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Developing bifunctional β -lactamase molecules with built-in target-recognizing module for prodrug therapy: identification of *Enterobacter Cloacae* P99 cephalosporinase loops suitable for randomization and phage-display selection

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Abstract

This study was focused on developing catalytically active β -lactamase enzyme molecules that have target-recognizing sites built within their scaffold. Using phage-display approach, nine libraries were constructed by inserting the randomized linear or cysteine-constrained heptapeptides in the five different loops on the outer surface of P99 β -lactamase molecule. The pIII signal peptide of Sec-pathway was employed for a periplasmic translocation of the β -lactamase fusion protein, which we found more efficient than the DsbA signal peptide of SRP-pathway. The randomized heptapeptide loops replaced native amino acids between positions ³⁴Y-³⁷K, ²³⁸M-²⁴⁶A, ²⁷⁵N-²⁸⁰A, ³⁰⁵A-³¹¹S, or ³²⁹I-³³⁴I of the P99 β -lactamase molecules for generating the loop-1 to -5 libraries, respectively. The diversity of each loop library was judged by counting the primary and β -lactamase-active clones. The linear peptide inserts in the loop-2 library showed the maximum number of the β -lactamase-active clones, followed by the loop-5, loop-3, and loop-4. The insertion of the cysteine-constrained loops exhibited a dramatic loss of the enzyme-active β -lactamase clones. The complexity of the loop-2 linear library, as determined by the frequency and diversity of amino acid distributions in the randomized region, appears consistent with the standards of other types of phage display library systems. The selection of the loop-2 linear library on streptavidin protein as a test target identified several β -lactamase clones that specifically bound to streptavidin. In conclusion, this study identified the suitability of the loop-2 of P99 β -lactamase for constructing a phage-display library of the β -lactamase enzyme-active molecules that can be selected against a target. This is an enabling step in our long-term goal of developing bifunctional β -lactamase molecules against cancer-specific targets for enzyme prodrug therapy of cancer.

Keywords

P99 β -lactamase; protein scaffold; loop randomization; functional enzyme libraries; phage display selection

INTRODUCTION

The success of prodrug therapy is based on the selective activation of a relatively low-toxic prodrug at the target site of drug action. In cancer prodrug therapy, such an activation of the

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prodrug delivers higher drug concentrations at the tumor site, resulting into better therapeutic efficacy and reduced systemic toxicity in comparison to the administration of a conventional chemotherapeutic drug itself (Shukla and Krag, 2006). A variety of strategies have been employed to achieve a selective prodrug activation in tumors, which include the following: (a) an exploitation of tumor-associated factors, such as enzymes, low extracellular pH, or hypoxia; or (b) a targeted delivery of exogenous enzymes to the tumors using antibodies, genes, viral vectors, or synthetic polymer as vehicles (Satchi-Fainaro *et al.*, 2003; Rooseboom *et al.*, 2004; Singh *et al.*, 2008). The therapy that uses monoclonal antibodies for a targeted delivery of exogenous enzymes at the tumor site is called antibody-directed enzyme prodrug therapy (ADEPT) (Bagshawe, 2006). In this strategy, the conjugate of a tumor antigen-specific antibody and a selected enzyme is administered to deliver the enzyme to the tumor. After the unbound conjugate is cleared from the circulation, the prodrug is infused intravenously. The presence of enzyme predominantly at the tumor site amplifies an availability of the active drug concentrations locally. A number of clinical and preclinical ADEPT studies have revealed some very important challenges related to the large size of tumor-targeting antibodies, which include a slow conjugate clearance from blood and normal tissues and limited delivery in the poorly vascularized tumors (Martin *et al.*, 1997; Denny and Wilson, 1998; Senter and Springer, 2001). Animal studies with tumor xenograft models have shown that the enzyme conjugates of smaller size, in which the tumor antigen recognition module is a scFv, nanobody, or peptide, may overcome some of the limitations related to antibodies (Michael *et al.*, 1996; Bhatia *et al.*, 2000; de Graaf *et al.*, 2002; Cortez-Retamozo *et al.*, 2004).

The emerging field of protein engineering led to different non-immunoglobulin scaffolds with diverse origins and characteristics for constructing protein libraries and grafting CDRs for developing molecules alternative to antibodies (Hosse *et al.*, 2006; Seelig and Szostak, 2007). Recently, we made an attempt to develop β -lactamase molecules that have target-recognition sites built within active enzyme molecules (Shukla *et al.*, 2007). Such bifunctional enzyme molecules are the smallest possible units that could be utilized in enzyme prodrug therapy. β -Lactamase (β -lactam hydrolase, E.C. # 3.5.2.6) has several desirable characteristics that make it an ideal enzyme for pro-drug therapy, which include high turnover rate, broad substrate specificities, and its ability to release a wide range of mechanistically distinct chemotherapeutic agents (Melton and Sherwood, 1996; Senter and Springer, 2001; Kuhn *et al.*, 2004). β -Lactamase enzyme in conjugation with antibodies has been employed in many experimental ADEPT studies using cephalosporin prodrugs (Meyer *et al.*, 1993; Kerr *et al.*, 1995; Svensson *et al.*, 1995; Senter and Springer, 2001). β -Lactamases catalyze the hydrolysis of the β -lactam ring. Four classes of β -lactamase, namely A, B, C, and D, differing in their size, protein structure, inhibitors, or mechanism of action, have been reported (Majiduddin *et al.*, 2002; Jacoby and Munoz-Price, 2005). We developed bifunctional β -lactamase molecules by selecting a β -lactamase library, composed of randomized linear octapeptide-inserts (X_8) in an outer loop (loop-1) of the *Enterobacter cloacae* P99 cephalosporinase (P99 β -lactamase), on human cancer cells (Shukla *et al.*, 2007). P99 β -lactamase is a class C enzyme from *E. cloacae* strain P99. The phage-display approach was used for preparing this library. The selected β -lactamase molecules exhibited the tumor cell-specificity and the enzyme activity. We extend this study further to identify other possible loops of this enzyme molecule that can be suitably randomized for the library preparation. An emphasis was placed on finding suitable loops on one outer face of the molecule such that additional loops could also be used for randomization from that same face of the molecule. The notion was that multiple target-recognition sites on one face could act in coordination for a higher affinity binding and greater specificity. The present communication deals with the randomization of four new loops of P99 β -lactamase with the linear (X_7) and cysteine-constrained (CX_7C) heptapeptides and the loop-1 with the cysteine-constrained heptapeptides using phage-display technology. Of these nine libraries, the library which showed an acceptable degree of randomization in the β -lactamase-active clones was selected against a protein target.

