Novel ceftazidime-resistance β-lactamases generated by a codon-based mutagenesis method and selection

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ABSTRACT

Four known and nine new ceftazidime-resistance β-lactamases were generated by a novel, contaminating codon-based mutagenesis approach. In this method, wild-type codons are spiked with a set of mutant codons during oligonucleotide synthesis, generating random combinatorial libraries of primers that contain few codon replacements per variant. Mutant codons are assembled by tandem addition of a diluted mixture of five Fmoc-dimer amidites to the growing oligo and a mixture of four DMTr-monomer amidites to generate 20 trinucleotides that encode a set of 18 amino acids. Wild-type codons are assembled with conventional chemistry and the whole process takes place in only one synthesis column, making its automation feasible. The random and binomial behavior of this approach was tested in the polylinker region of plasmid pUC19 by the synthesis of three oligonucleotide libraries mutagenized at different rates and cloned as mutagenic cassettes. Additionally, the method was biologically assessed by mutating six contiguous codons that encode amino acids 237–243 (ABL numbering) of the TEM_pUC19 β-lactamase, which is functionally equivalent to the clinically important TEM-1 β-lactamase. The best ceftazidime-recognizing variant was a triple mutant, R164H:E240K:R241A, displaying a 333-fold higher resistance than the wild-type enzyme.

INTRODUCTION

Many methods have been developed to accelerate the artificial evolution of proteins, either by enzymatic mutagenesis of the genes that encode them or by amplification from mutant primers. Enzymatic methods are mainly based on PCR techniques [e.g. error-prone PCR (1), DNA shuffling (2) and StEP (3)], taking advantage of the low fidelity of some thermostable DNA polymerases under special amplification conditions. For instance, Tag DNA polymerase may exhibit base substitution error rates ranging from 10^{-2} to 10^{-5} (4). However, these approaches are limited to point mutations and each wild-type amino acid will only change to a fraction of the other 20 (5). A similar effect is seen with libraries of spiked oligos (6), where each wild-type nucleotide is contaminated with the other three bases during oligonucleotide assembly. In order to cover all the sequence space, several schemes of saturation mutagenesis have been described. The most popular are the ‘NNN’ and ‘NNG/C’ approaches (7), which generate pools of 61 and 31 sense codons, respectively, that encode the 20 amino acids. Unfortunately, when a region of some amino acids has to be sampled, these strategies only allow the complete exploration of a maximum of four amino acids due to the restriction imposed by the practical transformation efficiency of 10^{7} variants (7). Additionally, the frequency of mutants generated with these saturation methods is strongly biased towards those sequences encoded by redundant codons. Codon redundancy has been circumvented by the use of sets of DMTr-trimer amidites (DMTr represents the 4,4'-dimethoxytrityl protecting group) that encode all 20 amino acids (8) or a subset of them (9,10). However, in contrast to enzymatic and spiked methods, all common saturation schemes of mutagenesis generate libraries enriched with variants bearing multiple amino acid substitutions and single mutants are poorly represented. The enrichment of libraries with variants carrying few codon replacements in codon-based mutagenesis approaches can be performed by an appropriate contamination of the wild-type codon with a specific mutant codon (11).

In 1998, Neuner et al. (12) described an ingenious codon-based saturation mutagenesis method that combines the use of DMTr-dimer amidites and a resin-splitting approach (13) as a means to eliminate codon redundancy in random oligonucleotide libraries. In this method, subsets of different codons with the sequence B_{1}B_{2}B_{3} can be generated if during oligonucleotide synthesis, proceeding in the common 3'→5' direction, the growing oligo is split into four fractions and each of these is coupled to a specific mix of DMTr-dimer amidites bearing the sequences B_{2}B_{3}, corresponding to the second and third nucleotide positions of the mutant codons. Mutant codons are completed by coupling a specific DMTr-monomer amidite to each separated fraction to define the first nucleotidic position B_{1}. Disappointingly, this scheme of mutagenesis requires the use of 11 DMTr-dimer amidites and falls into the resin-splitting procedures, which are very tedious, laborious and impractical to automate.

We took this concept of dimers, employing the fact that the Fmoc (9-fluorenylmethoxycarbonyl) and DMTr protecting groups are mutually orthogonal (11) (Fmoc is labile to base and stable to acid, while DMTr is the converse) to develop a novel codon-based mutagenesis method executed in a contaminating approach. The randomness of the method lies in the
combination of five Fmoc-dimer amidites and four DMTr-monomer amidites to generate 20 trinucleotides that encode a set of 18 amino acids, as illustrated in Figure 1. In this method, the growing oligonucleotide is contaminated (spiked) with a diluted mix of Fmoc-dimer amidites, the wild-type codon is assembled with conventional DMTr-monomer amidites and the set of mutant codons is completed with a mix of DMTr-monomer amidites, as shown in Figure 2. This mutagenic cycle of synthesis can be repeated for each wild-type codon where variability is to be explored.

The randomness and the binomial behavior of this approach were evaluated in the polylinker region of plasmid pUC19 by the synthesis of three oligonucleotide libraries mutagenized at different rates and cloned as mutant cassettes. Additionally, the method was biologically assessed by mutating a region of six contiguous amino acids [237–243, ABL numbering (14)] located between the end of the B3 β-strand and at the beginning of the B4 β-strand of the TEM<sub>UC19</sub> β-lactamase (15) and selecting mutants with a marked change of specificity from ampicillin towards cefazidime (CAZ). TEM<sub>UC19</sub> β-lactamase (16) was chosen as the model protein because this enzyme is found in the world wide spread plasmid pUC19, because it is enzymologically equivalent to the well known TEM-1 enzyme, in that it contains only two neutral amino acids replacements, and because numerous natural (www.lahey.org/studies/tentable.htm) and laboratory mutants of TEM-1 (17–20) have demonstrated that significant determinants of the specificity change from ampicillin to CAZ are located in the randomized region.

**MATERIALS AND METHODS**

<sup>31</sup>P NMR analysis was performed at 162 MHz, using 85% triphosphoric acid as the external reference. The oligonucleotide libraries were assembled on two DNA synthesizers (model 391) from Applied Biosystems using a 0.2 μmol standard protocol as recommended by the manufacturer. HPLC analyses were performed on a System Gold chromatograph and an ultraspHERE C18 analytical column (4.6 × 250 mm), both from Beckman, with detection at 260 nm. N-acyl-deoxynucleosides and thymidine were purchased from Chemogenes and DMTr-deoxynucleoside β-cyanoethyl phosphoramidites (DMTr-monomer amidites) from Glen Research. 9-Fluorenylmethoxycarbonyl chloride (Fmoc-Cl), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), N,N-diisopropylethylamine, t-butyl hydroperoxide and all ancillary reagents for oligonucleotide synthesis were purchased from Aldrich. Anhydrous tetrahydrofuran (THF) was purchased from Fisher, while anhydrous acetonitrile was from American Bioanalytical. All chemical reactions in solution phase were followed by thin layer chromatography (TLC) on aluminum-backed silica gel 60 F<sub>254</sub> sheets (Merck) using a mix of ethyl acetate/dichloromethane/triethylamine (45:45:10 v/v) as the elution system. 5'-Fmoc-monomers were synthesized and recovered by the method described by Lehmann et al. (21). 3'-DMTr-monomers were prepared by our reported procedure (11). The phosphorylating agent chloro(diisopropylamino)ethoxyphosphine (EtOPCINP<sub>2</sub>) was used in the synthesis of Fmoc-dimers and their corresponding amidites was prepared according to Atkinson and Smith (22), using ethoxydichlorophosphite instead of methoxydichlorophosphite.

