.

MATERIALS AND METHODS

DNAs and Transformation. Plasmid pBR322 (18) was grown in *E. coli* strain LS1 (*pro, leu, thi, rpsL20, hsdR, hsdM, ara-14, galK2, xyl-5, mtlA, supE44*), a derivative of HB101 (27). Plasmid DNA was prepared according to the method of Birnboim and Doly (28) and supercoils were further purified by chromatography on acridine yellow ED gel (Boehringer Mannheim) as

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Abbreviations: NaCl/EDTA/Tris, 0.15 M NaCl/1 mM EDTA/0.03 M Tris·HCl, pH 8.0; NaCl/Cit, 0.15 M NaCl/0.015 M sodium citrate, pH 7.0.

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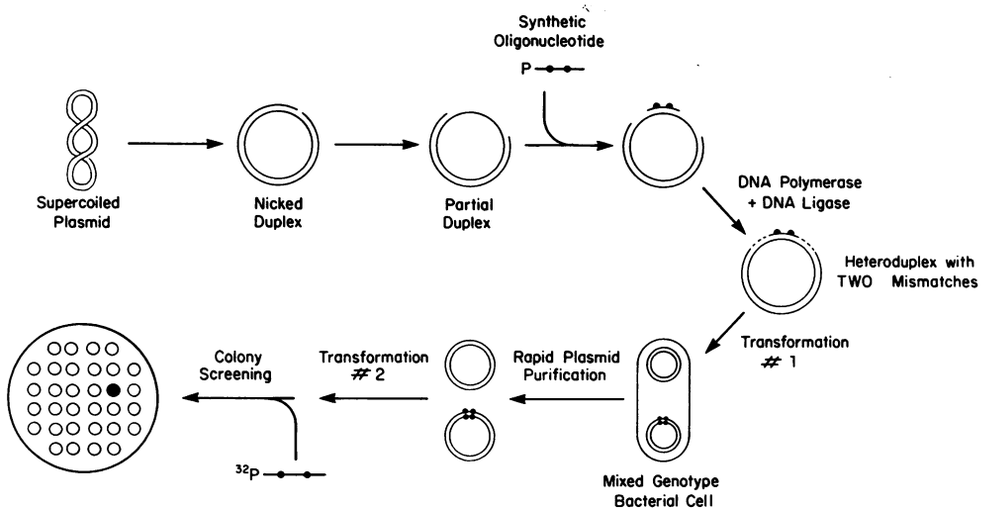

FIG. 1. Overall scheme for oligonucleotide-directed mutagenesis of double-stranded circular plasmid DNA. Supercoiled plasmid circles are nicked in one strand and rendered partially single-stranded by treatment with exonuclease. The gapped circles are hybridized with a homologous oligodeoxynucleotide carrying, by design, some mismatches. *In vitro* DNA synthesis, primed in part by the oligodeoxynucleotide, leads to heteroduplex plasmid circles. Molecular cloning and *in vivo* DNA replication generates homoduplexes, some of which have the DNA sequence of the primer oligodeoxynucleotide. Colony screening, with the same oligodeoxynucleotide labeled with ^{32}P as a hybridization probe, allows identification of the desired mutant colony regardless of its phenotype.

described by Vincent and Goldstein (29) or, alternatively, by ethidium bromide centrifugation followed by gel filtration (30). It probably is important that the plasmid DNA be free of oligodeoxynucleotides that often contaminate DNA preparations. For secondary transformations, where *in vitro* DNA synthesis is not involved, the rapid plasmid DNA isolation procedure of Ish-Horowicz and Burke (31) is convenient. Competent cells were prepared and transformations were done according to Hanahan (32). Selection was for resistance to tetracycline at 20 μg/ml.

The 16-base oligodeoxynucleotide 5'-A-A-T-G-A-T-G-A-C-C-T-C-T-T-T-T-3' was synthesized from dinucleotides by the triester method on a solid-phase support (33).

Enzymes. *E. coli* exonuclease III, *E. coli* DNA polymerase 1 (large fragment), phage T4 ligase, and the restriction enzyme *Hpa* II were obtained from Bethesda Research Laboratories. T4 polynucleotide kinase was obtained from Boehringer Mannheim.