MATERIALS AND METHODS

Selection of P99 β -lactamase regions for insertion of the randomized loop libraries

The regions of the P99 β -lactamase molecule amenable to substitution without a significant effect on the catalytic activity were identified by inspecting its secondary protein structure (PDB code 1BLS) (Lobkovsky *et al.*, 1994). The selected regions for this purpose appeared to be on the surface and not involved in the defined secondary structure or catalytic activity. It is generally assumed that the sites for such replacement loops are quite restricted based on the overall fold of the protein, making 3D models of the protein useful in constructing libraries. We selected the five exposed loops of P99 β -lactamase on one face of the protein molecule for replacements with the randomized loops for constructing the phage displayed libraries (Figure 1).

Phagemid vector

The phagemid vector that we used in our earlier study (Shukla *et al.*, 2007) was modified to create different loop libraries. Our earlier vector expresses a truncated pIII representing only the CT domain of the protein. In the first step, the vector was digested with restriction enzymes PstI and BamHI and the stuffer was replaced by a nucleotide sequence that would complete the geneIII for an expression of full pIII protein containing functional N1, N2, and CT domains.

A cassette-based approach was applied in constructing different loop libraries. The P99 β -lactamase gene (Lobkovsky *et al.*, 1994) was altered to accommodate the restriction sites flanking the loop-insertion positions for convenient addition, removal, and modification of different loop-inserts. The mutations were introduced at the selected locations in the gene for creating single cutter restriction sites for cloning the randomized loop insertions and the signal peptides. The silent mutations were used wherever possible for creating these restriction sites; however, at positions 42, 286, and 343 mutations resulted into the amino acid substitutions. The modified P99 β -lactamase gene with required HindIII and PstI compatible ends was synthesized (DNA 2, Menlo Park, California) and following purification it was ligated to our vector. The relevant portions of the P99 β -lactamase molecule showing native amino acids that were selected for a replacement with the randomized heptapeptide loops, and amino acid substitutions resulted from restriction site insertions are presented in Figure 2. The FLAG (DYKDDDDK) and 6 $\times$ His tags separated by a small linker (GAA) were also cloned at the C-terminal end of the β -lactamase molecule. The addition of these tags was helpful in the purification and immunodetection of the β -lactamase molecules. An amber stop (tag) was introduced between the 6 $\times$ His tag and protease cleavable linker encoding sequences, for free β -lactamase-FLAG-6 $\times$ His protein expression in a non-suppressor host. The presence of chloramphenicol acetyl transferase (CAT) and P99 β -lactamase genes provided a resistance to the vector-transformed bacterial host against chloramphenicol and cefotaxime, respectively. The presence of a protease-cleavable linker (PAGLSEGSTIEGRGAHE) at the N-terminal end of pIII has been helpful in post-panning phage particle elution with trypsin. Figure 3 shows schematic presentation of phagemid vector design and features in detail.

DsbA signal sequence cloning

The N-terminal presence of a signal peptide helps in the periplasmic translocation of desired fusion proteins during filamentous phage assembly. It is believed that the Sec-system is responsible for the translocation of most proteins to periplasm and therefore probably responsible for the display of proteins on the phage. However, in a recent study, the DsbA signal peptide, a cotranslational signal recognition particles (SRP)-dependent signal sequence, was reported to dramatically improve the phage display levels of their protein of interest when used in place of the Sec-translocation pathway (Steiner *et al.*, 2006). In the present study, we compared the efficiencies of the pIII (MKKLLFAIPLVVPFYSHS) and DsbA

(MKKIWLALAGLVLAFSAS) signal sequences for a periplasmic translocation of the β -lactamase-pIII fusion protein. The vectors containing the DsbA or pIII signal sequences were cloned as follows. The phagemid vector was digested with HindIII and AgeI (New England Biolabs, Ipswich, Massachusetts) and the annealed oligonucleotide sequences with the compatible ends were ligated with T4 DNA ligase (New England Biolabs). The oligonucleotides used for the DsbA signal sequence were 5'-P-agcttaagaaggagatatacatat-gaaaaagattggctggcgctggctggttagtttagcgttttagcgcatcgcgaca3' (forward) and 5'-P-ccggtgtc gccgatgcgctaaacgctaaactaaaccagc-cagcgccagccaaatctttcatatgtatatctcc ttctta3' (complement). The oligonucleotides used for pIII signal sequence were 5'-P-agcttaagaaggagatatacatatgaaaaagtactattcgcaattccactagtcgttcc-vtttctattctcactctcgaca3' (forward) and 5'-P-ccggtgtcgcagagtga-gaatagaaaggaacgactagtggaattgcgaatagtaactttttcatatgtatatctctt-tctta3' (complement). The ligated DNA was electroporated into the Electro-competent TG1 *E. coli* cells (Stratagene, La Jolla, California) and plated on the agar plates containing chloramphenicol (10 μ g/ml) and cefotaxime (0.1 μ g/ml). The colonies were randomly picked and their vector DNA sequences determined. The colonies with correct pIII and DsbA signal sequences were amplified for phage production using KM13 helper phage super infection (Kristensen and Winter, 1998).

Western blot analysis comparing fusion protein translocation by pIII and DsbA signal peptides

The phage proteins ($\sim 1 \times 10^{10}$ cfu/well, protein normalized) from the vectors expressing the pIII or DsbA signal peptide at the N-terminal end of the β -lactamase-pIII fusion protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The molecular weight markers were also included in the run. The proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, California). After blocking the membrane with 5% defatted milk powder, immunodetection of the pIII fusion protein was done using 2000 times diluted mouse anti-pIII antibody (MoBiTec, Göttingen, Germany) or mouse anti-FLAG[®] M2 antibody (Sigma Chemical Co., St. Louis, Missouri). The anti-mouse IgG antibody-HRP conjugate (Santa Cruz Biotechnology, Santa Cruz, California) was used as a secondary antibody. The luminescence was developed with ECL reagent (GE Health Care, Little Chalfont, UK) before the film (Hyperfilm[™] ECL) exposure. The densitometric analyses of the bands were done using Bio-Rad Quantity One 1D analysis software (Hercules, California). The membranes were also stained with MemCode[™] (Thermo Fisher Scientific, Rockford, Illinois), a reversible protein stain kit for PVDF membranes, for comparing the gel loading and membrane-transfer of the phage proteins.