The antibiotics cefotaxime (CTX), ampicillin (AMP) and kanamycin (KAN) were bought from Sigma Chemical Co., while CAZ was from Glaxo. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs, while Taq DNA polymerase, plasmid pUC19 and deoxyxynucleoside triphosphates (dNTPs) were bought from Boehringer Mannheim and used according to standard protocols. Plasmid pT4Blu was the parent plasmid of all random libraries. It is a β-lactamase promoter derivative from cloning vector pT4 (kindly donated by Peter Kast and Ying Tang, ETH, Zurich, Switzerland), containing 3.2 kb that include the KAN resistance gene and also the ColE1 and φ1 origins of DNA replication. *Escherichia coli* XL1-Blue *(recA<sup>1</sup>, endA<sup>1</sup>, gyrA<sup>96</sup>, thi-1, hsdR<sup>17</sup>, supE<sup>44</sup>, relA<sup>1</sup>, lac [F<sup>+</sup> proAB, lacP lacZ<sup>AM15</sup>, Tn10(tet<sup>1</sup>)]) was obtained from Stratagene and used for the cloning and plasmid purification procedures.

**Synthesis of Fmoc-dimer amidites**

Synthesis of Fmoc-dimers, precursor synthons of the Fmoc-dimer, amides, proceeded in the 3'→5' direction in a completely protected scheme to ensure the exclusive formation of 3'-5' phosphodiester bonds and in a 'one pot' approach to avoid isolation of the intermediate phosphoramidite (Scheme 1). We describe here the procedure used for synthesis of the Fmoc-<i>T</i><sub>4</sub> dimer and its corresponding amide. The other four Fmoc-dimer amidites, AA, AT, CG, and GC, were prepared with the same general procedure.

3'-O-(9-fluorenylmethoxycarbonyl)thymidine-3'-yl-ethylyphosphate-5'-yl-thymidine (Fmoc-<i>T</i><sub>4</sub> dimer) 3'-O-(4,4'-dimethoxytrityl)thymidine (1.53 g, 3.3 mmol) was dried by co-evaporation with anhydrous tetrahydrofuran (2 × 10 ml) and redisolved in 25 ml of the same solvent. 3-N-diisopropylethylylamine (0.68 ml, 3.9 mmol) and the phosphorylating agent (0.84 ml, 3.9 mmol) were added using anhydrous solvent under nitrogen atmosphere and with magnetic stirring. After 30 min reaction, TLC analysis showed complete conversion of the nucleoside to its phosphotriester bond and in a 'one pot' approach to avoid isolation of the intermediate phosphoramidite (Scheme 1). We describe here the procedure used for synthesis of the Fmoc-<i>T</i><sub>4</sub> dimer and its corresponding amide. The other four Fmoc-dimer amidites, AA, AT, CG, and GC, were prepared with the same general procedure.

3'-O-(9-fluorenylmethoxycarbonyl)thymidine (2.2 g, 4 mmol) and tetranto (1.05 g, 15 mmol), previously co-evaporated with THF (2 × 10 ml). After 60 min of the second reaction, the TLC analysis showed complete disappearance of the intermediate phosphoramidite (limiting reagent) and an excess of t-butyl hydroperoxide was added to oxidize the recently formed phosphate-triester bond to the more stable phosphate-triester.
white powder after precipitation over n-hexane and vacuum drying.

5′-O-(9-fluorenylmethoxycarbonyl)thymidine-3′-yl
ethylphosphate-5′-yl-thymidine-3′-yl-O-ethyl-N,N-
diisopropylaminophosphoramidite (Fmoc-TT amidite)

The Fmoc-TT dimer (1.19 g, 1.5 mmol) was dried by co-
evaporation with anhydrous THF (2 × 10 ml) and was redissolved in 15 ml of the same solvent. N,N-diisopropyl-
ethylamine (0.43 ml, 2.5 mmol) and the phosphitylating agent
(0.47 ml, 2.25 mmol) were added using independent dispos-
sible syringes under a nitrogen atmosphere and with magnetic
stirring. After 35 min reaction an abundant amount of precipitate corresponding to N,N-diisopropylhexammonium
chloride arose, but the reaction was continued for an additional
25 min. TLC analysis showed a complete transformation of
the starting dimer to a more hydrophobic product correspond-
ing to its phosphoramidite. The reaction was quenched with a
saturated solution of sodium bicarbonate (5 ml), diluted with
dichloromethane and washed as before. The crude compound
was finally recovered as a white powder after precipitation
over n-hexane and vacuum drying.

Random oligonucleotide libraries

Three oligonucleotides of sequence agtgccaggtgga-GCC/B1
B3-TCT/B1B2B3-gccgattgtaatc were assembled at different
levels of mutagenesis containing the mutant codons B1B2B3
represented in Figure 1. The wild-type codons subjected to
mutagenesis are those marked in capital letters. It is worth
noting that the wild-type nucleotides of the sequence were
added by common DMTr-monomer amidites while the codons
B1B2B3 were incorporated by a mix of the five Fmoc-dimer
amidites described above and an equimolar mixture of four
DMTr-monomer amidites, as shown in Figure 2. The flanking
arms included two restriction sites, HindIII and EcoRI.

