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An efficient method for generating proteins with altered enzymatic properties: Application to β-lactamase

(drug resistance/protein engineering/mutagenesis/antibiotics/random selection)

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ABSTRACT **Random-sequence or highly degenerate oligonucleotides have been useful for defining functionally important sequences both in proteins and in nucleic acids. In this approach, such oligonucleotides are used to replace a segment of DNA required for a desired function, and functional sequences are identified by an appropriate genetic or biochemical selection. Here, a collection of 10^5 altered β-lactamase proteins was generated by cloning a mixed-base oligonucleotide in place of the sequences coding for a 17-amino acid portion of the enzyme's active site. Approximately 2000 enzymes from this collection were able to confer ampicillin resistance on *Escherichia coli*. Fifty-eight of these were chosen for further study after characterization with various β-lactam substrates. β-Lactamases having altered specificity against different antibiotics, resistance to the suicide inhibitors clavulanic acid and sulbactam, and temperature-dependent activities were obtained. The amino acid residues responsible for these altered properties as well as for basic enzyme activity are defined. This approach should prove to be an effective and general tool for creating proteins with novel properties, especially in situations in which a high-resolution structure of the protein is not known.**

A frequent goal of genetic engineering is to create proteins with novel functional properties. Although x-ray crystallography often provides an accurate definition of protein structure, the relationship of a protein's structure to its function is still poorly understood. Mutagenesis of a gene allows one to generate subtle variants of an enzyme or to design proteins with new properties. However, with an incomplete understanding of structure–function relationships, it is often difficult to choose variants that will be informative or have a desired property. In contrast, the immune system creates binding antibodies, without foreknowledge of the antigens, by generating many potentially functional structures and selecting and amplifying those that demonstrate the desired function. In this way, antibodies have been generated that recognize reaction intermediates and can function as enzymes (1, 2).

The structural diversity required to generate a particular function can also be created *in vitro* by replacing wild-type DNA sequences with a random-sequence or highly degenerate oligonucleotide (3–9). Many functional structures can be obtained from such a collection by using genetic or biochemical selections. A comparison of these structures allows one to define a consensus sequence analogous to those obtained more typically for DNA elements. This "random selection" approach has distinct advantages over comparing evolutionarily related enzymes because many more variants can be studied and their function and structure is relatively independent of the evolutionary history of the organism.

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The basic principle of random selection should also permit the isolation of proteins with novel properties that would otherwise be difficult to obtain by conventional mutagenesis or by evolution. In this paper, we apply this principle to a gene conferring resistance to β-lactam antibiotics such as is found in many hospital isolates of pathogenic bacteria (10). Specifically, we have created variants in the active site of the TEM β-lactamase (EC 3.5.2.6), a protein encoded by many common *Escherichia coli* cloning vectors. Although this enzyme is only defined at a resolution of 4.0 Å (11), its similarity in sequence and structure to the *Staphylococcus aureus* β-lactamase that is solved at 2.4-Å resolution (12) allows for comparison. By this method, β-lactamases having altered specificity for antibiotics, resistance to suicide inhibitors, and temperature-sensitive activity were obtained. The advantages and disadvantages of this approach are compared to methods involving site-directed or classical mutagenesis.

MATERIALS AND METHODS

Replacing the Region Encoding the Active Site of β-Lactamase by a Degenerate Oligodeoxynucleotide. The *Eco*RI–*Dra* I fragment of plasmid pBR322 containing the TEM β-lactamase gene was cloned in a vector consisting of the *Dra* I–*Hae* II fragment containing the origin of replication from pUC18 and a *Hae* II fragment having the chloramphenicol-resistance gene from pACYC184. These *Hae* II sites were blunted to clone the chloramphenicol gene and are no longer present. To introduce the required restriction enzyme sites around the β-lactamase active site and to create a nonfunctional protein, the oligonucleotide containing *Hin*dIII and *Hae* II sites (AACACGGGATAATACGGCGCCTAGAAGCTTTCAAGGATCC) was cloned by mutually primed synthesis (3, 4, 13) between the *Xho* II and *Hin*cII sites in the β-lactamase gene (Fig. 1).