Randomization of selected P99 β -lactamase loops for the construction different libraries

Nine randomized β -lactamase libraries were constructed. Four P99 β -lactamase loops, namely the loop-2, loop-3, loop-4, and loop-5, were randomized with both the linear (X₇) and cysteine-constrained (CX₇C) heptapeptides. The loop-1 of the P99 β -lactamase molecule was randomized with the CX₇C only, as successful randomization of this loop with the linear octapeptides has been reported earlier (Shukla *et al.*, 2007). The vector expressing the pIII signal peptide was purified using Qiagen plasmid purification kit (Valencia, California). Purified plasmids were digested with selected restriction enzymes and the small stuffers were separated from the cut vector by gel electrophoresis. The bands of the vector arms were cut and purified using Qiagen gel extracting column kit (Valencia, California). The selected sites in the different loops were randomized using the nnk-scheme (n = a, t, c, or g; k = g or t) of cloning. The half-site cloning method (Cwirla *et al.*, 1990) was employed in the construction of the P99 β -lactamase loop libraries. In this strategy, a direct strand oligonucleotide is complemented with two oligonucleotides at the 5' and 3' ends leaving a gap of the random region (nnk nnk nnk nnk nnk nnk nnk; nnk₇) to be filled with the complementary nucleotides (nnm; m = c or a) following a bacterial transformation. The forward oligonucleotide sequences,

their right and left complement oligonucleotides, and the restriction sites of cloning for different loop libraries are presented in Table 1. Three oligonucleotides, forward and both complementary half sequences, for each cloning were annealed at equal concentrations (1:1:1) in the annealing buffer (10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, pH 7.5) by heating at 95°C in a heating block for 5 min. The tubes were allowed to cool down slowly in the block. The required oligonucleotides were synthesized and gel-purified by Bio synthesis (Lewisville, Texas). The restriction enzymes used in the cloning were obtained from New England Biolabs.

In order to avoid clones without heptapeptide inserts, the loop randomization was achieved in two steps. In the first step, the oligonucleotides containing stop-codons (taa and tga), in place of the random region (nnk)₇, were cloned. These oligonucleotides for a given loop were exactly the same as given in Table 1, except that the forward oligonucleotide had taa tga taa tga taa tga taa sequence in place of (nnk)₇. In the second step, the vector containing stop-codons in the P99 β -lactamase gene was restriction-digested, gel-purified, and cloned with the oligonucleotide with a random region (nnk)₇ for insertion of the linear heptapeptides (X₇) in a selected P99 β -lactamase loop. For the cysteine-constrained (CX₇C) heptapeptide loop inserts, the oligonucleotides were the same except that the random region was flanked by cysteine codons on the both sides [tgt(nnk)₇tgc]. The ligation of the vector to annealed oligonucleotides was done in a 1:5 ratio using T4 DNA ligase enzyme (New England Biolabs). After column purification (Qiagen), the ligated DNA was used for transformation of the electroporation-competent TG1 *E. coli* cells ($\geq 1 \times 10^{10}$ transformations/ μ g) (Stratagene) using *E. coli* Pulser (Bio-Rad) with 0.1-cm gap electroporation cuvettes at 1800 V according to the supplier's instructions. For each loop library, 25–30 transformations were performed. The transformed bacteria were plated on LB/agar containing 10 μ g/ml chloramphenicol and/or 0.1 μ g/ml cefotaxime (Sigma Chemical Company) in 245 $\times$ 245 $\times$ 18 mm³ bio-dishes (BD Biosciences, Acton, Massachusetts). The numbers of total primary and the β -lactamase-active primary clones were estimated by counting the colonies that appeared on chloramphenicol and cefotaxime +chloramphenicol plates, respectively. Phage libraries of the β -lactamase active clones were produced by the super-infection with KM13 helper phage (Kristensen and Winter, 1998).

Panning of phage-displayed β -lactamase loop library

The loop-2 linear library of the β -lactamase-active molecules, which showed considerable diversity, was panned against a purified protein target for the selection of binders. Streptavidin was used as a test target due to its availability in various forms, including 96-well coated plates that were assumed to facilitate a large-scale post-panning clone screening, if needed. The phage library was subtracted for the plastic and SuperBlock (Thermo Fisher Scientific) binders and then panned on the streptavidin that was immobilized in MaxiSorpTM polystyrene Immuno plate wells (Nulge Nunc, Roskilde, Denmark). The wells of a 96-well microplate were coated with streptavidin by overnight incubation of 100 μ l streptavidin solution (10 μ g/ml PBS; phosphate-buffered saline) in cold. The wells were washed three times with PBST (PBS containing 0.05% Tween-20) and blocked with SuperBlock for 1 h at room temperature. The blocked wells were incubated with 100 μ l subtracted phage sample ($\sim 1 \times 10^{12}$ cfu) for 2 h at room temperature. The wells were washed eight times (5 min each), six times with PBST, and two times with PBS. The DNA of the bound phages was amplified by the rolling circle amplification using TempliPhi Amplification kit (GE Health Care), as described earlier (Shukla *et al.*, 2007). Briefly, the bound phages were eluted in 50 μ l sample buffer by heating the samples at 95°C for 5 min. The samples were then mixed with 50 μ l reaction buffer and 2.5 μ l ϕ 29 DNA polymerase and incubated overnight at 30°C. DNA polymerase was inactivated by heating the samples at 65°C for 10 min. A portion of the amplified phage DNA was digested with BstAPI restriction enzyme (New England Biolabs), according to supplier's protocol. Following the heat-inactivation at 80°C for 20 min, the circular phage DNA was recovered by the ligation

reaction using T4 DNA ligase (New England Biolabs). The non-suppressor electro-competent TOP10 *E. coli* cells (Invitrogen, Carlsbad, California) were transformed with the ligated DNA by electroporation using *E. coli* Pulser (Bio-Rad) with 0.1-cm gap electroporation cuvettes at 1800 V according to the supplier's instructions. The transformed bacteria were plated on the agar plates containing cefotaxime (0.1 µg/ml) and chloramphenicol (10 µg/ml). The transformed bacterial host recognizes the amber stop at the 3' end of the 6×His-tag sequence, thus generating free β -lactamase-FLAG-6×His protein suitable for purification and further analysis.