The assembly process required the use of two DNA
synthesizers, labeled A and B (Fig. 2). Synthesizer A was
fitted out with common reagents for oligonucleotide synthesis,
including clean 50 mM DMTr-monomer amidites. At position
X (fifth vial from left to right) a mix B2B3 of the five Fmoc-
dimer amidites TT:AA:AT:GC:CG in the relative molar ratio
1.0:1.03:1.03:1.03:1.23 and at a total 50 mM concentration in
anhydrous acetonitrile was installed for assembly of the
library polylinker100. Synthesizer B was prepared with the
common activating, capping and oxidizing solutions, but
the detritylating reagent was substituted for a ‘defluroenylat-
ing’ reagent made up of 0.1 M DBU in acetonitrile. This
solution was used to remove the Fmoc protecting groups of the
incorporated dimers. At position X (fifth vial from left to right)
an equimolar 100 mM acetonitrile solution of the four DMTr-
monomer amidites (A, C, G and T) was installed to complete
the mutant codons. This mixture of monomers is usually
designated N, but here is named B1 because these monomers
will define the first 5′ nucleotide position of the mutant
codons. With regard to the synthesis program, co-delivery of
amidites and tetrazol to the column was accomplished by three
pulses of 3 s (~400 µl), as opposed to the conventional pulses
of 3 × 2 s. Under these conditions, clean DMTr-monomer
amidites were coupled with an efficiency of 98%, while Fmoc-
dimer amidites were coupled with an efficiency of 95%. The
assembly of polylinker100 (mutated at saturation, 100%)
started on synthesizer A with synthesis of the sequence
5′-(B2B3)-gcggattgtaat-3′ in the trityl-on mode. Next, the
column was transferred to synthesizer B, the Fmoc protecting
groups were removed and one coupling with the mix B1 was
performed to complete the mutant codons B1B2B3, in trityl-on
mode. The column was returned to synthesizer A for one
coupling with the mix Fmoc-B2B3 and then to synthesizer B
to complete the mutant codons. This process was repeated four
more times and finally the sequence agtgccaggtgga was
added on synthesizer A, in trityl-off mode, to complete the
library and remove the last DMTr protecting group.

For the second library, originally expected to be mutated at
50%, the assembly started on synthesizer A programming the
sequence TC(T/B2B3)-gcggattgtaat in the trityl-on mode.
The bases in parentheses were automatically co-delivered to
the growing chain. During this step the wild-type sequence
grew by only 1 nt, while the pool of mutant sequences grew by
2 nt. In the next two cycles of synthesis, the wild-type codon
was completed with addition of clean DMTr-monomer
amidites, while the mutant segments bearing the Fmoc
protecting group were in standby. As before, the column
was transferred to synthesizer B to complete the set B1B2B3.
The column was returned to synthesizer A for three coupling
cycles to add the sequence 5′-GG(G/B1B2B3)-3′ and then the
mutant codons were completed on synthesizer B. The cycling
processes were repeated for each wild-type codon to be
explored. Finally, the 5′ flanking region gataaatctgga was
added on synthesizer A. The level of mutagenesis per codon,
indicated by the incorporation of Fmoc-B2B3, was determined
by the difference in absorbance of the DMTr cation released
from the previous nucleotide to the mutagenized codon and
the DmTr-monomer amide incorporated with the mix Fmoc-
B2B3. This library was mutated at an average 48.9% per codon
and was named polylinker49.

For the third library, 100 mM solutions of clean DMTr-
monomer amidites were installed in synthesizer A and a
20 mM solution of Fmoc-B2B3 in the X position. This oligo
was synthesized as was polylinker49 and mutated at an
average 20.5% per codon. It was named polylinker20.

Two additional libraries of sequences 5′-gtttatctgt-
ataaatctgga-GCC/B1B2B3-GGT/GT/B2B3-GAG/B1B2B3-CGT/
B1B2B3-GGG/B1B2B3-TCT/B1B2B3-gccgattgtaatc were assembled to mutate the region 237–243 of the
TEmpGC19 β-lactamase. The first library was named Blamat63
and the second Blamat42, to reflect their respective lengths.
The main differences between the two libraries were the sizes of the flanking regions (marked in lower case), the relative
ratio of components in the mutagenic mix and the level of
mutagenesis. Blamat63 was assembled using 100 mM DMTr-
monomer amidites and the mix Fmoc-B2B3 at 40 mM in the
same ratio of components as was used for the polylinker
libraries. Blamat42 was also synthesized with 100 mM
DMTr-monomer amidites, but Fmoc-B2B3 was at 50 mM
and the ratio of the components was 1.0:1.0:1.1:1.8:2.3 for
TT:AA:AT:GC:CG, respectively.

The solid supports containing the libraries were subjected to
treatment with 0.5 ml of thiophenol/triethylamine (3:1 v/v) for
2 h at 55°C to remove the internucleotidic ethyl groups of the
mutant codons. The supernatant was removed by filtration and each support was subjected to an overnight treatment with concentrated ammonium hydroxide to remove all remaining protecting groups and release the oligonucleotides from the supports. Libraries were purified on 15% polyacrylamide gels containing 8 M urea and recovered in deionized water after n-butanol desalting.

**Relative reactivity of Fmoc-dimer amidites**

Each mix Fmoc-B$_2$B$_3$ used for assembly of the libraries was tested by coupling to a solid support carrying dA$_{30}$ in order to generate a pool of five trimers with the sequences TTA, AAA, ATG, CGA and GCA. Previous experiments performed by conventional chemistry had demonstrated that such a pool was well resolved by HPLC, using a gradient of 5–12% acetonitrile in 20 min, at a flow of 1 ml/min, and 0.1 M triethylammonium acetate, pH 7.0, as the aqueous buffer. These experiments were performed in duplicate and the relative reactivities were determined by correcting the integrated areas of each component using calculated molar extinction coefficients (23).

**Cloning of mutant cassettes**

Duplexes of the libraries polylinker20, polylinker49 and polylinker100 were generated by extension of the complementary primer 5'-gattaaggatcgg-3' and the Klenow fragment (3'-5' exo-) of DNA polymerase I as described earlier (11,24). The products were double digested with the restriction enzymes HindIII and EcoRI and cloned into the previously opened plasmid pUC19. These recombinant plasmids were electroporated into XL1-Blue cells and one-thousandth of the electroporation mixture was plated on LB agar containing AMP at 200 μg/ml. The plates were incubated overnight at 37°C and many clones per library were sequenced to complete 32 in-phase sequences.

**Amplification and cloning of libraries Blamut63 and Blamut42**

Each oligonucleotide library and a primer of sequence 5'-agcgcttgtgctgctgctgtaattcatactgtaattcagc-3', which included the underlined cleavage site XhoI, were used to amplify a 195 bp fragment (megaprimer) by conventional PCR, using as template the gene bla$_{pUC19}$. These megaprimer were purified in agarose gels and on QG columns from Qiagen. They were independently combined with a primer of sequence 5'-tagaaggatcgg-3', containing the underlined cleavage site NcoI, and plasmid pUC19 to yield 873 bp fragments by PCR. These fragments containing the complete mutant genes of bla$_{pUC19}$ were subjected to double digestion with the enzymes NcoI and XhoI and the fragments were purified as before and ligated overnight to plasmid pT4Bla, using T4 DNA ligase. The ligation reaction was desalted with n-butanol and one-quarter of it was electroporated into 30 μl of electrocompetent *E.coli* XL1-Blue cells. After a 1 h recovery of the transformed cells in 1 ml of SOC medium, 1 μl of this mixture was plated on a solid LB plate containing 25 μg/ml KAN and was incubated overnight at 37°C to quantify the library size. The remaining solution was diluted with 19 ml of liquid LB containing 26.3 μg/ml KAN and was incubated overnight at 37°C. Each library arising from Blamut63 and Blamut42 was recovered as a population of mutant plasmids using miniprep columns from Qiagen.

