Supporting Online Material for

Design and Evolution of New Catalytic Activity With an Existing Protein Scaffold

Hee-Sung Park, Sung-Hun Nam, Jin Kak Lee, Chang No Yoon, Bengt Mannervik, Stephen J. Benkovic, Hak-Sung Kim*

*To whom correspondence should be addressed. E-mail: hskim76@kaist.ac.kr

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Materials and Methods

Reagents

The restriction enzymes, T4 DNA ligase, thermophilic polymerases, and other PCR reagents were purchased from New England Biolabs (Beverly, MA). The oligonucleotides used were synthesized by Bioneer (Chungwon, Korea). All other molecular biology reagents were purchased from commercial sources and were of analytical grade.

Plasmids

For the periplasmic and cytoplasmic expression of proteins in fusion with the maltose binding protein (MBP), cloning plasmids were constructed from pMALp2x, and expression plasmids were made from pMALc2x. The TEM-1 β-lactamase gene encoded in pMAL vectors was replaced with the aminoglycoside 3'-phosphotransferase (kanamycin-resistance) gene from pACYC177 using PCR with the primer pairs Nmal/Cmal and Nkan/Ckan. This resulted in the plasmids p2KP and p2KC (6.1-kb). Substitution of the antibiotics-resistance gene was verified by sequencing of the entire vector, phenotypic resistance on kanamycin, and sensitivity on ampicillin.

Oligonucleotides

The following ologinucleotides in this work were used: Nmal, 5'-CCCGGTACCGGGCGCGTAAAAGGATCT-3'; Cmal, 5'-CCCGGTACCTCGACTGAGCCTTTCGTT-3'; Nkan, 5'-GCGGGTACCTGATCTGATCCTTCAACT-3'; Ckan, 5'-GCGGGTACCCTCAGCAAAAGTTCGATT-3'; NT1, 5'-CCCGAATTCATGAAGGTAGAGGTGCTG-3'; CT1, 5'-CCCAAGCTTTTAGATGGTGTACTCGTGGCC-3'; N59, 5'-ACCCACCACCACTGGGACTGTGCTGGCGGGAATGAG-3'; C59, 5'-GTCCCAGTGGTGGTGGGT-3'; N134, 5'-CCTGCCGTGTTCACAGGTTGTACCTTGTTTGTGGCTGGC-3'; C134, 5'-ACCTGTGAACACGGCAGG-3'; Nznst, 5'-CTGAACGTCAAGTGCCTGTATACCGGGCCGTGCCACACTACAGACCACATTTGTTACTTCGTG-3'; Cznst, 5'-CAGGCACTTGACGTTCAG-3' ; Nlp1, 5'-GAGGTGCTGCCTGCCCTGNNSNNSGTTNNSGGGTGGGGCNNSGTACCTTCCAACGGGTACCTGGTCATTGATGAT-3'; Clp1, 5'-CAGGGCAGGCAGCACCTC-3'; Nlp2, 5'-GAGGCTGCCATTGTGGATACTCCATTTACGGATNNSNNSACTGAAAAGTTAGTGGACGCGGCGAGAAAG-3'; Clp2, 5'-ATCCACAATGGCAGCCTC-3'; Nlp4, 5'-