A library of 500,000 independent *E. coli* colonies containing potentially functional β-lactamase molecules was made by cloning a degenerate oligonucleotide between newly created *Hin*dIII and *Hae* II restriction endonuclease sites in the gene and selecting for chloramphenicol-resistant transformants (Fig. 1). The region of the oligonucleotide coding for the 17-amino acid portion of the active site contained about 80% wild-type nucleotides and a mixture of the other three. The single-stranded oligonucleotide was converted to the double-stranded form by mutually primed synthesis (3, 4, 13), cleaved with *Hin*dIII and *Hae* II, and then inserted into the vector DNA.

To characterize the degenerate oligonucleotide, 10 clones were chosen without selection and sequenced. Eighty-five non-wild-type nucleotides were observed, an averaged of 8.5 per oligonucleotide, or approximately 6 amino acid substitutions per enzyme. Eleven bases were changed to guanine, 25 to thymine, 40 to adenine, and 9 to cytosine, indicating an unwanted bias against guanine and cytosine that was probably introduced either in the mixing of the nucleotides for synthesis or by an unequal potency in the synthesis reaction.

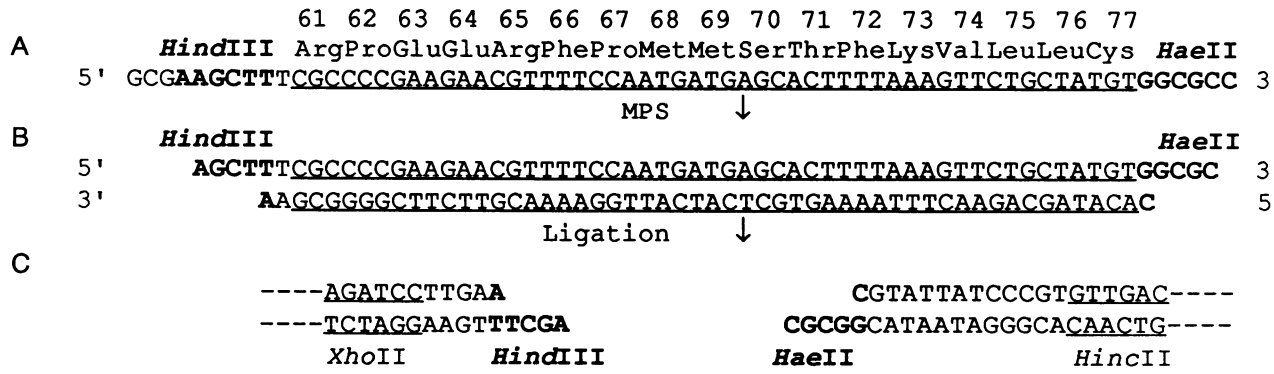

FIG. 1. Replacing the region encoding the active site of β-lactamase by a degenerate oligonucleotide. (*A*) The oligonucleotide used to replace the region coding for a portion of the active site of β-lactamase (residues 61–77). The degenerate bases, containing 80% wild-type nucleotides, are underlined. (*B*) The double-stranded form of the degenerate oligonucleotide produced by mutually primed synthesis (MPS) and subsequent cleavage with *Hin*dIII and *Hae* II. (*C*) The sequence of the vector in the region encoding the β-lactamase active site, showing the newly introduced *Hin*dIII and *Hae* II sites for cloning the degenerate oligonucleotide and the *Xho* II and *Hin*cII sites used for generating the vector (see *Materials and Methods*).

Identification and Characterization of Functional β-Lactamases. Bacteria containing functional proteins were selected from this library by plating in the presence of various concentrations of a set of β-lactam antibiotics. The antibiotics included those of the penicillin type, containing the 5-membered thiazolidine ring (ampicillin and piperacillin); the cephalosporin type, containing the 6-membered dihydrothiazine ring (cephaloridine and cephalothin); and the suicide inhibitor clavulanic acid (used in combination with ampicillin). Plasmids were isolated from 58 resistant strains and sequenced on both strands by the dideoxy chain-termination method.

Bacterial clones containing the 58 different functional enzymes were assayed for growth in the presence of various β-lactam antibiotics and ranked in comparison with the wild-type enzyme. These drug-sensitivity assays were done by transforming plasmids into *E. coli* strain HMS174, a *recA1* derivative of W3110. For the drugs ampicillin, piperacillin, clavulanic acid, and sulbactam, sensitivities were determined by patching cells on plates containing 2-fold increasing concentrations of the drugs. Nitrocefin assays were carried out on whole-cell lysates by measuring the change in A_{495} as described (14). For the remaining drugs, assays were done using the BBL Sensi-Disk procedure (BBL Microbiology Systems, Division of Becton Dickinson). Paper disks impregnated with the indicated amount of antibiotic were placed on a lawn of growing cells, and the area of inhibited growth around the disks was measured.