**Selection of active mutants**

For selection of functional mutants again CAZ, each library of mutant plasmids arising from Blamut63 and Blamut42 was used to transform *E.coli* XL1-Blue cells by electroporation. The mixture was plated on five LB plates (200 μl each) containing CAZ at 12.5 μg/ml and incubated for 16–20 h at 37°C. Resistant mutants that arose on these plates were inoculated into 3 ml of liquid LB (containing 25 μg/ml KAN) and were incubated overnight at 37°C to recover plasmid from each of them. Plasmids were retransformed, subcloned and sequenced.

**Antibiotic susceptibility**

Minimum inhibitory concentrations (MICs) of *E.coli* XL1-Blue cells carrying mutant plasmids obtained by CAZ selection were assessed in solid LB plates containing 2-fold increasing concentrations of the antibiotics AMP and CTX. For CAZ, we used linear increasing concentrations, starting with three low concentrations (0.10, 0.125 and 0.150 μg/ml) to determine the MIC of the wild-type TEM$_{pUC19}$ β-lactamase and ending with six higher concentrations (10, 15, 20, 30, 40 and 50 μg/ml) for the mutants. Assay of each mutant was performed at three different dilutions (10$^2$, 10$^3$ and 10$^6$ c.f.u./drop). Spotting of cells was done with a multipipette device, inoculating 5 μl per dilution. Plates were incubated at 37°C for 16–20 h and the presence of colonies in the most diluted spot was interpreted as a signal of resistance.

**RESULTS**

**Fmoc-dimer amidites**

Chemical synthesis of five Fmoc-dimers with the sequences TT, AA, AT, GC and CG, precursors of the target Fmoc-dimer amidites, was accomplished in two stages, as shown in Scheme 1A. In the first stage, fully protected dimers were obtained through a ‘one pot’ reaction where activation of the 5'-nucleoside, coupling with the 5'-nucleoside and oxidation of the internucleotidic phosphite took place without isolation of any intermediate material. In the second stage, each crude fully protected dimer was detritylated and purified by flash column chromatography. In general, Fmoc-dimers were obtained in modest to good yields, but with very high standards of purity as assessed by HPLC and $^{31}$P NMR (Table 1). It is worth noting that each Fmoc-dimer was obtained as a mixture of two diastereoisomers due to the chiral center formed in the internucleotidic phosphotriester, accounting for the pair of peaks that appeared in HPLC and $^{31}$P NMR. Table 1 also shows the appropriate protected starting nucleosides required to assemble each dimer.

Fmoc-dimer amidites were obtained by phosphylation of the corresponding Fmoc-dimers (Scheme 1B) and were recovered in a crude form to avoid chromatographic purification of such reactive species, a step that is frequently deleterious to the overall yield. In spite of this, the four Fmoc-dimer amidites (TT, AA, AT, GC) were highly pure, as indicated in Table 1, and only CG resulted in low purity due to formation of a large amount of an H-phosphonate subproduct.
Although this subproduct reduces the purity of the expected amidite, it is innocuous during the coupling reaction. Each Fmoc-dimer amidite was obtained as a mixture of four diastereoisomers because two chiral phosphorus atoms were generated per molecule, one corresponding to the internucleotide phosphotriester and another one to the phosphoramidite function. Such a diastereoisomeric mixture was evident in the HPLC analysis of the compounds AA, GC and CG, where four peaks appeared. \(^{31}\)P NMR analysis revealed two highly differentiated sets of signals, one of them centered around 148 p.p.m., corresponding to the phosphoramidite function, and the other one around –1.0 p.p.m., corresponding to the internucleotidic phosphate.

**Oligonucleotide libraries**

Performance of the dimer approach for the non-saturating codon-based mutagenesis method was assessed by the assembly of five libraries. These included a mutagenic window of six contiguous codons that encode amino acids Ala237, Gly238, Glu240, Arg241, Gly242 and Ser243 of the
TEM<sub>UC19</sub> β-lactamase. Three libraries were constructed to demonstrate the robustness and reliability of the method, while the other two were applied to a real problem, the search for mutants displaying a change of specificity from AMP towards CAZ that could emerge in nature due to the increasing use of this cephalosporin in clinical treatments.

**Polylinker libraries**

The first three libraries of sequence agtgcgaagcttga-GCC-GGT-GAG-CGT-GGG-TCT-cgc were mutated at three experimental rates (20.5, 48.9 and 100%), substituting each wild-type codon marked in capital letters with the pool of trimers B<sub>1</sub>B<sub>2</sub>B<sub>3</sub> shown in Figure 1. Since these oligos were designed to be cloned in the polylinker region of plasmid pUC19 through the HindIII and EcoRI sites, they were named polylinker20, polylinker49 and polylinker100. In fact, synthesis of polylinker100 was very simple because the growing oligo was saturated with a mix of the five Fmoc-dimer amidites (Fmoc-B<sub>2</sub>B<sub>3</sub>), the unreacted chains were blocked by acetylation, the Fmoc group was removed and the mutant codons were completed with a mixture of the four DMTr-monomer amidites (DMTr-B<sub>1</sub>). For assembly of polylinker49, we originally expected 50% mutagenesis per codon and, knowing of the similarity of reactivity between monomers and dimers (12,25), a 50 mM mix Fmoc-B<sub>2</sub>B<sub>3</sub> and 50 mM clean DMTr-monomer amidites were co-delivered to the synthesis column, obtaining a substitution near to that wanted. At this point, we must remember that each wild-type codon was completed by two successive couplings with clean DMTr-monomer amidites while the mutant codons were completed with the mix B<sub>1</sub>, as shown in Figure 2. Polylinker20 was planned to be mutated at 17%, however, under the conditions used for polylinker100 and polylinker50, the mix Fmoc-B<sub>2</sub>B<sub>3</sub> would have to be at 10 mM, and with co-delivery with the 50 mM DMTr-monomer amidites the resultant mixture would
have been 30 mM, and a poor coupling efficiency would have been obtained. For this reason the 50 mM clean DMTr-monomer amidites were substituted for 100 mM solutions and the mix Fmoc-B2B3 was at 20 mM. A slightly higher mutagenesis rate resulted, but it is not experimentally significant. The three libraries were subjected to a two-step deprotection and were purified by polyacrylamide gel electrophoresis. The Fmoc-B2B3 solutions used for these libraries contained the Fmoc-dimer amidites TT:AA:AT:GC:CG in the molar ratio 1.0/1.03/1.03/1.03/1.23, a combination that yielded an even frequency of trimers (TTA, AAA, ATA, GCA and CGA) when coupled to a solid support carrying dA, as shown in Figure 3A.