Preparation of Nicked DNA. Fifteen micrograms of pure supercoiled pBR322 DNA and 9.0 μg of ethidium bromide in 150 μl of 20 mM Tris·HCl, pH 7.4/1 mM dithiothreitol/7 mM $MgCl_2$ were treated in the dark at room temperature with 7.5 units of *Hpa* II for a time estimated to produce about 75% nicked circles and 25% linear molecules (about 1 hr). The time and concentrations should be determined empirically on a smaller scale with the same reagents. The reaction was assayed by electrophoresis on 1.2% agarose in 40 mM Tris acetate, pH 7.8/10 mM EDTA (conditions that separate linear, circular, and supercoiled DNA molecules). We used *Hpa* II for nicking because 52 *Hpa* II sites are in pBR322 (one of them is near the Ser-70 region). Moreover, *Hpa* II is appropriately inhibited by ethidium bromide, in contrast to some other restriction enzymes (e.g., *Pst* I). DNase 1 could also have been used (13), because the precise location of the nick probably is not critical.

The reaction was stopped by adding 0.1 vol of 0.1 M EDTA and the solution was extracted twice with phenol, once with chloroform, and then twice with diethyl ether. These extractions removed both protein and ethidium bromide. The DNA was precipitated by adding 0.1 vol of 3 M ammonium acetate and 2 vol of ethanol. After centrifugation, the pellet was washed with 70% (vol/vol) ethanol, dried, and dissolved in 15 μl of sterile water.

Exonuclease Treatment, Heteroduplex Formation, and Transformation. About 1 μg (0.4 pmol) of the nicked DNA was treated for 15 min at room temperature with 6 units of *E. coli* exonuclease III in 50 mM Tris·HCl, pH 8.0/5 mM $MgCl_2$/10 mM 2-mercaptoethanol (total volume, 3 μl). The enzyme was inactivated by heating to 60°C in the presence of chloroform, which was then removed by evaporation under reduced pressure. The nicked and exonuclease-treated DNA was heated to 65°C for 5 min in 0.5 M NaCl in the presence of 80 ng of the synthetic oligonucleotide (18 pmol) in a 5-μl volume and allowed to stand at room temperature for 1 hr.

Buffer and salt concentrations were adjusted to 100 mM NaCl, 20 mM Tris·HCl at pH 7.5, 10 mM dithiothreitol, 10 mM $MgCl_2$, each of the four deoxynucleoside triphosphates at about 400 μM, and 500 μM ATP. Sixteen units of *E. coli* DNA polymerase I (large fragment) and 40 units of T4 DNA ligase were added to yield a final reaction volume of 50 μl. Four microliters of this reaction mixture was used directly to transform competent *E. coli* LS1. A second cycle of transformation was usually done after a quick plasmid preparation (31) from an overnight 10-ml broth culture containing tetracycline at 20 μg/ml and inoculated with the original transformation mix.

Colony Screening by Primer-Probe Hybridization. Gergen *et al.* (34) described a microtiter dish colony collection procedure and a procedure for making filter paper replicas of the colony collection. By following this procedure, a colony collection was made, transferred to agar with a 96-prong replicator, and grown for 18 hr. The colonies were "lifted" onto Whatman 541 filter paper for amplification on 250/μg/ml chloramphenicol plates for 24 hr, followed by alkali lysis and neutralization (34). The filters prepared in this way were then prehybridized for 3 hr at 65°C in 6× NaCl/EDTA/Tris, 0.5% Nonidet P-40, and denatured, sonicated salmon sperm DNA at 100 μg/ml (1× NaCl/EDTA/Tris is 0.15 M NaCl/1 mM EDTA/0.03 M Tris·HCl, pH 8.0). Hybridization with ^{32}P-labeled oligonucleotide was done at room temperature for 16–18 hr in 6× NaCl/EDTA/Tris, 0.5% Nonidet P-40, 250 μg of yeast tRNA per ml, and 1.6 ng of ^{32}P-labeled oligodeoxynucleotide per ml (at