RESULTS

Selection for Functional β-Lactamases. The DNA sequence encoding a 17-amino acid region containing the active site of TEM β-lactamase was replaced by a degenerate oligonucleotide in which this region was mutagenized at a level of ≈20% per base pair (Fig. 1). From a library of 10^5 altered β-lactamases, ≈2000 were able to confer ampicillin resistance on *E. coli*. These functional β-lactamases were characterized in more detail by their ability to permit growth in the presence of a variety of β-lactam antibiotics. The properties and primary sequences of 58 functional enzymes are shown in Fig. 2.

The most interesting variants are those which show unique patterns of activity with various substrates. Even though the majority of the enzymes described here contain multiple amino acid changes from the wild type, most of the functional differences can be attributed to a single amino acid change either because of the existence of an enzyme having a single substitution and/or because of the functional similarity of several enzymes having a common amino acid change. Although multiple changes are not ideal for a detailed structure–function analysis, they are not a problem when generation of novel function is the goal.

As expected, most variants from the wild type lowered the activity against all of the substrates tested. This can be explained either by a general inactivation of enzyme function or by degradation of the enzyme *in vivo*. Some of the positions allow only limited diversity of a particular amino acid type. This diversity, and considerations of its degree and type, provides useful information concerning the enzyme's structure and its relationship to function.

β-Lactamases Resistant to Suicide Inhibitors. Some of the β-lactamases obtained in these experiments show an increased resistance to the suicide inhibitors clavulanate and sulbactam, a property not previously described. Enzymes with changes at position 69 from methionine to the aliphatic hydrophobic amino acids leucine, isoleucine, and valine (Fig. 2, C01–C14) demonstrate resistance to clavulanate at concentrations up to 16-fold higher than the wild-type enzyme. The changes to threonine-69 (D15) and to tyrosine-72 (D05, -10, -11, -13, -17, and -18) also show a relative increase in clavulanate resistance in comparison to their activities against other β-lactam drugs. In contrast, the change to asparagine at position 69 (A02) does not increase resistance to the suicide inhibitors. Although higher concentrations of sulbactam are required to maintain an effect comparable to clavulanate, the majority of the enzymes respond similarly to both drugs. Exceptions may be a preference for tyrosine-72 (D10) over leucine-72 (B04, D02, and D03) in activity against clavulanate and a preference for valine-69 (C08) in activity against sulbactam. It is interesting that the derivatives resistant to these suicide inhibitors also show relatively more resistance to cefoperazone than to the other cephalosporin-type antibiotics. Biochemical analysis of these enzymes should be helpful in further understanding the complex mechanism of β-lactamase inactivation by clavulanate and sulbactam (15–17).

Temperature-Dependent Enzymes. Other interesting derivatives function poorly at low or high temperatures compared to the wild-type enzyme. Even though the change to tyrosine at position 72 (D10) is detrimental to overall enzyme function, bacteria harboring this protein are more resistant to ampicillin at 37°C than at 30°C. Preliminary biochemical analysis of this enzyme shows a 100-fold lower k_{cat} and a 3-fold lower K_m than the wild-type enzyme. The more common and opposite effect is seen with the leucine-72 substitution (B04), which causes a minor decrease in general enzyme activity and an increase in heat susceptibility.