### Cloning of the libraries polylinker20, polylinker49 and polylinker100

These mutagenic oligonucleotides were converted into their corresponding DNA duplexes by extension of the complementary primer gattacgaattcgcg under mild polymerization conditions, using the Klenow fragment of DNA polymerase that lacks 3’→5’ and 5’→3’ exonuclease activity, in order to avoid any additional mutagenic condition. The duplexes were double digested with the enzymes HindIII and EcoRI and cloned into the polylinker region of plasmid pUC19 as mutagenic cassettes. With this modification the AMP resistance gene tem of pUC19 remained intact and the electroporated XL1-Blue cells were plated on solid LB containing AMP to generate three libraries of mutants (11,24). From the libraries polylinker20, polylinker49 and polylinker100, 36, 38 and 41 clones, respectively, were sequenced (see Supplementary Material) in order to complete sets of 32 in-phase mutants. The additional sequences contained 4, 6 or 9 nt insertions or deletions, respectively, and were not considered for the statistical analysis. In-phase mutants followed a binomial behavior predicted by the experimental level of mutagenesis as seen in Table 2 and Figure 4. In this sense the robustness and reproducibility of the method has been completely demonstrated. It is worth noting that the rate of aberrant

### Table 2. Expected ($P_{\text{Theor.}}$) and experimental ($P_{\text{exp.}}$) frequency of in-phase mutants generated with the five oligonucleotide libraries assembled in this work, under no selective conditions

<table>
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<tr>
<th>Mutant codons per variant</th>
<th>Polylinker20 ($\alpha = 20.5%$)</th>
<th>Polylinker49 ($\alpha = 48.9%$)</th>
<th>Polylinker100 ($\alpha = 100%$)</th>
<th>Blamut63 ($\alpha = 29.0%$)</th>
<th>Blamut42 ($\alpha = 40.5%$)</th>
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<td>0.000 (0)</td>
<td>0.234</td>
<td>0.187 (6)</td>
<td>0.000</td>
</tr>
<tr>
<td>5</td>
<td>0.001</td>
<td>0.000 (0)</td>
<td>0.094</td>
<td>0.125 (4)</td>
<td>0.000</td>
</tr>
<tr>
<td>6</td>
<td>0.000</td>
<td>0.000 (0)</td>
<td>0.016</td>
<td>0.062 (2)</td>
<td>1.000</td>
</tr>
</tbody>
</table>

$\alpha$ represents the experimental average level of mutagenesis. $P_{\text{Theor.}}$ was calculated by the equation of binomial distribution (24). The kinds of mutants found in each library are indicated in parentheses. The sequences of libraries polylinker20, polylinker49 and polylinker100 are available as Supplementary Material.
mutations increased with the level of mutagenesis, indicating the likely existence of contaminants in the Fmoc-dimer amidites that are magnified as the concentration is increased, or perhaps due to incomplete removal of the Fmoc group. We do not consider the observed proportion of aberrant sequences as significant for the purpose of library creation, especially at the lower level of mutagenesis.

**β-Lactamase libraries**

A library of sequence 5'-gtgtcgtgcgataaatcctga-GGC-GGT-GAG-CGT-GGG-TCT-cgcgttaattgcgacactgg-3' was assembled using 100 mM clean DMTr-monomer amidites and a 40 mM mixture of B2B3. The ratio of components for this mix was the same as that used for the polylinker libraries. Under these conditions of reaction we expected to obtain a 28.6% level of mutagenesis. Experimentally we obtained an average 29.0% per codon. This mutagenic primer contains 63 nt and was named Blamut63.

The second library assembled for mutagenesis of β-lactamase was synthesized with the sequence 5'-ggttcgatctgtaaatcctga-GCC-GGT-GAG-CGT-GGG-TCT-cgcgttaattgcgacactgg-3' and named Blamut42. This oligo was implemented with shorter flanking arms as compared to Blamut63 and was mutated with 50 mM Fmoc-B2B3 mix and 100 mM DMTr-monomer amidites. In contrast to the mix Fmoc-B2B3 used for library Blamut63, which was ~20% more concentrated in the dimer CG to compensate for its minor reactivity with regard to the other dimers, the mix used for this experiment was 80 and 130% more concentrated in the dimers GC and CG with regard to the other components. When this mix was coupled to dAβz, a pool of the trinucleotides TTA, AAA, ATA, GCA and CGA in the molar ratio 1.01/1.10/1.00/1.60/1.91 was obtained by HPLC analysis (Fig. 3B). With regard to the level of mutagenesis, we expected to obtain a 33% value based on the concentration of reagents, however, the experimental result was 40.5%. The only explanation we find for this bias is a probable mis-adjustment in the flow rate of the amidites and a large amount of Fmoc-B2B3 must have been added to the column in comparison to the DMTr-monomer amidites. The libraries were deprotected and purified as for the polylinker libraries.

**Cloning and selection of libraries Blamut42 and Blamut63**

Since the region 237–243 of the TEM<sub>pUC19</sub> β-lactamase to be mutated is encoded by nucleotides 703–720 of the gene <i>tem</i><sub>pUC19</sub>, composed of 858 nt, and the flanking sequences did not contain putative restriction sites for the enzymes N<sub>coI</sub> and X<sub>hoI</sub>, available in the recipient plasmid pT4Bla, it was evident that the oligonucleotide libraries could not be introduced as cassettes, and the process would require two rounds of PCR to complete the mutant genes.

The oligonucleotide libraries Blamut63 and Blamut42 and a primer complementary to the 3' position of the <i>bla</i><sub>pUC19</sub> gene were used to generate two megaprimer (26) by PCR using as template the <i>bla</i><sub>pUC19</sub> gene contained in plasmid pUC19. In a second PCR, these megaprimer were independently used with another primer complementary to the 5' position of the <i>bla</i><sub>pUC19</sub> gene to generate complete mutant genes, again using plasmid pUC19 as template. After a double digestion with the restriction enzymes N<sub>coI</sub> and X<sub>hoI</sub>, the fragments were cloned into plasmid pT4Bla, which also contained the KAN resistance gene, and the resultant ligations were used to transform <i>E. coli</i> XL1-Blue cells. One-thousandth of each electroporation mixture was plated on solid LB containing KAN to quantify the library size, giving in both cases an average size of 1.5 × 10<sup>6</sup> variants. Twenty clones of each library were selected at random and labeled with a sequential number and either the prefix 63M or 42M, indicating the library from which they came. Their plasmid DNA was isolated and subjected to DNA sequencing to analyze the distribution of variants in the mutagenic window, with no selection for β-lactamase function. Among them, three Blamut63 and one Blamut42 were contaminated with other sequences and were not included in the analysis. Three clones per library resulted with nucleotide insertions or deletions, while six wild-type sequences occurred in Blamut63 and eight in Blamut42. The remaining sequences were in-phase mutants and are shown in the upper sections of Tables 3 and 4. The sequence analysis of the amplified region found between the megaprimer and the 5' primer of several clones showed that a 0.095% base substitution error rate was introduced by the PCR. A similar result (0.11%) was reported by Shafikhani et al. (4).