Lysate preparation

The randomly selected individual isolated colonies of transformed Top10 bacteria were amplified in 2×YT medium with shaking at 260 rpm for 30 h at 30°C. The cultures were centrifuged in cold at 3000× g for 10 min. The bacterial pellets were frozen at -80°C with BPER-II (Invitrogen) and the bacterial protease inhibitor cocktail (Sigma Chemical Company). The lysates were prepared by incubating the thawed pellets at room temperature for 90 min with slow shaking. The lysed bacterial preparations were centrifuged at 15 000× g in cold for 20 min. The supernatants were used for the β -lactamase purification, binding screening, and protein and enzyme activity assays.

β -Lactamase enzyme activity assay

The enzyme activity of β -lactamase was assayed by the colorimetric or fluorometric procedures using nitrocefin (Oxoid, Basingstoke, UK) and soluble fluorocillin green (Invitrogen), respectively, as substrates. For the colorimetric procedure, 20 µl of the enzyme samples in a 96-well microplate were mixed with 100 µl of nitrocefin solution (100 µg/ml) that was made in PBS containing 0.125% n-octyl- β -D-glucopyranoside (Sigma Chemical Company), a non-ionic surfactant (Shukla *et al.*, 2007). The optical density was measured at 490 nm. For the fluorometric procedure, 100 µl fluorocillin green (10 µg/ml) solution in PBS was added to the microplate wells containing β -lactamase. The fluorescence was measured at the wavelengths of 495 nm (excitation) and 525 nm (emission). The initial experiments were run with different amounts of the β -lactamase enzyme with both substrates for determining the linear range of the two assays. The colorimetric and fluorometric readings were taken at 2-min intervals for 10 min using Synergy-HT microplate reader (Biotek Instruments, Winooski, Vermont).

ELISA of β -lactamase

The presence of the 6×His and FLAG tags at the C-terminal end of the β -lactamase was utilized for measuring the enzyme protein in the lysates. Different dilutions of 100 µl β -lactamase clone lysates were incubated in the 96-well Ni-NTA microplates (HisSorb™; Qiagen) overnight in cold. The next day, the wells were washed four times with PBST and blocked with casein (Thermo Fisher Scientific) for 1 h at room temperature. The wells were washed two times with PBS and incubated with the 20 000 times-diluted FLAG antibody-HRP conjugate (Sigma Chemical Company) for 1 h at room temperature. After washing the wells four times with PBST and two times with PBS, the horse-radish peroxidase activity was measured using 1:1 mixture of the stable peroxidase and luminol/enhancer from Super-Signal® ELISA kit (Thermo Fisher Scientific). The luminescence was read using GloRunner™ microplate luminometer (Turner BioSystems, Sunnyvale, CA). The estimation of the β -lactamase protein helped us to normalize the β -lactamase levels of different clones independent of their enzyme activity.

Screening of selected clones for their target-specific binding

Streptavidin and bovine serum albumin (BSA) were immobilized in the MaxiSorp™ polystyrene Immunoplate wells (Nulge Nunc, Roskilde, Denmark) by incubating 100 µl protein solutions (10 µg/ml in PBS) overnight at 4°C. The next day, the wells were washed three times

with PBST and blocked with SuperBlock (Thermo Fisher Scientific) for 1 h at room temperature. The wells were washed two times with PBS and incubated with 100 μ l of selected β -lactamase clone samples. These samples were normalized for their β -lactamase levels by dilution in PBS solution containing bacterial protease inhibitor cocktail (Sigma Chemical Company). Following 2 h of incubation with slow shaking at room temperature, the wells were washed four times with PBST and once with PBS. The activity of the bound β -lactamase was measured by using the fluorocillin green substrate as described earlier.

DNA sequence analysis and bioinformatics

The DNA of the vectors after each cloning and that of randomized inserts in the selected clones were sequenced. The bacterial pellet from 4-ml cultures of each selected clone was processed for the plasmid purification using QIAprep[®] miniprep columns (Valencia, California), according to the supplier's instructions. The cycle sequencing reactions were performed using BigDye[®] Terminator version 3.1 kit (Applied Biosystems, Foster City, California) using appropriate primers. Vermont Cancer Center DNA Analysis Facility at the University of Vermont carried out the automated DNA sequencing using ABI Prism[®] 3130xl Genetic Analyzer. Sixty β -lactamase-active clones, each from loop-2 linear and loop-5 linear primary libraries (unamplified and unselected), were sequenced for their random oligonucleotide-inserts. The frequency and diversity of amino acid distributions in the random region of the loop-2 linear library of β -lactamase-active clones were determined by using Receptor Ligands Contacts (RELIC) program (Mandava *et al.*, 2004; Rodi *et al.*, 2004). All the 22 streptavidin-selected β -lactamase clones from the loop-2 linear library were also sequenced for their DNA inserts and the translated amino acid sequences were searched for significant motifs using the IBM Sequence Pattern Discovery Tool software program based on TEIRESIAS algorithm (Rigoutsos and Floratos, 1998).

RESULTS

Vector DNA sequences, cloned proteins, and β -lactamase activity

The vector DNA sequences showed no unintended alterations. Western blotting experiments conducted using appropriate antibodies confirmed the expression of the cloned proteins and tags during each step of the cloning process. The synthetic P99 β -lactamase in fusion with the FLAG and 6 $\times$ His tags showed $27 \pm 3\%$ loss in the enzyme activity as compared to the wild-type β -lactamase.

Comparison of P99 β -lactamase translocation by pIII and DsbA signal peptide sequences

The phage preparations from both pIII and DsbA signal peptide groups showed a high β -lactamase enzyme activity without any significant difference in nitrocefin cleavage. The proteins of both phage preparations were separated by SDS-PAGE. The gel loading and membrane-transfer of the proteins were evaluated by membrane staining of the proteins. The comparable intensities of pVIII and other visible protein bands in pIII-phage and DsbAss-phage lanes indicate equal gel loading and membrane-transfer (Figure 4B). The immuno-blotting of the membrane with anti-pIII antibody detected two bands in each lane (Figure 4A). The lower immuno-reactive bands at ~43 kDa represent pIII protein and the upper bands at ~87 kDa represent P99 β -lactamase-pIII fusion protein with linkers and tags. Based on the densitometric analyses of the immuno-reactive bands, the percentage of pIII that has P99 β -lactamase as fusion protein was calculated (Figure 4C). The data showed significantly higher (23%, $p \leq 0.05$) levels of P99 β -lactamase-pIII fusion protein in the phage generated by the vector using pIII signal peptide for fusion protein translocation in comparison to levels in the phage produced by the vector using DsbA signal peptide. Based on these data, the calculated number of the pIII molecules that presented P99 β -lactamase fusion protein was ~1.43 (out of 5) in the phage generated from the pIIIss-vector. The phage from the DsbAss-vector had the fusion

protein presentation in ~ 1.17 pIII protein molecules/phage. The results from the anti-FLAG antibody, which detected the C-terminal FLAG-tag of the fully matured β -lactamase only, also confirmed the higher fusion protein in the phage generated from the pIIIss-vector (data not presented). We used the pIII signal peptide for the P99 β -lactamase translocation in our loop libraries, as it was found to be a little more efficient than DsbA signal peptide.