GGGGGTGACGACCGTATCGAGCTCGCCAAGAAANNSGGGNNSGGGGCCCTGACT CACAAG-3’; Clp4, 5’-GATACGGTCGTCACCCCC-3’ Nlp6-1, 5’-CCTGCCGTGTTCACAGGTTGTTTTATTAAAGCGNNSNNSNNSGGCAATNNSNNSG ACGCAACTGCGGATGAGATGTGT-3’; Clp6, 5’-ACCTGTGAACACGGCAGG-3’; Nlp6-2, 5’-CCTGCCGTGTTCACAGGTTGTACCTTGAAAGCGNNSNNSNNSGGCAATNNSNNSG ACGCAACTGCGGATGAGATGTGT-3’; Nlp6-3, 5’-CCTGCCGTGTTCACAGGTTGTNNSTTGAAANNSNNSNNSGCCNNSNNSTTGGGCA ATNNSNNSGACGCAACTGCGGATGAGATGTGT-3’; 66N, 5’-ACAGTGTCCGCCACCGCCTGGCACTGGGACTGT-3’; 66C, 5’-GGTGGCGGACACTGT-3’; 68N, 5’-TCCGCCACCCACTGGGCCTGGGACTGTGATGGC-3’; 68C, 5’-CCAGTGGGTGGCGGA-3’; 70N, 5’-ACCCACTGGCACTGGGCCTGTGATGGCGGGAAT-3’; 70C, 5’-CCAGTGCCAGTGGGT-3’; 131N, 5’-TATCCAGGCCCGGGAGCCTCTACCGACGATATT-3’; 131C, 5’-TCCCGGGCCTGGATA-3’; 155AN, 5’-CCCGTGTTCCGTGGTGCCTTTATTAAACCGTGC-3’; 155C, 5’-ACCACGGAACACGGG-3’; 194C, 5’-CCCAAGCTTTTAGATGGTGTACTCGGCGCCATAGTAGACTCT-3’; 71N, 5’-CACTGGCACTGGGACCACGATGGCGGGAAT-3’; 71C, 5’-GTCCCAGTGCCAGTG-3’; 155DN, 5’-CCCGTGTTCCGTGGTGACTTTATTAAACCGTGC-3’.

Construction of a library by the SIAFE process

The gene encoding human glyoxalase II (GlyII, 260 amino acids) was subcloned from plasmid pKHGII (1). Template T1 was constructed through deletion of the glutathione binding domain in GlyII by PCR (94 °C, 1 min; 55 °C, 30 sec; 72 °C, 30 sec; 30 cycles) with the primers NT1 and CT1. Catalytic elements were then grafted onto template T1 by overlapping PCR with the following primers, which resulted in T2: for H59C, N59 and C59; for D134C, N134 and C134; for Y157, G159, T164, and D165, NZnst and CZnst. A library was constructed from T2 by incorporating the substrate binding elements including the newly designed four sets of loops. These loops consisted of loop1 (XXVXGWGXVPSNG), loop2 (TPFTDXXTEKL), loop4 (ELAKKXGX), and loop6 (FIKAXXXGNXXDA, TLKAXXXGNXXDA and XLKXXXAXXLGNXXDA). Overlapping PCR was conducted with the following specific oligonucleotides: for loop1, Nlp1 and Clp1; for loop2, Nlp2 and Clp2, for loop4, Nlp4 and Clp4; for loop6, Nlp6-1, Nlp6-2, or Nlp-3 and Clp6.

For efficient incorporation of the designed loops, the following sets of gene fragments containing loop portions were amplified from T2 by using Vent polymerase and specific primer pairs (NT1/Clp1, Nlp1/Clp2, Nlp2/Clp4, Nlp4/Clp6, Nlp6-1/CT1, Nlp6-2/CT1, and Nlp6-3/CT1). The resulting gene fragments were isolated and reassembled to form a full length gene using the primers, NT1 and CT1. At this point, additional random mutation throughout the gene was induced by reducing the fidelity of Taq polymerase during cyclic amplification. Assembly of gene fragments was carried out in a reaction mixture of 100 μl containing the following components: seven gene fragments (~1 pg), 1X Taq polymerase buffer (75 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20, 1.25 mM $MgCl_2$), dNTP (dATP and dGTP, 1.0 mM; dCTP and dTTP, 0.2 mM), 0.1 ~ 1.0 mM $MnCl_2$, 2.5 U of Taq polymerase, and 100 pmol of NT1 and NC1 primers. Thirty five cycles were conducted at 94^{o}C for 30 sec, 50^{o}C for 30 sec and 72^{o}C for 30 sec. To optimize the assembly yield of gene fragments and the frequency of mis-incorporation of random nucleotides into the entire gene, mutation rates were precisely controlled ranging from 1% to 3% by modulating the concentrations of dNTP and $MnCl_2$. The resulting PCR products were used for the construction of a library through the following procedure: digestion of the PCR products by *Eco*RI and *Hind*III, ligation into p2KP cloning vector for periplasmic expression, and transformation into *E. coli* XLI-Blue using electroporation. A library of 1.5 x 10^8 transformants was screened on selective agar plates containing 0.05 mM isopropyl-β-D-thiogalactoside (IPTG), 0.2 mM $ZnCl_2$, 50 μg/ml kanamycin, and 0.2 μg/ml cefotaxime. After incubation at 30^{o}C for 48 ~ 56 h, colonies were picked and transferred onto the same selective plates followed by incubation for 48 h. In order to isolate true positives, plasmids of primary clones were isolated and transferred into fresh *E. coli* XL1-Blue, and the resulting clones were checked for resistance against cefotaxime on the same selective agar plates