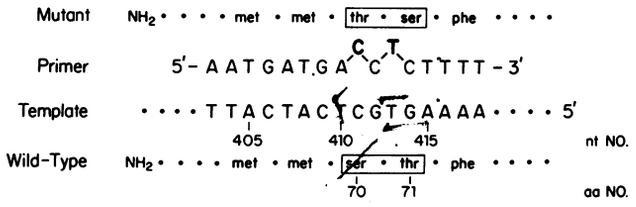

FIG. 2. Nucleotide sequence of the synthetic oligodeoxynucleotide used as a specific mutagen and of the complementary wild-type strand. The amino acids encoded by both mutant and wild-type sequences are shown. nt NO is the nucleotide number in pBR322 (19). aa NO is the amino acid number in β-lactamase (26).

least 10^7 cpm/μg). The filters were washed three times in 25–50 ml per filter of 6× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) for 5 min at room temperature and autoradiographed. The temperature of the hybridization and washing is important and will vary with the nucleotide sequence and length. We have followed empirical rules developed by Suggs *et al.* (35) for oligodeoxynucleotide hybridizations on Southern blots and colony screens. One estimates the apparent melting point (t_d) to be the sum of 2°C per A·T base pair and 4°C for each G·C base pair when the hybridizations and washes are done in 6× NaCl/EDTA/Tris or 6× NaCl/Cit. We do the hybridizations at least 5°C below the calculated t_d and do a series of washes and autoradiography, first at 5°C and then at higher temperatures to ensure passing through the temperature that best distinguishes mutant from wild type.

RESULTS

Fig. 1 illustrates our use of a 16-base synthetic oligodeoxynucleotide with two deliberate base pair mismatches to prime *in vitro* DNA synthesis on pBR322 DNA containing single-stranded regions that had been generated by exonuclease treatment of nicked, double-stranded circles. The two mismatches in the primer d(A-A-T-G-A-T-G-A-C-C-T-C-T-T-T-T) at the bases denoted (*) will accomplish the double mutation Ser-70 → Thr and Thr-71 → Ser (Fig. 2). For generality, the method is designed for screening at the DNA level rather than depending on the phenotype of the mutant protein. Therefore, we have used colony hybridization with a ^{32}P-labeled oligodeoxynucleotide to select mutants. Conveniently, the same oligodeoxynucleotide used as a primer in the *in vitro* synthesis can be labeled with ^{32}P and also used as a hybridization probe. Those colonies containing the mutant gene will match perfectly with the synthetic probe and therefore hybridize more strongly than will wild-type colonies, which have two base pair mismatches.

Fig. 3 shows the result of a colony screen; a mutant can be clearly identified. In this experiment, colonies were picked and distributed into microtiter dishes to provide an ordered array and a permanent library (34). After growth, a filter replica of the colonies was made and used for colony screening (13, 34).

The microtiter dish technique allowed convenient screening of a few thousand colonies and was used to isolate the first mutant (22G9). However, when it became apparent that the frequency of the desired mutation was only about one in a thousand, we began to use a random colony screening procedure, which allows the rapid evaluation of tens of thousands of colonies. Fig. 4 shows the results of such a screen: the bottom shows

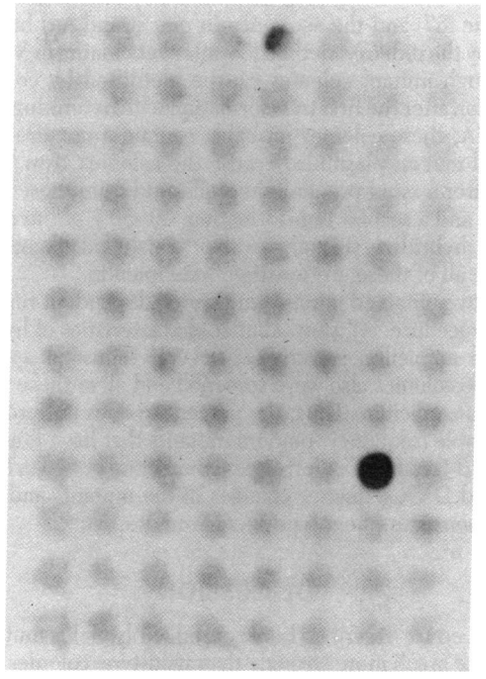

FIG. 3. Autoradiogram showing the result of a colony screen. Colonies from a second transformation (see Fig. 1) were picked and grown in a microtiter dish to provide a convenient permanent library. Filter paper replicas of the library, prepared as described in text and ref. 34, were hybridized with the ^{32}P-labeled oligodeoxynucleotide, washed, and autoradiographed. The darkest colony, designated 22G9, proved to be the desired mutant.