Once the variability of both libraries had been assessed, we arbitrarily selected a wild-type clone from one of the libraries to use as a control. The identities of the wild-type gene <i>bla</i><sub>pUC19</sub> and the promoter <i>bla</i> were confirmed by complete DNA sequencing. The bacterial background resistance to CAZ was considered at 0.15 μg/ml, although the precise minimum concentration at which we still observed some growth was 0.125 μg/ml. In order to isolate mutants with at least a
100-fold increase in resistance with respect to the wild-type clone, each library was retransformed and the transformation mixture was distributed on five LB plates containing 12.5 µg/ml CAZ. After incubation, 13 colonies arose for Blamut63 (Table 3, lower section) and 11 for blamut42 (Table 4, lower section). As these resistance mutants were generated by CAZ selection, they were numerically ordered and labeled with the prefix 63caz or 42caz. Each colony was independently inoculated in liquid LB containing KAN and the plasmid was recovered. After analysis on agarose gels, mutants 63caz10 and 42caz3 turned out to be false positives and were discarded. Each plasmid containing a mutant gene was retransformed, subcloned and sequenced in order to ensure that the specificity changes of the isolated mutants towards CAZ were indeed due to a change in their amino acid sequence and not to additional mechanisms of resistance of the cells, such as generation of mutant penicillin-binding proteins insensitive to the antibiotic or mutant porins that prevent entry of the antibiotic into the periplasmic area (27). All clones were grouped according to their sequence and analyzed for resistance against AMP, CAZ and CTX. The results for both libraries are shown in Table 5.

**DISCUSSION**

In this work we describe the benefits of a novel codon-based mutagenesis method for the random modification of a region of amino acids that is supposed to be implicated in a protein function. This approach reduces codon redundancy by contaminating wild-type codons with a set of mutant codons, as applied to enrich libraries with variants carrying few replacements. The resultant libraries allow a more complete exploration of the sequence space concerning single, double and triple mutants in long mutagenic windows as opposed to conventional saturation methods.

Considering that a pool of 20 trinucleotides may be generated by combination of five dimers and four monomers, as illustrated in Figure 1, the rationale for the codon-based mutagenesis method we developed, as shown in Figure 2, is that wild-type codons may be contaminated with a pool of mutant codons if during oligonucleotide synthesis a diluted mix of Fmoc-dimer amidites (Fmoc-B2B3) and a concentrated mixture of a common DMTr-monomer amidite (DMTr-w3) are co-delivered and coupled to the growing oligonucleotide. Many chains will react with the monomer DMTr-w3, growing by only 1 nt, while few chains will react with the mix Fmoc-B2B3, growing by 2 nt. Since the Fmoc and DMTr protecting groups are mutually orthogonal (11), two additional cycles of synthesis can be executed with conventional DMTr-monomer amidites to complete the wild-type codon while the mutant segments stay arrested. Next, the Fmoc group is removed, while the wild-type codon is in standby, and a mix of DMTr-monomer amidites (DMTr-B1) is coupled to the mutant segments to complete the set of codons B1B2B3. This mutagenic cycle can be repeated for each wild-type codon to be explored. Such an approach requires six positions for phosphoramidites, four for clean DMTr-monomer amidites, one for the mutagenic mix Fmoc-B2B3 and the last one for the mix DMTr-B1, and seven ancillary bottles. Since the instruments at our disposal are equipped with only five vials for phosphoramidites and six ancillary bottles, two DNA synthesizers, labeled A and B (Fig. 2), were necessary to assemble the libraries. In fact, most of the assembly process takes place in synthesizer A and the synthesis column is only transferred to synthesizer B to complete each set of mutant

### Table 3. DNA sequence of the region 237–243 of clones generated with the random oligonucleotide library Blamut63 with and without CAZ selection

<table>
<thead>
<tr>
<th>Clone</th>
<th>Amino acid and nucleotide sequence</th>
<th>Wild-type</th>
<th>No selection</th>
<th>CAZ selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>63M1</td>
<td>GCC GGT GAG CGT GGG TCT</td>
<td></td>
<td>ATT</td>
<td></td>
</tr>
<tr>
<td>63M2</td>
<td>CAT GCG CAA TAA CGC TCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>63M4</td>
<td>GCG CAA AGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>63M5</td>
<td>TTT GAT GGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>63M7</td>
<td>GTT GAT TAT GGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>63M8</td>
<td>CAZ CAA AGC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Empty cells represent wild-type codons.

### Table 4. DNA sequence of the region 237–243 of clones generated with the random oligonucleotide library Blamut42 with and without CAZ selection

<table>
<thead>
<tr>
<th>Clone</th>
<th>Amino acid and nucleotide sequence</th>
<th>Wild-type</th>
<th>No selection</th>
<th>CAZ selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>42M1</td>
<td>GCC GGT GAG CGT GGG TCT</td>
<td></td>
<td>ATT</td>
<td></td>
</tr>
<tr>
<td>42M2</td>
<td>CGC TCG GAG TGA GAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42M3</td>
<td>CGC GCA GAG GGA AGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42M4</td>
<td>ACG GGC GAG GGA AGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42M5</td>
<td>CGC TGC AGC GAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42M6</td>
<td>GCG TGC AGC GAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42M7</td>
<td>GCG TGC AGC GAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42M8</td>
<td>GCT TCG GAT GCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42M9</td>
<td>AAT AAG CAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42M10</td>
<td>AAT AAG GGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42M11</td>
<td>AAT GGT GAA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Empty cells represent wild-type codons.

42caz2 and 42caz5 correspond to codon-deletion mutant where GAG and GTG were replaced with GCG.
codons. It should be noted that the method can be implemented in only one synthesizer, completing the mutant codons by co-delivery of the four clean DMTr-monomer amidites to the column, with the concomitant price of obtaining a bias towards G and A residues (P. Gaytán, personal results), and syringe removal of the Fmoc group. Better automation could be achieved with other synthesizer models equipped with eight phosphoramidite vials, such as the 394 DNA/RNA synthesizer from Applied Biosystems (Foster City, CA) or the ASM-800 DNA synthesizer from Biosset Ltd (Novosibirsk, Russia), and an appropriate synthesis program.