	AMP	CT	AmC		CD	NIT	CEC	MA	HT		61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77
	PIP	SUL	TIM	CF	CZ	CFP	CID	SUM			ARG	PRO	GLU	GLU	ARG	PHE	PRO	MET	MET	SER	THR	PHE	LYS	VAL	LEU	LEU	CYS
A01	7	7	2	1	5	5	6	7	3	6	7	6	6	6	5	6		---	THR	---	---	---	---	---	---	---	---	---	---	---	---	---	---
A02	7	7	2	1	4	4	6	7	2	5	5	5	5	5	5	5		---	---	LYS	---	---	---	---	---	ASN	---	---	---	---	---	---	---
A03	7	7	2	2	5	5	5	5	5	6	7	5	5	5	6	5		---	HIS	---	---	---	---	---	---	---	---	---	---	---	---	---	---
A04	7	7	4	3	5	5	6	6	4	6	7	5	5	5	6	5		---	ALA	---	---	---	---	---	---	---	---	---	---	---	---	---	ALA
A05	7	7	3	2	6	5	6	6	5	6	7	5	5	5	5	5		---	SER	TYR	---	---	---	---	---	---	---	---	---	---	---	---	---
A06	7	7	3	2	5	5	7	5	4	6	6	6	5	5	5	5		---	---	---	---	---	---	---	---	---	---	---	---	---	---	MET	---	---
A07	7	7	4	2	5	5	6	6	5	4	3	4	5	5	7	5		---	---	ALA	---	---	---	---	---	---	---	---	---	---	---	---	---
A09	7	7	3	2	5	5	6	5	5	5	7	6	5	5	6	5		---	---	ASP	---	---	---	---	---	---	---	---	---	---	---	---	---
A10	7	7	3	3	5	5	6	5	4	6	6	5	5	5	5	5		---	---	ASP	---	---	---	---	---	---	---	---	---	---	---	---	VAL
A11	7	7	2	2	5	5	6	5	5	6	5	5	5	5	5	5		---	SER	LYS	---	---	---	---	---	---	---	---	---	---	---	---	---
A12	7	5	1	2	5	4	4	4	4	5	6	4	4	4	5	4		---	---	---	---	---	---	---	---	---	---	---	---	---	PRO	---	---	VAL
A13	7	7	2	3	5	6	7	5	5	6	6	5	5	5	5	5		---	---	ASN	---	---	---	---	---	---	---	---	---	---	---	---	---
B01	7	7	4	3	5	5	3	4	3	4	3	6	5	4	5	4		---	SER	---	---	---	---	---	---	ILE	---	---	---	---	---	---	---
B02	6	5	0	0	3	2	3	1	0	3	2	4	3	3	3	3		---	---	---	---	---	---	---	---	ILE	---	---	---	---	---	---	---
B03	7	6	1	1	4	3	4	3	2	4	5	6	4	4	4	4		---	---	---	---	---	---	---	---	---	---	---	---	---	ILE	---	---	---
B04	5	3	0	1	1	1	3	2	0	3	5	4	4	3	3	3		---	---	---	---	---	---	---	---	---	---	---	---	LEU	---	---	---	---
B05	7	5	1	2	3	2	5	4	1	4	2	4	4	4	4	4		---	---	---	---	---	ILE	---	---	---	---	---	---	---	---	---	---	---
B06	7	7	2	2	5	4	5	4	2	4	4	4	4	5	5	4		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	ILE
B07	7	6	2	2	4	5	5	4	4	5	7	6	5	5	5	5		---	---	ASP	---	---	---	---	---	---	---	---	---	---	---	ILE	---	---
B08	6	5	1	1	3	2	3	2	1	5	0	3	4	5	4	3		HIS	SER	ASP	---	---	---	---	VAL	---	---	---	---	---	---	---	---	---
B09	7	7	3	1	4	4	5	5	2	4	4	5	4	4	6	4		---	SER	GLY	---	---	---	---	---	---	---	---	---	---	---	PHE	---	---
B10	5	6	0	0	4	2	3	1	1	3	5	5	3	3	4	3		---	ARG	---	GLN	---	---	---	---	---	---	---	---	---	---	---	---	---
B11	6	6	0	0	3	1	5	2	0	3	1	3	4	4	4	3		---	---	---	---	---	---	---	---	---	---	---	---	---	HIS	---	---	---
B12	7	7	1	0	4	3	3	2	1	3	5	6	4	3	1	4		---	---	---	---	---	---	---	---	ILE	---	---	---	---	---	---	---	---
B13	7	7	2	1	5	3	3	5	1	4	3	6	4	4	2	4		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	ILE	---
B14	7	7	2	2	5	5	5	4	3	4	7	6	4	4	5	5		---	---	---	---	---	---	---	---	VAL	---	---	---	---	---	---	---	---
B16	7	7	3	3	5	5	5	4	3	5	6	6	5	4	5	5		---	---	ASP	---	---	---	---	---	ILE	---	---	---	---	---	---	---	---
C01	7	6	5	5	7	7	3	2	1	3	7	7	3	3	4	4		---	---	---	---	---	---	---	---	ILE	LEU	---	---	---	---	---	---	---
C02	6	5	5	4	6	6	3	2	1	3	3	6	3	3	5	3		---	THR	---	---	---	---	---	---	---	LEU	---	---	---	---	---	---	---