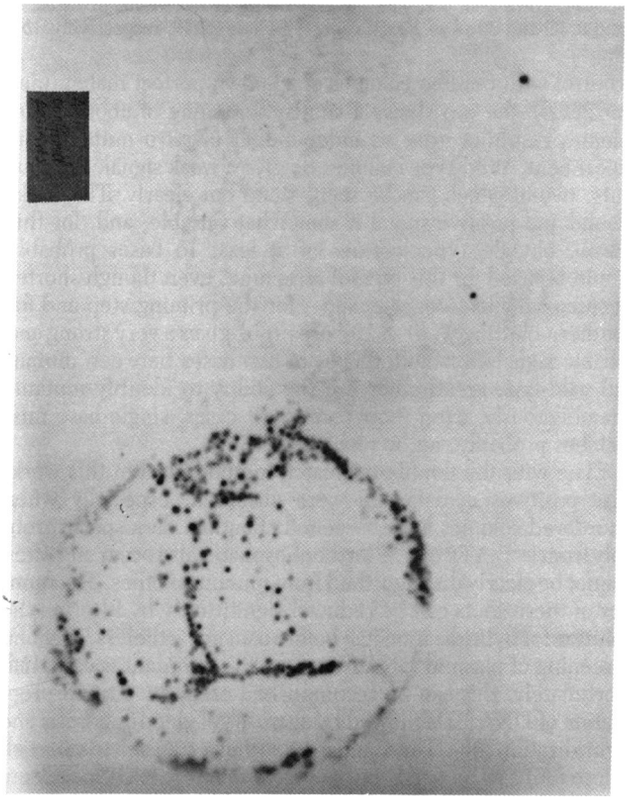

FIG. 4. Autoradiogram showing the results of a colony screen of randomly spread colonies. The upper circle shows cells from a second transformation spread randomly on the surface of 15-cm L agar plates. About 2,000 tetracycline-sensitive colonies were expected from preliminary experiments. After overnight growth, when the colonies were about 1 mm in diameter, they were transferred to 541 filter papers and then treated as described in the text and ref. 34. The lower circle is a control of randomly spread cells containing a perfect match (mutant 22G9), grown and treated as described above.