In order to assess the method, five Fmoc-dimer amidites of sequences TT, AA, AT, GC and CG were synthesized as shown in Scheme 1. These compounds were chosen because on combination in the 5' position with any of the other monomers (A, T, G or C) 19 sense codons and one nonsense codon are generated, most of them of preferential use in E. coli. These 19 trimers encode 18 amino acids and there is only one redundancy, for serine. Methionine and tryptophan were excluded from the library because their inclusion would have required the use of two additional Fmoc-dimer amidites, increasing codon redundancy. Preliminary coupling results with each independent Fmoc-dimer amitide revealed that they were as reactive as conventional DMTr-monomer amidites, in agreement with previous reports on DMTr-dimer amidites (12,25). Such a result suggested that the level of mutagenesis could be controlled by appropriate selection of the clean DMTr-monomer amidites and the mix Fmoc-B2B3 concentrations if these were co-delivered to the synthesis column.

In order to demonstrate the robustness and reproducibility of the method, three oligonucleotide libraries of sequence agtgcagagctggaga-GCC-GGT-GAG-CGT-GGG-TCT-cgcgaatt- of the method, three oligonucleotide libraries of sequence trations if these were co-delivered to the synthesis column. the reactivities of the components and the level of mutagenesis. The libraries were labeled polylinker20, polylinker49 and polylinker100, respectively, and cloned into the polylinker region of plasmid pUC19 as mutant cassettes (11,24). As shown in Table 2 and Figure 4, the analysis of 32 in-phase sequences per library (see Supplementary Material) from random selected clones showed that the least contaminated library was enriched with single mutants, the half-mutated library was enriched with double and triple mutants and the saturated library contained only multiple mutants. The three libraries followed a binomial distribution and the expected frequency of variants per library was well correlated with the experimental results, demonstrating the confidence of the method.

Biological assessment of the approach was carried out by mutating six contiguous codons in the **bla**<sub>pUC19</sub> gene that encode the amino acids Ala237, Gly238, Glu240, Arg241, Gly242 and Ser243 of the TEM<sub>b</sub>-lactamase, a region shown in Figure 5. Residue 239 does not exist in TEM-type β-lactamases according with Ambler numbering (14). This region comprises important determinants of the specificity change from AMP to CAZ, as exemplified by the replacement of the Glu240 residue by a positively charged amino acid such as lysine or arginine (18,20).

We tested two oligonucleotide libraries at different levels of mutagenesis, spiking the codons 5’-GCC-GGT-GAG-CGT-GGG-TCT-3’ with the mutant codons B<sub>1</sub>B<sub>2</sub>B<sub>3</sub>. These libraries were named Blamut63 and Blamut42, according to their nucleotide composition. Blamut63 was synthesized with longer flanking arms than those assembled in Blamut42. The libraries were mutated at 29 and 40%, respectively, with two mutagenic mixes containing different ratios of the five Fmoc-dimer amidites. Each library was converted to a megaprimer by a first PCR and into complete mutant genes by a second PCR. These gene libraries were cloned and the variants that emerged with and without CAZ selection were analyzed by DNA sequencing.

Only 8 of 14 clones of the non-selected Blamut63 library were mutant (Tables 2 and 3), representing a 57% efficiency of mutagenesis. However, based on the 29% level of mutagenesis, Blamut63 should contain 12.8% wild-type clones as
calculated by a binomial distribution (24). This result indicates that a 30% difference in wild-type sequence exists between the cloned library and the expected oligonucleotide library. A similar bias (45%) was also observed with the non-selected mutants of library Blamut42 (Tables 2 and 4). In comparison with the frequency of variants observed in the polylinker libraries, it is evident that enrichment of the wild-type sequence arose during the PCRs, either due to better hybridization of the most template-similar sequences (28) or by in vitro recombination of incomplete fragments (3). Returning to the non-selected mutants of Blamut63, it is worth noting its enrichment in double mutants correlating with the level of mutagenesis. Another remarkable result is represented by the set of 14 mutant codons obtained in these variants, which encoded 12 different amino acids, most of them represented only once, a result that is in agreement with an even frequency of trimers (Fig. 3A) generated with the mutagenic mix used for this library. Therefore the frequency of amino acids in the libraries is predictable from the reactivities obtained through HPLC analysis of trimers generated with the mix. Apparently the best combination to ensure an even frequency of mutant amino acids must be equimolar in TT, AA, AT and GC because they exhibit similar reactivities and CG must be used in a 0.23 molar excess due to its lower reactivity. Mutants for the codon GGG were not found in this library, because experimentally this was poorly contaminated and the analyzed sample was very small, however, it did occur in Blamut42.

In the non-selected mutants of Blamut42 (Table 4) all codons of the target window were explored and mainly triple mutants were obtained bearing alternating mutant and wild-type codons, a result only accessible through this kind of contaminating method or with cumbersome resin-splitting methods (13). This library was biased towards mutant codons assembled with the GC and CG dimers, as a consequence of the major concentration of these compounds in the mutagenic mix. This result is interesting, because one could deliberately enrich libraries with different subsets of amino acids without excluding the others.

Some deletions of 1 and 2 nt also occurred in both libraries. Such mutations seem to be inherent subproducts generated during conventional synthesis, perhaps as a consequence of incomplete capping or detritylation during the cycles of synthesis. Such a hypothesis is supported because one of them fell in a 5′ flanking region and another one in a wild-type codon located in the mutagenic window and exclusively assembled with conventional DMTr-monomer amidites. Frameshift mutations of this kind have also been reported with other oligonucleotide-based mutagenesis methods (9,10,29,30), but their precise origin is not clear.

Another surprising result found in Blamut42 was the emergence of mutant codons that were not generated with the mix of dimers. They were point mutants generated by Taq DNA polymerase errors. For instance, the wild-type codon GAG was changed to GTG, GGG and AAG. The analysis of non-selected clones from Blamut63 and Blamut42, in a region located between the megaprimer and the 5′ primer, allowed us to calculate an error rate of 0.095%. On average, 1 nt was substituted per mutant gene and these point mutations were distributed along the genes in an apparently random mode.

The libraries were selected over a high concentration of CAZ, 100-fold higher than the wild-type TEMpUC19 β-lactamase. After sequencing, the resultant mutants, 12 for Blamut63 and 10 for Blamut42, shown in the lower section of Tables 3 and 4, respectively, were grouped and subjected to susceptibility tests against the penicillin antibiotic AMP and the cephalosporin compounds CAZ and CTX (Table 4).

The first point to note from Tables 3–5 is that residues Ala237, Gly242 and Ser243 were always conserved under CAZ selection, suggesting that they are the optimal amino acids for efficient hydrolysis of this cephalosporin. A similar result was previously found only for Ala237 (18), through conventional NNG/C mutagenesis of the three contiguous amino acids Ala237, Gly238 and Glu240, but has never been described for the amino acids Gly242 and Ser243. The method was also able to reproduce the double mutants G238S:E240R and G238S:E240K and the triple mutant G238S:E240K:R241G that emerged in two independent libraries (17,20). The triple mutants G238N:E240K:R241Q and G238N:E240K:R241E, although new, are essentially similar to the double mutant G238N:E240K and mutant G238S:E240K (18).