C03	7	7	5	3	6	6	3	2	1	4	4	5	3	2	7	3		---	SER	ASN	---	---	---	---	---	---	LEU	---	---	---	---	ILE	---	---
C05	6	4	6	2	7	6	1	0	0	0	0	4	1	0	7	2		---	---	ASP	---	---	---	---	---	---	ILE	---	---	---	---	---	---	---
C06	7	5	5	3	7	7	3	1	1	3	4	7	3	3	4	3		---	SER	---	---	---	---	---	---	---	LEU	---	---	---	---	SER	---	---
C07	6	4	5	2	7	5	1	0	0	1	3	5	1	1	5	2		---	---	LEU	---	---	---	---	---	---	ILE	---	---	---	---	---	---	---
C08	6	4	5	1	6	5	1	1	0	1	1	4	2	1	5	2		---	---	---	GLN	---	---	---	---	---	VAL	---	---	---	---	---	---	---
C09	6	4	5	4	7	5	1	0	0	1	0	4	1	0	4	2		---	ALA	---	---	---	---	---	---	ILE	ILE	---	---	---	---	---	---	---
C10	6	6	5	4	6	6	3	1	1	2	2	5	3	3	5	3		---	---	---	---	---	---	---	---	---	LEU	---	---	---	---	---	---	---
C11	5	4	6	2	6	4	0	0	0	0	0	3	1	0	5	1		---	---	LYS	---	---	---	---	---	---	ILE	---	---	---	---	---	---	---
C12	6	4	5	2	6	5	1	1	0	1	2	4	2	1	6	2		---	---	ASP	---	---	---	---	---	LEU	ILE	---	---	---	---	---	---	---
C13	6	6	5	5	6	6	2	1	1	2	0	4	2	2	5	2		---	---	---	---	---	---	---	---	---	LEU	---	---	---	---	---	PRO	---	---
C14	6	6	5	3	6	5	2	1	1	3	2	5	3	3	5	3		HIS	SER	LYS	---	---	---	---	---	---	LEU	---	---	---	---	---	---	---	---
D01	1	0	0	0	1	1	0	1	0	1	0	0	0	0	0	0		---	---	---	VAL	---	---	---	---	ILE	---	---	---	MET	---	ILE	PRO	---	---
D02	2	0	0	2	1	0	2	2	0	1	5	0	1	1	2	1		---	ARG	---	---	---	---	---	---	---	---	---	---	LEU	---	---	---	---	---
D03	4	0	1	4	1	0	3	1	0	2	2	3	3	1	4	2		---	---	GLY	---	---	---	---	---	---	---	---	---	LEU	---	---	---	---	---
D04	1	0	0	0	1	1	1	0	0	0	0	0	1	0	2	0		---	---	TYR	---	SER	---	---	---	---	---	---	---	---	---	---	---	---	---
D05	2	2	1	0	4	1	1	1	0	1	1	1	1	0	6	1		HIS	---	---	LYS	---	---	---	---	---	---	---	---	TYR	---	---	---	---	---
D06	1	0	0	0	1	0	2	1	0	1	1	0	2	0	1	1		---	---	---	SER	---	TYR	---	---	---	---	---	---	---	---	---	---	---	---
D07	5	0	0	3	2	0	2	1	0	1	0	1	2	1	3	1		ASN	---	---	ASP	---	---	---	---	---	---	---	---	---	---	---	---	---	SER
D08	2	0	2	0	4	1	1	1	0	1	2	1	1	1	3	1		---	---	---	---	---	---	---	---	THR	LEU	---	---	---	---	---	---	ILE	---
D09	3	0	0	0	2	0	1	0	0	1	3	2	1	1	0	1		---	---	VAL	---	PRO	---	---	ILE	---	---	---	---	---	---	---	---	---	---
D10	3	3	2	0	5	3	1	0	0	1	2	3	1	0	6	1		---	---	---	---	---	---	---	---	---	---	---	---	TYR	---	---	---	---	---
D11	1	2	1	0	4	0	0	0	0	0	0	1	0	0	6	0		---	THR	---	VAL	---	---	---	---	---	---	---	---	TYR	---	---	---	---	---
D12	6	6	4	1	5	1	0	0	0	0	2	1	0	0	5	1		---	SER	---	---	---	---	---	---	---	---	---	PRO	---	---	---	---	---	---
D13	3	3	2	0	4	3	0	0	0	0	0	2	1	0	6	1		---	---	VAL	---	---	---	---	---	---	---	---	---	TYR	---	---	---	---	---
D14	3	2	2	1	3	2	0	0	0	0	0	1	0	0	7	1		---	---	GLN	---	---	---	---	---	ILE	---	---	---	---	---	ILE	---	---	---
D15	3	3	2	0	5	2	0	0	0	0	0	2	1	0	7	1		---	HIS	LYS	---	---	---	---	---	---	THR	---	---	---	---	---	---	---	---
D16	5	4	4	1	6	1	0	0	0	0	0	1	0	0	4	1		---	---	---	---	---	---	---	---	LEU	LEU	---	---	---	---	ILE	---	---	---
D17	3	4	1	0	4	2	1	0	0	0	1	2	1	0	5	1		---	HIS	ASP	---	---	---	---	---	ILE	---	---	---	---	TYR	---	---	---	---
D18	3	4	1	0	4	2	1	0	0	0	2	3	1	0	7	1		---	---	GLY	---	---	---	---	---	---	---	---	---	---	TYR	---	---	---	---
WT	7	7	2	2	5	5	5	5	5	5	5	5	5	5	5	5		ARG	PRO	GLU	GLU	ARG	PHE	PRO	MET	MET	SER	THR	PHE	LYS	VAL	LEU	LEU	CYS