DNA shuffling

Shuffling of the pool of genes from each round of directed evolution was carried out using 50 ~ 100 bp fragments, and reassembled by PCR as described (2). The resulting DNA fragments were cloned into p2KP, and introduced into *E. coli* XL1-Blue by using electroporation. A library of 2.5 ~ 7.1 x 10^7 transformants was screened on selective LB agar plates as described above with increasing concentrations of cefotaxime (0.2 ~ 4.5 μg/ml). Colonies grown at a given selection pressure were picked and confirmed by retransformation into a fresh host. A mixture of plasmids from confirmed positive clones was prepared and used for the next round of DNA shuffling. At the fourth round, to increase the diversity of the library, additional mutagenic PCR was conducted with error rates of 1 ~ 3%. The nucleotide sequence of the finally selected evMBL8 was determined (GenBank accession number; DQ307739).

Expression of wild type and evolved enzymes

Wild type IMP-1 (3) and GlyII, and evMBL mutants were amplified by PCR and cloned into a p2KC vector for cytoplasmic expression. *E. coli* XL1-Blue cells containing recombinant plasmids were grown at 37^{o}C in LB broth containing 50 μg/ml kanamycin. When the optical density reached 0.6 at 600nm, 0.1 mM IPTG and 0.2 mM $ZnCl_2$ were added to the culture medium followed by further growth at 30^{o}C for 6 ~ 9 h. Cells were harvested, resuspended in lysis buffer (50 mM Hepes buffer pH 7.4, 200 mM NaCl), and subjected to sonication. The lysate was centrifuged at 10,000 X g, and the supernatant was applied to an amylose resin column. Further purification was performed according to the manufacturer's instruction. Protein concentration was determined by the Bradford method.

Analysis of cell growth in liquid media

Cells from overnight cultures were diluted into 5ml LB broth containing 0.05 mM IPTG, 0.2 mM $ZnCl_2$, 50 μg/ml kanamycin, and various concentrations of cefotaxime (0.02 ~ 2.0 μg/ml). Cells were grown at 30^{o}C in the test tube from a starting optical density of 0.04 at 600nm. Optical density was checked every 2 h.

Assay of enzyme activity

β-Lactamase activity was assayed by monitoring the decrease in absorbance at 260nm using a UV/Vis spectrophotometer (4). The reaction mixture contained 1 ~ 2 μM wild type or 1 ~ 2 mM evolved enzymes, 50 mM Hepes (pH 7.4), and 0.02 ~ 2.0 mM cefotaxime in a reaction volume of 1ml. β-Lactamase activity was also measured using high-performance liquid chromatography (HPLC) equipped with a C_{18} analytical column at a flow rate of 1mL/min. The solvent used was 10% acetonitrile/0.01% TFA, and the elution was monitored at 260nm. To determine the kinetic parameters, the initial velocity at each substrate concentration (0.02 ~ 0.2 mM for wild type, and 0.2 ~ 2.0 mM for evolved mutants) was measured, and $(k_{cat})^{app}$ and $(K_m)^{app}$ values were calculated from the reciprocal plot of the Michaelis-Menten equation.