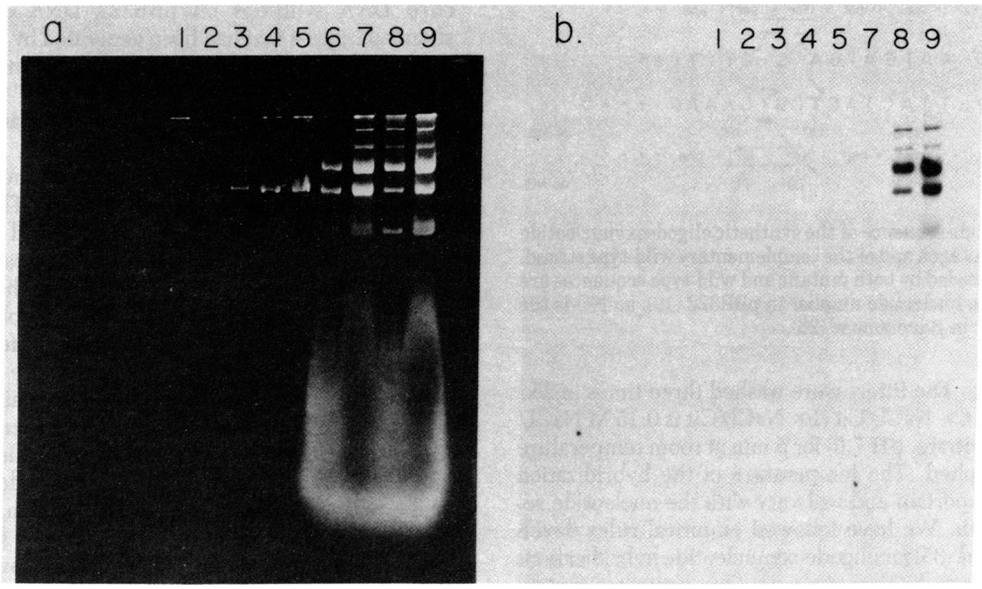

FIG. 5. Hybridization of ^{32}P-labeled oligodeoxynucleotide with gel-purified plasmid DNAs. Plasmid DNAs, prepared from 7-ml overnight unamplified cultures as described by Ish-Horowicz and Burke (31), were each digested with *Eco*RI in a reaction volume of 65 μl. Aliquots were electrophoresed in 1.2% agarose. The gel was stained with ethidium bromide and photographed. Then it was treated for 20 min at room temperature with 0.15 M NaCl and 0.5 M NaOH and neutralized in 0.15 M NaCl and 0.5 M Tris·HCl, pH 7.4. The gel was dried in a gel dryer (Bio-Rad) and the resulting membrane was hybridized overnight at room temperature in 6× NaCl/EDTA/Tris, 0.5% Nonidet P-40, 250 μg of tRNA per ml, and 1.6 ng of ^{32}P-labeled oligodeoxynucleotide per ml. No prehybridization is necessary. The gel membrane was then washed three times for 10 min each at 4°C in 6× NaCl/Cit followed by three washes for 5 min each at 37°C in 6× NaCl/Cit. (*a*) Ethidium bromide-stained gel. Lane 1, 0.4 μg of pBR322 standard; lanes 2–5, 0.1, 0.2, 0.5, and 1.0 μg of *Eco*RI-cut pBR322; lanes 6 and 7, 10 and 30 μl of *Eco*RI digest of clone 38G3, respectively; lanes 8 and 9, 10 and 30 μl of *Eco*RI digest of clone 22G9, respectively. (*b*) Autoradiogram of the dried gel after hybridization.

a control with random colonies of a known perfect match (mutant 22G9); the top shows a similar screening of about 1,800 colonies resulting from an independent *in vitro* mutagenesis experiment. Wild-type colonies do give a weak signal, but mutants, two of which can be seen, stand out clearly. The background and positive signal is somewhat variable, and, for this reason, oligodeoxynucleotides of at least 15 bases probably should be used for this type of screening, even though shorter oligodeoxynucleotides may serve for the priming step and for Southern blotting (7, 8). A 19-base probe gives a very strong and reliable signal (13). Differences of two bases between mutant and wild-type greatly increase the ability to identify mutants unambiguously, even though, in most cases, single-base mismatches probably can be identified (13).

Even with the double-mismatch method used in this work, false positives occasionally were observed, especially when unordered colonies were screened. Random dark spots, probably from [^{32}P]ATP or [^{32}P]orthophosphate, also occur and often cannot be clearly distinguished from mutant colonies. The number of these spots can be reduced significantly by filtering the solution for hybridization just before use; nevertheless, a second screening of plasmid DNA from a putative mutant is essential. Fortunately, this can be accomplished easily by a rapid preparation of DNA (31) followed by agarose gel electrophoresis and hybridization (36). The standard Southern transfer to nitrocellulose (36) can be used, but we have found it even more convenient to simply dry the agarose gel and use the resulting agarose membrane directly for hybridization with the ^{32}P-labeled oligodeoxynucleotide (S. G. S. Tsao, C. F. Brunk, and R. Pearlman; personal communication). Fig. 5 illustrates the unambiguous discrimination between wild-type and mutant colonies. This analysis also discriminates single base mismatches (ref. 13 and unpublished data) and should show differences between single- and double-base mismatches, possibly because any such mutants were not picked at the earlier colony screening step (the faint colony in Fig. 3 has not yet been analyzed).

To show that the expected two-base change has actually been made, the sequence of the DNA in the region of the active site (Ser-70 and Thr-71) has been determined by the Maxam–Gilbert technique (37) and the sequence in one strand has been confirmed by the dideoxy method (38) after subcloning in M13mp7.