Total primary and β -lactamase-active primary clones in the randomized-loop libraries

Table 2 shows the numbers of total primary clones and the β -lactamase-active primary clones in the different loop libraries. The colonies of the transformed bacteria that grew on the chloramphenicol plates were counted as total primary clones; however, those on the plates containing both chloramphenicol and cefotaxime were considered to be the β -lactamase-active clones. The total number of the primary clones that represented the efficiency of cloning for a given library was in the range of 1.13×10^8 – 2.30×10^8 among different loop libraries. However, the numbers of the β -lactamase-active primary clones in the different libraries showed greater variation, ranging between 6.62×10^2 and 5.41×10^6 . The DNA sequence analyses of 60 β -lactamase-active primary clones each from loop-2 and loop-5 linear libraries showed that all of them were unique, indicating no major problem related to the occurrence of multiple clones of identical sequences in the primary libraries. In general, the numbers of β -lactamase-active primary clones in the linear loop libraries were higher in comparison to their corresponding cysteine-constrained loop libraries. While the loop-2 library exhibited the highest numbers of the β -lactamase-active primary clones in both linear and cysteine-constrained categories, the loop-4 libraries presented the lowest numbers of the catalytically active clones. The loop-2 linear library that presented the highest number of enzyme-active primary β -lactamase clones was further studied for its suitability for target specific selections.

Overall and positional representation of amino acids in randomized loop-2 linear library

The analyses of frequencies of amino acid occurrence in the random region of the loop-2 linear library of the β -lactamase-active clones showed that the observed frequencies of alanine, cysteine, aspartic acid, glutamine, histidine, isoleucine, leucine, methionine, asparagine, serine, threonine, and tyrosine were close ($\pm 30\%$) to their expected frequencies. However, a little overrepresentation of proline (+69%), glycine (+52%), and valine (+56%), and underrepresentation of phenylalanine (–50%), lysine (–54%), glutamine (–46%), arginine (–34%), and tryptophan (–39%) were observed. The expected frequencies were calculated based on the number of codon(s) available for a given amino acid under the nnk-scheme of cloning. The diversity of different amino acids at each position (1 through 7) of the loop-2 linear library of the β -lactamase-active clones was analyzed. Diversity is a statistical measure of the proportion of the 20 possible amino acids that are observed at a given position. If a given position in all analyzed sequences is populated in equal proportions by all 20 amino acids, it was scored 1.0 (20/20). The positions 2, 3, and 4 showed very high diversity (>0.9), followed by positions 5, 6 (~ 0.7), 7 (~ 0.6), and 1 (~ 0.5). These results showed a fair representation of all the amino acids in the random region.

Binding studies of the target-selected clones from the loop-2 linear library

The data presented in Figure 5 show the binding results of 96 randomly selected clones from the panning of the randomized (linear) loop-2 β -lactamase library on streptavidin. The clone samples normalized for their β -lactamase levels were studied for their comparative binding to streptavidin and BSA. The samples from the unselected loop-2 linear library, representing the pool of library clones, and those from non-randomized β -lactamase were also run in parallel as negative controls. Of the 96 screened clones, 22 were found to show significantly higher binding to streptavidin in comparison to BSA. The negative control β -lactamase clones did not

have higher binding to streptavidin. Furthermore, the streptavidin-binding clones did not show significant binding to casein and well-plastic (data not presented).

Identification of target-binding clones, their occurrence frequency and motif-sharing

The loop-2 insert DNA segments of streptavidin-binding clones were sequenced. The translated amino acid sequences of the insert loop-2 and their frequency of occurrences among positive clones are presented in Table 3. These β -lactamase clones are represented by their insert heptapeptides in the loop-2. Two clones appeared multiple times. The frequency of occurrence of β -lactamase clone that expresses heptapeptide GSCFYAP in the loop-2 was 10. Another clone expressing heptapeptide GLWGEPA appeared two times. A total of 12 streptavidin-binding β -lactamase clones were unique. The enzyme assays of lysates from streptavidin-binding clones revealed lower activities of 4 β -lactamase clones (GSCFYAP, GLWGEPA, QGRSGIA, SPRLALS), as compared to the eight other binding clones or to an unselected pool of the library clones. However, when the enzyme activity data were normalized with their β -lactamase protein contents, there was no significant difference in the specific activities among these clones. It showed that the lower enzyme activities in the lysate of these four clones were the result of lower levels of β -lactamase protein in their lysates, indicating a lower expression/solubility of these β -lactamase clones.

The insert heptapeptides from positive clones were aligned to determine if they share any consensus motif. Table 4 presents some of the motifs these heptapeptides share. Three insert-peptides in β -lactamase clone numbers 1260, 1248 (or 1252), and 1251 share the LXXXP motif. The other 3 clones (1103, 1260, and 1238) share the VXP motif. Some other motifs for example G(I/L)VXP, VLR, (K/R)VXP, GLXXXP, LXX(D/E)P, LXX(M/L)P, LX(V/M)XP, and C(V/L)P, which are shared by two insert-peptides, were also identified. The clone with GSCFYAP peptide sequence, which appeared 10 times, did not share a motif with any of the insert-peptides from the streptavidin-binding clones.