FIG. 2. Properties and primary sequences of functional β-lactamases. Column abbreviations: AMP, ampicillin; PIP, piperacillin; CT, clavulanic acid; SUL, sulbactam; AmC, Augmentin (amoxicillin, 20 μg; clavulanic acid, 10 μg); TIM, Timentin (ticarcillin, 75 μg; clavulanic acid, 10 μg); CD, cephaloridine (30 μg); CF, cephalothin (30 μg); NIT, nitrocefin; CZ, cefazolin (30 μg); CEC, cefaclor (30 μg); CFP, cefoperazone (75 μg); MA, moxalactam (30 μg); CID, cefonicid (30 μg); HT, heat stability; SUM, averaged sum of all drug susceptibilities. Numbers in columns AMP, PIP, CT, and SUL indicate the concentration of drug allowing growth on plates containing 2-fold increasing concentrations. In the AMP column, a 1 indicates growth on 50-μg/ml plates while a 7 indicates growth at 3200 μg/ml. PIP plates contained 25–1600 μg/ml; a 0 in the PIP column indicates no growth at 25 μg/ml while a 7 indicates growth at 1600 μg/ml. CT plates contained ampicillin at 50 μg/ml and CT at 0.75–25 μg/ml. SUL plates contained ampicillin at 50 μg/ml and SUL at 12.5–200 μg/ml. Numbers in the remaining drug columns refer to the diameters of inhibited growth in the BBL Sensi-Disk assays. Diameters in millimeters for cells containing wild-type and no enzyme, respectively, were as follows: AmC, 23, 13; TIM, 30, 17; CD, 22, 12; CF, 25, 17; CZ, 25, 17; CEC, 28, 23; CFP, 35, 22; MA, 33, 16; CID, 31, 16. The heat stability (HT) column represents the combined difference in resistance to the drugs AMP, PIP, CD, and CF at 30°C versus 37°C. The enzymes that are most heat-labile are labeled 0. The SUM column is the sum of the drugs in the preceding columns.