Although mutant colonies can be identified by colony hybridization after the first transformation with heteroduplex plasmid DNA, these colonies probably contain a mixture of wild-type and mutant plasmids because the colonies grow in ampicillin. After a rapid preparation of plasmid from these "mixed" colonies and a second transformation, 30–50% of the resulting colonies hybridize strongly to the ^{32}P-labeled oligodeoxynucleotide; all of these are sensitive to ampicillin.

We have obtained mutants in three independent repetitions of the procedure. All show similar characteristics of hybridization and ampicillin sensitivity. Several thousand apparently wild-type colonies also were screened and all of these were resistant to ampicillin. Thus, the procedure does not produce, at a detectable frequency, random mutants that have functionally inactive β-lactamase. A fortuitous connection between the demonstrated DNA sequence changes in the mutants and the ampicillin-sensitive phenotype seems remote.

DISCUSSION

The procedure described here reproducibly yields mutants that hybridize much more strongly than wild-type colonies with the synthetic oligodeoxynucleotide probe that is used first as the primer for *in vitro* synthesis of heteroduplex plasmid and then as a probe for identifying mutants. The plasmid DNA of one mutant has been characterized in the region of the mutation and shown to have the desired sequence.

All of the mutants that show strong hybridization with the synthetic probe are also sensitive to ampicillin. The inversion

of Ser-Thr to Thr-Ser was expected to affect catalytic activity because, for example, the substitution of the primary alcohol of Ser-70 by the secondary alcohol of Thr might sterically hinder nucleophilic attack by the hydroxyl group; the amino acid changes also might cause conformational rearrangement within the active site. Particularly interesting in this respect will be studies on the ability of the mutant protein to bind penicillins and related substrates, as distinct from its ability to act as a catalyst. Also of interest will be the effects of the single changes Ser-70 → Thr and Thr-71 → Ser. These mutants might have arisen by partial repair during the mutagenesis procedure, but so far we have isolated no such single-base-change mutants. Although preliminary studies on cell extracts suggest that the mutant protein is catalytically inactive, other reasons for the absence of ampicillin resistance, such as more rapid proteolytic degradation of the mutant protein, remain a formal possibility.

The present yield of mutants, about one per thousand, should be raised significantly by further improvements. However, a major point is that improvements are not required because of the rapidity and reliability of the screening procedure (13). The screening procedure depends only on DNA sequence and not on any phenotypic difference between mutant and wild type. This feature allows easy detection of mutants even with phenotypically silent changes. For example, even though the Ser-70 → Thr and Thr-71 → Ser mutant of β-lactamase created in this work is functionally inactive, one could as easily have identified a mutant that retains normal activity, has acquired enhanced catalytic effectiveness, or displays altered specificities for various substrates. The use of double-base mismatches to facilitate identification of mutants, even when one of these base changes causes no amino acid change, should be emphasized. The degeneracy of the genetic code makes the double-mismatch approach applicable even for single amino acid changes. These features of the method, together with the ability to create specific mutations at any region of the genome, should encourage its widespread application.

The generation of a specifically restructured mutant protein generally will require six steps: (*i*) cloning of the relevant gene in an appropriate vector, (*ii*) expression of the cloned gene, (*iii*) determination of the DNA sequence of the gene, (*iv*) chemical synthesis of an oligodeoxynucleotide, (*v*) oligonucleotide-directed *in vitro* mutagenesis, and (*vi*) identification of mutant colonies. This work has focused on steps *v* and *vi*, which, together with present and rapidly improving techniques for accomplishing steps *i–iv*, should allow the restructuring of proteins to become a common undertaking and thereby increase our understanding of the relationship between primary structure and biological function. Moreover, such techniques also will allow the synthesis of new proteins with useful properties not hitherto available.

Note Added in Proof. Using this technique, we have generated a one-base mutant, Ser-70 → Thr [d(...A-T-G-A-T-G-A-Ĉ-C-A-C-T...)], which is catalytically inactive. We have also obtained Thr-71 → Ser [d(...A-T-G-A-T-G-A-G-C-T̂-C-T...)], which has a low level of activity.

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