DISCUSSION

A variety of proteins of different sizes, structures, and functionalities have been successfully expressed on filamentous phage. However, there are also reports showing very poor phage-display of numerous proteins (Wilson and Finlay, 1998). An incompatible signal peptide directing an inefficient periplasmic translocation of an expressed protein has been reported to be one of important factors that could possibly lead to poor phage-display of a protein (Rodi *et al.*, 2002). Filamentous phage biology requires proteins to translocate across the cytoplasmic membrane into periplasm for their incorporation into the phage particles. Steiner *et al.* (2006) reported that exchanging the post-translational Sec-dependent pathway, which is utilized for the translocation of a majority of secreted *E. coli* proteins and phage pIII (Rapoza and Webster, 1993), with cotranslational SRP pathway improved the phage display of their protein of interest dramatically. They found an approximately 700-fold increase in the phage presentation of their protein using the DsbA signal peptide of SRP pathway in comparison to the signal peptides of Sec-pathway. In the present study, we compared the efficiencies of the DsbA and pIII signal peptides on the phage presentation of the β -lactamase and found no advantage of using this SRP pathway signal peptide over pIII. The DsbA signal peptide of SRP pathway has been shown to produce superior phage display particularly of fast-folding proteins (Steiner *et al.*, 2006). Our results indicate that the P99 β -lactamase may not be a fast-folding protein and the pIII signal peptide of Sec-pathway translocates this protein with a little better efficiency than the DsbA signal peptide. We therefore employed the pIII signal peptide-mediated P99 β -lactamase translocation in our libraries.

Nine loop libraries were prepared by inserting the random linear or cysteine-constrained heptapeptides in the five different loops of the P99 β -lactamase molecule. The selected loops

were on one face of the outer surface of the protein molecule. The random heptapeptide-inserts replaced native amino acids between positions 34–37, 238–246, 275–280, 305–311, or 329–334 of the P99 β -lactamase molecules for generating the loop-1, loop-2, loop-3, loop-4, or loop-5 library, respectively. The diversity of the each loop library was judged by counting the primary and the β -lactamase-active clones. The numbers of the primary clones (1.13×10^8 – 2.30×10^8) of these libraries show a considerable diversity, indicating the cloning and transformation success. The diversity of the clones that retained enzyme activity is an important factor that determines the amenability of a particular loop for randomization and subsequent utility as a bifunctional molecule. Of the loop locations evaluated, the linear peptide inserts in the loop-2 library showed the maximum number of β -lactamase-active clones with a proportion of 1 enzyme-active clone in the ~90 primary clones. The second largest numbers of the enzyme-active clones were in the linear loop-5 and loop-3 libraries, presenting one active clone each in ~2100 and ~4500 primary clones, respectively. The loop-4 linear library presented the poorest ratio of the β -lactamase-active clones among the linear insert libraries, with one active clone in ~19 000 primary clones. The amenability of a loop for randomization without losing the enzyme activity may depend on several factors, including the position of that loop in the enzyme structure and the number of amino acid residues being replaced. We observed the highest numbers of the β -lactamase-active clones in the loop-2 linear library, where the native loop-length was not changed. In the present study, insertion of the randomized linear heptapeptides increased the native loop-lengths by five residues in the loop-1 library, by three residues in the loop-3 and –5 libraries, and by two residues in the loop-4 library. In the cysteine-constrained libraries, the loop-lengths were further increased by two cysteine residues flanking the heptapeptides. All of these substitutions resulted in decreased numbers of the β -lactamase-active clones. However, the loop-length changes cannot be generalized as the determining factor, because in our earlier study (Shukla *et al.*, 2007) we observed a high diversity of β -lactamase-active clones in the loop-1 library in which only two amino acids were replaced by the linear octapeptides, thus increasing the loop length by six residues. It is interesting to note that the loop-4 libraries, which were constructed by replacing the amino acids closest to the enzyme active site ($K^{315}T^{316}S^{318}$) (Lobkovsky *et al.*, 1994), presented the poorest diversity of the β -lactamase-active clones. Although the 3D modeling of P99 β -lactamase structure suggested that loop-4 is away from the active site, it is possible that increasing the loop-length from 5 residues (wild-type) to 7 (libraries) might have brought this loop close enough to adversely affect the active site. We observed that the insertion of the cysteine-constrained random peptides in any of the five selected loops produced fewer numbers of the β -lactamase-active clones as compared to the respective loop libraries having the linear inserts. The ratio of the β -lactamase-active clones to the primary (total) clones was the highest in loop-2 (1 in ~6200) and the lowest in loop-4 (1 in $\sim 2.1 \times 10^5$) of the cysteine-constrained libraries. These results are contrary to our speculation that the cysteine-constrain might help in controlling an undesired interaction of the insert loops with other amino acids in the molecule. It is possible that the presence of a disulfide bond in the constrained loops somehow reduces the flexibility of the β -lactamase molecules in maintaining the desirable structure for its catalytic activity. The wild-type P99 β -lactamase molecule does not contain cysteine in its structure (Lobkovsky *et al.*, 1994).

The quality analysis of the loop-2 linear library showed a good overall representation of amino acid residues in its randomized insert. It is interesting to note that the cysteine representation, which is reported to be very poor in some other filamentous phage libraries (Rodi *et al.*, 2002), was very good and close to the expected levels in this library. The amino acid diversity at each of seven random positions was also good; however, a lower diversity at position 1 indicated the preference for certain amino acids at this position. The analyses of the amino acid diversity and distributions at different insert positions showed an acceptable quality of the linear loop-2 β -lactamase library that is comparable to several other reported libraries of other phage-display systems (Rodi *et al.*, 2002; Shukla *et al.*, 2007). In addition, the presence of the

higher number of enzyme-active clones in the loop-2 linear library indicates a lesser interference with the proper folding of the enzyme molecules in this library. Therefore, the loop-2 linear library of the P99 β -molecules was chosen for further work, using streptavidin protein as a test target. The selection of the loop-2 linear library was done on streptavidin protein using single-round panning coupled with the β -lactamase activity-based screening. This strategy offered several advantages over the conventional panning and the phage immunodetection-based screening procedures (Shukla *et al.*, 2007). The successful single-round panning, involving a phage recovery from the amplified DNA, has been reported by others also (Tanaka *et al.*, 2002; Naik *et al.*, 2004). We identified 22 clones (out of 96; ~23%) that were found to bind streptavidin specifically. The DNA analysis showed multiple clones with identical insert amino acid sequences among binding clones, indicating a successful panning strategy. The alignments of the streptavidin-binding clones revealed several significant motifs. Of particular interest were LXXXP and VXP motifs, each of which is shared by the three peptides. The search of literature on the streptavidin-binding peptide motifs did not reveal any match, which is not surprising as these motifs are not from small free or phage-bound peptides but are a part of the larger enzyme-active P99 β -lactamase scaffold.