Analysis of metal content

Metal content of purified wild type and evolved enzymes was measured with a Varian inductively coupled plasma spectrometer (ICP) with atomic emission spectroscopy detection as described elsewhere (5)

Molecular modeling of evMBL8

A molecular model of evMBL8 was constructed using CcrA (PDB entry: 1A7T) and GlyII (PDB entry: 1QH5) as templates. These were selected from a profile-based search

using sequence homology. Comparative modeling was performed by using the protein structure modeling program, NanoModel (Nanormics Inc.) (6). The modeling procedure is as follows. The first step was to construct the model of evMBL8 through the replacement of the corresponding residues in sequence structure alignment with the template. Insertion/deletion parts in the alignment were modeled using similar loop fragments from Protein Data Bank. As the next step, the side chains of conserved residues were fixed and those of non-conserved residues were adjusted by using a rotamer library. Finally, the structures were refined by an energy-minimized algorithm. The steepest descents for 300 steps and conjugate gradients for 2000 steps were used, with a distance-dependent dielectric constant (4*r). All of the molecular mechanic calculations were carried out using the Discover 2.98 program (Accelrys Inc.), with an AMBER forcefield, and using nonbonded cut off of 10 Å, running on a Silicon Graphics O2 workstation (Silicon Graphics, Mountain View, California, USA). The coordinate of the model was deposited in the protein data bank (PDB entry: 2F50). Autodock 3.0.3 was used to predict the evMBL8-cefotaxime complex structure as previously described (7).

Supporting Text

Investigation of possible contamination by wild type IMP-1 in the bacterial population carrying evMBL8

To confirm that the observed β-lactamase activity of evMBL8 is genuine, and not due to contamination of IMP-1, the following control experiments were conducted. First, we picked five distinct single colonies expressing evMBL8 and IMP-1, respectively, from freshly transformed plates and determined their resistance against antibiotics (0, 1, 2, and 20 μg/mL cefotaxime, and 50μg/mL ampicillin) simply plating around 10^5 cfu per plate. In case of cells expressing wild-type IMP-1, we observed about 10^5 cfu per plate containing high concentrations of antibiotics (20μg/mL of cefotaxime and 50 μg/mL of ampicillin). However, cells carrying evMBL8 gave rise to the same number of colonies as IMP-1 only at low concentrations of cefotaxime (~10^5 cfu on 1 and 2 μg/mL of cefotaxime), and no colonies were found at high concentration of cefotaxime (20μg/mL) and ampicillin (50 μg/mL). Based on this result, the possibility of contamination by IMP-1 in the bacterial population carrying evMBL8 was ruled out.

Mutagenesis study of evMBL8

We conducted side-directed mutagenesis of the evolved mutant evMBL8 to investigate the role of predicted metal ligands (H66, H68, and H131 for metal; D70, C155,

and H194 for metal 2) in catalytic reaction. All residues were mutated to Ala (one at a time) by using overlapping PCR with following mutagenic oligonucleotide primer sets (66N/66C for H66A, 68N/68C for H68A, 70N/70C for D70A, 131N/131C for H131A, 155AN/155C for C155A, and NT1/194C for H194A). When the resulting mutants were checked for their activities, it was revealed that all six individual mutants lost catalytic activities and gave no growth on antibiotic selective plate with cefotaxime concentration as low as 0.2 μg/mL. We also carried out two additional mutations (C71H and C155D) in an attempt to restore GlyII-like metal coordination (H59 and D134) in evMBL8 by using two sets of oligonucleotide primers (71N/71C for C71H and 155DN/155C for C155D). The two resultant mutants were found to lose catalytic activities, and did not show antibiotic resistance against cefotaxime. Thus, it is reasonable that evMBL8 has evolved to have β-lactamase activity, retaining the designed metal coordination of IMP-1 essential for catalysis.