As seen from the middle of Table 5, some very interesting double and triple mutants occurred in both libraries, where the mutations were separated by at least 74 amino acids. The substitution of Arg164 by either histidine or cysteine was clearly generated by a Taq DNA polymerase error, replacing a guanine for an adenine or a cytosine for a thymine, respectively, in the original wild-type codon. It has been demonstrated that substitution of the residue Arg164 by any of the
other 19 amino acids increases the resistance to CAZ (16). It has been suggested that this change of specificity from penicillins towards cephalosporins is due to disruption of a salt bridge formed between the highly conserved residues Arg164 and Asp179, relaxing the Ω-loop structure that covers the active site pocket and, therefore, allowing a better fit of bulkier antibiotics in the active site. In fact, mutation R164H has emerged in eight different clinical isolates (www.lahey.org/studies/tetable.htm) in combination with replacements at other positions. One example is the natural double mutant TEM-28, carrying the changes R164H:E240K, which also emerged in our libraries as mutant 42caz9. The question at this point is why Arg164 was replaced by histidine and cysteine and not by asparagine or serine if single mutants of these amino acids display higher CAZ resistance, as was demonstrated by Vakulenko et al. (16)? The reason is really simple, a change from Arg to Asn requires two base substitutions in the wild-type codon cgt, a very unlikely event in point mutation strategies. In the case of mutation R164S, a nucleotide transversion is required, which is less likely to occur as compared to the transitions that gave rise to the histidine and cysteine codons.

Analyzing the region mutagenized with the oligonucleotide libraries in all the CAZ-resistant mutants shown in Table 5, a preferential substitution of Glu240 by positively charged amino acids such as lysine or arginine was evident. This result is in agreement with the suggestion that a specificity change towards CAZ may also be obtained by formation of an electrostatic interaction between the side chain of the amino acid and the carboxyl group attached to the oximino chain of the antibiotic (31). In this sense, the increase in CAZ resistance of the double mutants R164H:E240K, R164H:E240R, R164C:E240R is a result of the combination of two effects: an expansion of the active site and an electrostatic interaction between the substrate and the mutant enzyme.

The best of our variants, displaying a 333-fold increase in CAZ resistance in comparison to the wild-type enzyme, was the triple mutant R164H:E240K:R241A, which combines the two effects described above. However, the additional substitution of Arg241 by alanine generates a 3-fold increase in resistance with respect to the double mutant R164H:E240K. A reduction in the volume of the side chain of residue 241 could make room to allow a better fit of the bulky and negatively charged 7β-chain of CAZ.

Other evidence that suggests a better fit of the 7β-chain of CAZ in the active site comes from the doubly isolated mutant 42caz2 and 42caz5. It corresponds to a substitution-deletion mutant in which two contiguous amino acids, Glu240 and Arg241, were replaced by one alanine. Such codon deletion may have occurred by incomplete removal of one Fmoc group incorporated with the dimers and incomplete capping or detritylation of one of the subsequently added nucleotides. This deletion might generate an expansion of the groove formed between the Ω-loop and the turn that joins β-strands B3 and B4 and could account for the 133-fold increase in CAZ resistance. This finding agrees with described specificity changes towards CAZ caused by expansions of the active site due to relaxation (16), reduction (32) or enlargement (33) of the Ω-loop structure (Fig. 5).

The double mutant R164H:E240V is interesting because both mutations can be generated by single point mutations of the original codons. However, the replacement of glutamate by valine requires a transversion from adenine to thymine, and this may be the reason why it has not yet emerged in clinical isolates. This mutant clearly developed CAZ resistance by the expansion (34) model of the active site pocket. In mutant clones containing G238S it has been proposed that expansion may be caused by steric hindrance of serine with the residue Met69 or Asn170, by shifting the position of either the B3 β-strand (34) or the Ω-loop (35). We speculate that, in contrast to the polar wild-type Glut240 residue, which is facing the aqueous solvent, the hydrophobic side chain of valine moves away from the aqueous medium and generates steric hindrance with any of the amino acids mentioned above, expanding the active site.

Another evidence that suggests a higher affinity for CAZ through formation of a hydrogen bond between the hydroxyl of the side chain and the carboxylate of the oximino moiety in the antibiotic. From a clinical point of view, the double mutant R164H:E240K:R241A is interesting because both mutations can be generated by single point mutations of the original codons. However, the replacement of glutamate by valine requires a transversion from adenine to thymine, and this may be the reason why it has not yet emerged in clinical isolates. This mutant clearly developed CAZ resistance by the expansion (34) model of the active site pocket. In mutant clones containing G238S it has been proposed that expansion may be caused by steric hindrance of serine with the residue Met69 or Asn170, by shifting the position of either the B3 β-strand (34) or the Ω-loop (35). We speculate that, in contrast to the polar wild-type Glut240 residue, which is facing the aqueous solvent, the hydrophobic side chain of valine moves away from the aqueous medium and generates steric hindrance with any of the amino acids mentioned above, expanding the active site.

From all these results, the versatility of the method may be highlighted, as different subsets of amino acids can be represented in the region modified with primers, modifying the mix of Fmoc-dimer amidites or the mix of DMTr-monomer amidites in the third position, as shown in Figure 1. The frequency of variants (few versus multiple codon replacements) can be controlled by the appropriate selection of the concentration of the mutagenic mix and, additionally, the contamination of the wild-type sequence may be performed by a pre-addition protocol (24) in order to optimize the use of the Fmoc-dimer amidites.

Here we have explored a window of six contiguous codons, but the method can be easily applied to windows of non-contiguous codons and/or larger windows of up to 15 amino acids per oligonucleotide, which can be reliably made up to 75 bases long. With a window of 15 codons and a recursive PCR approach (36) one could completely explore genes of the size of b1aCU19 (263 amino acids) with only 18 mutant oligonucleotides and a high likelihood of completing the search of most single and double mutants. Other multiple mutants would also be well represented in a manner unavailable by any other method.

The present requirement for non-commercial compounds may be easily overcome because we have demonstrated the reproducibility of the synthetic pathway for Fmoc-dimer amidites and, for instance, Monomer Sciences (New Market, AL) sells 5’-Fmoc-monomers while Chemgenes (Ashland, MA) offers services to synthesize dimers. Furthermore, the ethyl protecting group could be replaced by the common β-cyanoeethyl group in order to simplify the final oligonucleotide deprotection.

This mutagenesis method allowed us not only to find known β-lactamase mutants, but also new specificity mutants. The finding that expansion of the groove located between the Ω-loop structure and the turn joining the B3 and B4 β-strands through deletion of one amino acid located in this region also affords activity towards CAZ suggests the value of a method that deliberately introduces codon deletions. Such a method is currently unavailable, but can be conveniently implemented through orthogonal chemistry.
SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

ACKNOWLEDGEMENTS

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