A variety of non-immunoglobulin scaffolds with diverse origins and characteristics have been used for constructing polypeptide libraries (Skerra, 2000; Hosse *et al.*, 2006). However, the use of an enzyme scaffold for generating the library of catalytically active molecules suitable for panning has been limited to P99 and TEM-1 β -lactamases (Legendre *et al.*, 1999; Shukla *et al.*, 2007). TEM-1 is a class-A β -lactamase, which apart from being smaller in size (263 vs. 361 amino acids) has only 30% similarity with the P99 β -lactamase. The X-ray structures of TEM-1 β -lactamase have shown significant differences in the arrangement of secondary structure elements when compared to P99 β -lactamase (Oefner *et al.*, 1990; Lobkovsky *et al.*, 1993; Lobkovsky *et al.*, 1994). TEM-1 β -lactamase efficiently cleaves the penicillins and some cephalosporins; however, extended-spectrum cephalosporins are poor substrates for this enzyme (Cantu *et al.*, 1997). P99 β -lactamase has been reported to show versatility and very high activity towards broad- and extended-spectrum cephalosporins and their prodrug conjugates, some of which have been used in preclinical ADEPT studies (Meyer *et al.*, 1993; Vrudhula *et al.*, 1993; Kerr *et al.*, 1995; Svensson *et al.*, 1995; Vrudhula *et al.*, 1995). The development of catalytically active P99 β -lactamase molecules that have a built-in target-recognizing site should be a very effective alternative to the antibody- β -lactamase conjugates in the enzyme prodrug therapy. The smaller size of these molecules should allow them to have faster clearance from blood and normal tissue and increase penetration to a solid tumor, in comparison to big antibody-enzyme conjugates. β -Lactamase is a small single domain enzyme that tend to be very stable and easy to purify (Cartwright and Waley, 1984). β -Lactamase is highly specific to β -lactam, and being a non-mammalian (bacterial) enzyme with no human homologue, it is subject to minimal interference from inhibitors, enzyme substrates, or endogenous enzyme systems. Furthermore, P99 β -lactamase has shown high *in vivo* stability in preclinical ADEPT studies (Meyer *et al.*, 1993; Svensson *et al.*, 1995; Siemers *et al.*, 1997). Lactam-based drugs have been used for many years to treat bacterial infections and they exhibit low toxicities. Cephalosporin prodrugs of mechanistically diverse anticancer agents such as doxorubicin (Svensson *et al.*, 1995), taxol (Vrudhula *et al.*, 2003), platinum complexes (Hanessian and Wang, 1993), phenylenediamine mustard (Kerr *et al.*, 1995), and vinblastine (Meyer *et al.*, 1993) have been successfully used with β -lactamase in the experimental studies. The hydrolytic cleavage of the β -lactam ring by the β -lactamase enzyme causes a secondary reaction that triggers the release of the anticancer drug at the C-3' position of the cephalosporin nucleus. Although all these properties make β -lactamase an ideal candidate for the enzyme prodrug therapy, the bacterial proteins are likely to be immunogenic in humans. However, efforts to identify the P99 β -lactamase-specific CD4⁺ T-cell epitopes have been successful and their removal in a variant has been reported to be significantly low immunogenic following repeated administration in mice (Harding *et al.*, 2005).

The analyses of nine different loop-insert libraries of the P99 β -lactamase molecules in the present investigation have identified the loop-2 linear library (X₇) with acceptable diversity and selectivity against the target. Our previous study identified a loop-1 linear library (X₈) that met similar criteria (Shukla *et al.*, 2007). It is also observed that insertion of the cysteine-constrained peptides in any of the five chosen loops dramatically reduced the number of the catalytically active clones. Therefore, our future endeavors will be focused on examining the linear loop P99 β -lactamase libraries. It will also be interesting to find out if the randomization without changing the normal length of loop-3, -4, or -5 improves the numbers of enzyme-active clones in these libraries. The loops of enzyme-active clones from these libraries, even with limited diversity, can be combined with the loop-1 and/or loop-2 linear libraries. It is expected that the simultaneous presence of randomized loops on one outer face of the combined P99 β -lactamase library should help in selecting the molecules with antibody-like affinity.

In conclusion, this study identified the suitability of the loop-2 of P99 β -lactamase for constructing the phage-display library of the β -lactamase enzyme-active molecules that can be selected against a target. This is an enabling step in our long-term goal of developing bifunctional β -lactamase molecules against cancer-specific targets for the enzyme prodrug therapy of cancer.

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Figure 1. Randomized loops of β -lactamase. (A) Schematic representation of different loops (solid black) of the *Enterobacter cloacae* P99 cephalosporinase molecule, based on the published X-ray crystallographic structure (Lobkovsky *et al.*, 1994), that were selected for randomization. (B) The Molscript drawing of surface structure of the *E. cloacae* P99 cephalosporinase molecule showing that all the selected loops (black) are on one face of the outer surface (based on PDB, 1BLS).

Figure 2. Relevant portions of synthetic *E. cloacae* P99 cephalosporinase molecule showing native amino acid sequences that were replaced by random linear or cysteine-constrained heptapeptide inserts in different loops and also the mutations that resulted from the creation of cloning sites. The amino acid sequence of wild-type β -lactamase is based on the Protein Data Bank entry (1BLS). The amino acids which were replaced for construction of different loop libraries are highlighted and written in white. The mutated amino acids in synthetic β -lactamase molecules are highlighted and written in black. Amino acids are presented as single-letter codes.

Figure 3. Schematic presentation of phagemid vector design and features. The vector has the modified *E. cloacae* P99 cephalosporinase (BLA) gene that allows cloning of random peptide sequences in the different loops of the BLA molecule. Five random sites presented in the BLA gene correspond to the insertion sites of (nnk)₇ or tgt(nnk)₇tgc for the linear or the cysteine-constrained heptapeptides, respectively. The vector sequences were substituted at 356–3361 bp for loop-1, 3968–3988 bp for loop-2, 4079–4090 bp for loop-3, 4169–4183 bp for loop-4, or 4241–4252 bp for loop-5 library. CAT = Chloramphenicol acetyltransferase.

Figure 4.