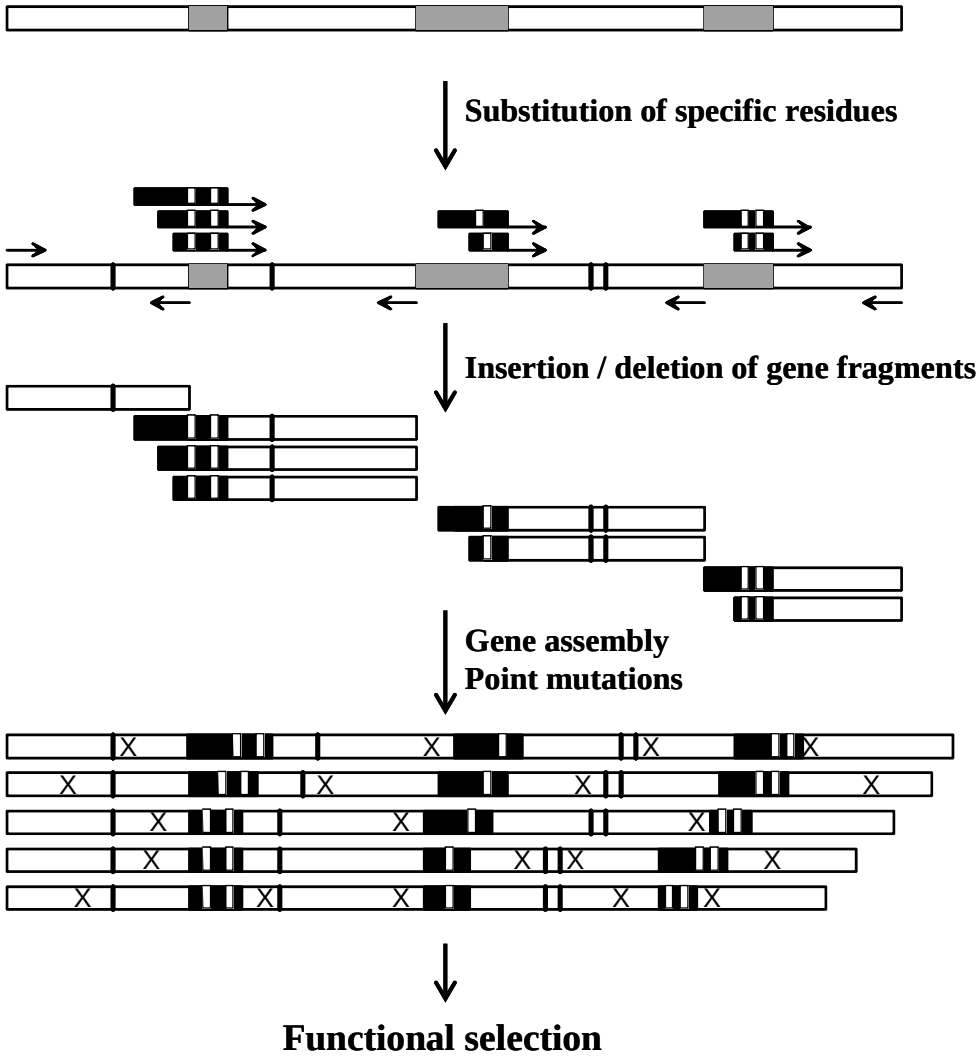


Fig. S1. Schematic diagram of the SIAFE process. The first step involves the substitution of specific residues required for new functions followed by the deletion of redundant or adverse gene segments. The next step is to replace preexisting gene segments of a target gene with new ones by using overlapping PCR. This step uses a mixture of pre-designed oligonucleotides of differing sizes and sequences containing some consensus and some random sequences. The resulting PCR fragments are assembled in a combinatorial way to produce a pool of full-length gene products with diverse gene sizes and sequences. Specific residues to be substituted are shown in vertical black lines, while gene segments to be deleted are shown in gray bars. Specifically designed oligonucleotides for insertion of gene segments are represented in black bars, where the consensus sequences are marked with open vertical lines. Cross signs indicate random point mutations generated by mutagenic PCR.

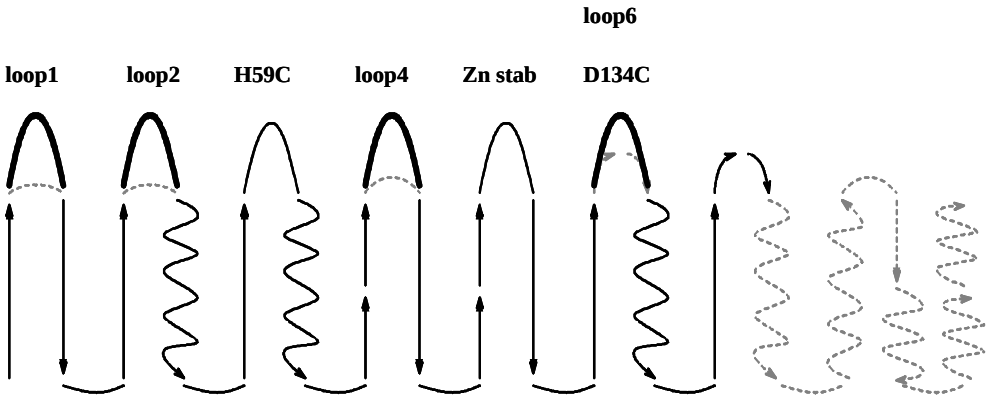
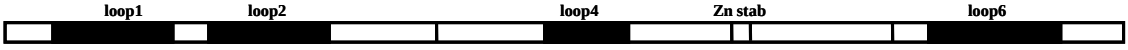