β-Lactamases with Altered Drug Specificity. Several classes of novel derivatives with an altered specificity to different cephalosporin-type substrates were also obtained. First, the threonine-62 derivative (A01) and the lysine-63 derivative (A02) have specifically lost activity against nitrocefin while maintaining activity against cephalothin and the other cephalosporin-type antibiotics. Second, derivatives with isoleucine-65 (B05) or histidine-75 (B11) have maintained wild-type levels of activity against cephaloridine while losing considerable activity against nitrocefin. These two classes might be expected, as they were initially selected by plating on cephalothin and cephaloridine, respectively. All of the enzymes studied showed little deviation from the wild-type preference for the penicillin-type antibiotics in comparison to the cephalosporin-type. However, in several cases (A01–A13), a small increase in activity against some of the cephalosporin-type drugs was observed.

Additional Observations. The amino acids immediately C-terminal to the active-site serine are involved in the second α-helix of the protein; however, prolines, which tend to disrupt α-helices, appear to have little negative effect in the four enzymes in which they are found (C13, D01, D12, and A12). Some possible explanations for this discrepancy are that the α-helical nature of the protein in this region is not important for function, and that the prolines would not interrupt the initiation of the α-helical region, and that interactions of the α-helix with other regions of the protein allow the required structures to be stably maintained in spite of the disruptive nature of the prolines.

Positions where no amino acid changes were observed are likely to be required to maintain a crucial structure or are involved in an interaction with the substrate. In the *S. aureus* protein it has been suggested (12) that lysine-73 forms a salt bridge with glutamate-166 and is involved in proton transfer during catalysis. The hydroxyl of the active-site serine-70 is involved in the initial attack on the β-lactam ring. It is thus not surprising that no alternative amino acids were found in these two positions. Position 67 contains an alanine in the related enzymes from *Bacillus cereus*, *Bacillus licheniformis*, and *S. aureus* (18), but no variants were seen in this experiment from the wild-type proline. Although the active-site serine-70 and lysine-73 are predicted to be conserved by structural and evolutionary analysis, the high diversity in positions 62 and 63 could not be predicted.

DISCUSSION

This paper describes a simple and potentially general method for creating functional proteins with altered enzymatic properties. The two key features of the approach are the use of highly degenerate or random-sequence oligonucleotides for mutagenesis and the ability to carry out an appropriate selection or screen for the desired variant. Thus, this method combines desirable features of oligonucleotide-directed mutagenesis and classical genetic selections and screens. In particular, the method has the potential to generate a wide variety of functional sequences not found in nature and to alter enzymatic function without precise knowledge of the protein structure.

It is useful to compare the approach described above with other methods for generating proteins with novel properties. Site-directed mutagenesis, perhaps the most commonly employed method, makes it possible to generate any desired protein sequence at will. However, given the large number of possible derivatives (for a 20-amino acid region there are 380 possible single mutants and $>10^5$ possible double mutants), it is completely impractical to alter protein function by directed mutation in the absence of detailed structural information. For example, in the case of the TEM β-lactamase, whose structure is unsolved, it would have been essentially impossible to predict which alterations would lead to resistance to suicide inhibitors.

Conventional genetic approaches, even those involving relatively localized mutagenesis, have significant limitations with respect to the frequency and diversity of variant proteins. For example, it would be difficult to obtain interesting variants requiring multiple amino acid changes or those requiring amino acid substitutions involving multiple nucleotide changes in the same codon. In contrast, the method described here can generate any possible amino acid change, and the mutation frequency can be set to any desired level up to complete randomization of the encoding DNA sequence. The frequency of specific protein variants will be influenced by the genetic code in conjunction with the codons chosen, the mutation frequency, and the length of the oligonucleotide. In addition, the very high mutation frequency should make it possible to utilize more complex assays to screen a set of variant proteins for a desired property.

One limitation to the approach described here is that alterations are confined to the relatively short region defined by the oligonucleotide. Thus, as is the case for TEM β-lactamase, the method is most useful when functionally important regions of the protein have been defined previously. However, by subdividing a protein into regions encoded by adjacent oligonucleotides, it might be possible to create interesting derivatives even in the absence of any specific functional or structural information.

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The numbers in the HT and SUM columns have been normalized such that cells containing wild-type enzyme are scored as 5 and cells containing a plasmid encoding no enzyme are scored as 0. The amino acids column headings and the row labeled WT depict the wild-type sequence in this region. Variants from these amino acids are shown with the appropriate three-letter code; dashes indicate the wild-type amino acid. Row labels indicate classes defined by the drug used in the initial selection. Those of the A series were selected by plating on CF; the B series, on CD; the C series, on CT; and the D series, on AMP.

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