Western blot analysis of phage proteins using anti-pIII antibody. (A) Immunodetection of SDS-PAGE-separated phage proteins ($\sim 1 \times 10^{10}$ phage/well, proteins normalized). Lane 1 represents the phage from vector using the pIII signal sequence (pIIIss) for the pIII- β -lactamase fusion translocation. Lane 2 represents phage from vector using the DsbA signal sequence (DsbAss) for the pIII- β -lactamase fusion translocation. (B) Protein staining of the membrane that was used for immunodetection of pIII (4A above). An equal gel loading and membrane-transfer of the proteins are evidenced by equal intensities of pVIII and other visible bands of phage proteins in the two lanes. (C) The data from densitometric analyses of the bands are plotted as the percentage of the total pIII molecules that present the β -lactamase as fusion protein. Each bar represents mean $\pm$ SE of three analyses. *The significance of difference between the groups was evaluated by Student's *t*-test; $p \leq 0.05$ was considered to be significant. White bar = phage from vector with pIIIss; black bar = phage from vector with DsbAss.

Figure 5.

Screening of β -lactamase clones selected from the loop-2 linear library panning for their binding to streptavidin (target) and BSA (control) proteins. The bars represent the mean $\pm$ SE of the two experiments done in triplicate. The binding was assessed by measuring the residual β -lactamase activity using Fluorocillin Green substrate. *The significance of differences between streptavidin and BSA bindings were evaluated by Student's *t*-test; $p \leq 0.05$ was considered to be significant. Clone numbers represent identity of the selected clones. NR-BLA = the lysate from bacteria transformed with the vector expressing the non-randomized β -lactamase loops; Library = the lysate from the unselected bacterial loop-2 linear library; RFU = relative fluorescence unit.

Table 1

Restriction sites and oligonucleotide-inserts used for randomizing amino acids in different loops of *E. cloacae* P99 cephalosporinase libraries

Libraries	Restriction sites and annealed oligonucleotide-inserts	
Loop-1 (CX ₇ C)	XmaI 5' P-ccgggatggcgggtggcgttattttattgt (nnk) ₇ tgcaaaccgcactattatgca 3' 3' ctaccgccaccggcaataaata 5' 3' tttggcgtgataat-P5'	NsiI
Loop-2 (X ₇)	MfeI 5' P-aattgggtcatggccaacatg (nnk) ₇ gcctcacttaagcagggcatcggttag 3' 3' ccagtagccggttgtag 5' 3' cgagtggaattcgtcccgtagcgc-P5'	BmtI
Loop-3 (X ₇)	AvrII 5' P-ctaggctgggagatgctcaac (nnk) ₇ gcaaacacgggtggtcagagc 3' 3' cgaccctctacgagttg 5' 3' cgtttggtgccaccagc-P5'	SacI
Loop-4 (X ₇)	Kasi 5' P-gcgccgttgctgtggcagaagtgaatccaccggct (nnk) ₇ tcctgggtccataaaaactggg 3' 3' gcaacggacacgctcttcacttaggtggccga 5' 3' aggaccaggtattttgaccagct-P5'	SaliI
Loop-5 (X ₇)	BsiWI 5' P-gtagctggcctttatt (nnk) ₇ atcggtattgtgatgctcggaatacagggtag 3' 3' caccggaaataa 5' 3' tagccataacactacgagcgcttatgtc-P5'	KpnI

The above oligonucleotide-inserts were used for constructing randomized loop-2, -3, -4, and -5 libraries with linear inserts (X₇). The oligonucleotide-inserts for cysteine-constrained (CX₇C) randomized loop libraries were the same except that (nnk)₇ flanked by cysteine codons on both sides "tgt (nnk)₇tgc," as presented above for Loop-1 library. (nnk)₇ = nnk nnk nnk nnk nnk nnk nnk; n = a, t, g or c; k = g or t; X = any amino acid.

Table 2Numbers of total primary and β -lactamase-active primary clones in different randomized-loop libraries

Libraries		No. of total primary clones ^a	No. of BLA-active primary clones ^b
Loop-1	CX ₇ C	$1.18 \pm 0.231 \times 10^8$	$1.34 \pm 0.180 \times 10^4$
Loop-2	X ₇	$2.12 \pm 0.164 \times 10^8$	$2.41 \pm 0.251 \times 10^6$
	CX ₇ C	$1.76 \pm 0.298 \times 10^8$	$2.83 \pm 0.436 \times 10^4$
Loop-3	X ₇	$1.51 \pm 0.277 \times 10^8$	$3.37 \pm 0.406 \times 10^4$
	CX ₇ C	$1.44 \pm 0.324 \times 10^8$	$1.99 \pm 0.338 \times 10^4$
Loop-4	X ₇	$1.13 \pm 0.145 \times 10^8$	$5.91 \pm 0.562 \times 10^3$
	CX ₇ C	$1.38 \pm 0.291 \times 10^8$	$6.62 \pm 0.588 \times 10^2$
Loop-5	X ₇	$2.30 \pm 0.416 \times 10^8$	$1.11 \pm 0.178 \times 10^5$
	CX ₇ C	$1.86 \pm 0.329 \times 10^8$	$5.21 \pm 0.555 \times 10^3$

Clone numbers are mean $\pm$ SE of three plates.BLA = β -lactamase; C = cysteine; X = random amino acid.^a Clones on chloramphenicol plates.^b Clones on chloramphenicol +cefotaxime plates.

Table 3Amino acid sequences of insert heptapeptides in the loop-2 of streptavidin-binding β -lactamase clones

Clone numbers	Insert loop sequence	Occurrence frequency
1120, 1143, 1223, 1226, 1229, 1230, 1235, 1250, 1253, 1266	GSCFYAP	10
1248, 1252	GLWGEPA	2
1096	GNVSGNF	1
1099	SWQRIRS	1
1103	GLRNVLP	1
1131	KPSCVPY	1
1238	LTERVLP	1
1242	QGRSGIA	1
1243	SPRLALS	1
1251	GLTMPPH	1
1255	VETLCLP	1
1260	GILKVDP	1

Amino acids are represented by single-letter codes.

Table 4

Alignment of amino acid sequences of the loop-2 insert region of streptavidin-binding β -lactamase clones for consensus sequence motif determination

Clone numbers	Sequence alignment	Clone numbers	Sequence alignment
1103	G L R N V L P	1260	G I L K V D P
1260	G I L K V D P	1248, 1252	G L W G E P A
1238	L T E R V L P	1251	G L T M P P H
1251	G L T M P P H	1242	Q G R S G I A
1255	V E T L C L P	1243	S P R L A L S
1131	K P S C V P Y		
1243	S P R L A L S	1238	L T E R V L P
1251	G L T M P P H	1243	S P R L A L S

Amino acids sharing consensus motifs are highlighted. Amino acids which are exactly the same are given in white bold letters and those represent conserved substitutions are in black bold letters. Amino acids are represented as single letter codes.