Fig. S2. Topology of template scaffold GlyII showing locations where the designed functional elements are incorporated. Loops and the C-terminal domain to be removed are marked with gray dotted lines, whereas designed loops to be incorporated are shown with thick lines. Zn stab indicates the substitution and insertion of residues (A106Y, insertion of glycine between T107 and P108, S112T, and G113D) which are involved in the stabilization of Zn coordination.



	XXVXGWGXVPSNG	TPFTDXXTEKL	C^{71}	ELAKKXGX	Y-G-TD	C^{155}	FXKAXXXGNXXDA

Protein	Mutation residues						
evMBL1	**ARVLGWGLVPSNG**	TPFTD**GI**TEKL **$Q^{54}A^{57}$**	C^{71} **R^{77}**	ELAKK**EGS**	Y-G-TD	C^{155}	FIKA**CVLGNVLDA**
evMBL1.1	**TTLLGWGAVPSNG**	TPFTDV**E**TEKL **$Q^{59}A^{62}$**	C^{71} **D^{90}**	ELAKK**IGL**	Y-G-TD	C^{155}	FIKA**CVLGNVLDA**
evMBL1.2	**ASVFGWGPVPSNG** **G^{34}**	TPFTDH**A**TEKL **S^{51}** **P^{63}**	C^{71} **K^{75}**	ELAKK**TGI**	Y-G-TD	C^{155}	FIKA**CVLGNVLDA**
evMBL1.3	**SRVAGWGRVPSNG**	TPFTDS**D**TEKL **A^{70}**	C^{71}	ELAKK**YGA**	Y-G-TD **D^{135}**	C^{155}	FIKA**CVLGNVLDA**
evMBL1.4	**GGVNGWGSVPSNG**	TPFTDA**C**TEKL **P^{63}**	C^{71}	ELAKK**LGG**	Y-G-TD **A^{140}**	C^{155}	F**T**KA**CVLGIVLDA**
evMBL1.5 **V^{4}**	**DAVQGWGGVPSNG** **T^{26}**	TPFTDV**I**TEKL **A^{62}**	C^{71} **S^{75}** **E^{89}**	ELAKK**RGT** **$T^{104}F^{109}$**	Y-G-TD	C^{155}	FIKA**CVLGNVLDA**
evMBL1.6	**PPVGGWGCVPSNG**	TPFTDG**R**TEKL **I^{48}**	C^{71} **R^{76}**	ELAKKA**GS** **H^{111}**	Y-G-TD **R^{137}**	C^{155}	FIKA**CVLGNVLDA**
evMBL1.7	**NGVAGWGYVPSNG** **D^{23}**	TPFTDL**P**TEKL **V^{52}** **P^{63}**	C^{71}	ELAKK**IGH**	Y-G-TD **G^{150}**	C^{155}	FIKA**CVLGKVLDV**
evMBL1.8 **K^{2}**	**SPVYGWGPVPSNG**	TPFTDS**I**TEKL **R^{58}** **A^{60}**	C^{71}	ELAKK**GGE**	Y-G-TD **N^{135}**	C^{155}	FIKA**CVLPNVLDP**
evMBL1.9 **V^{3}**	**TLVDGWGPVSSNG**	TPFTDA**E**TEKL **P^{59}**	C^{71}	ELAKK**NGL** **E^{107}** **D^{128}**	Y-G-TD	C^{155}	FIKA**CVLGSVLDA**
evMBL1.10	**DPVTGWGAVPSNG**	TPFTDY**V**TEKL	C^{71} **F^{79}**	ELAKK**SGL** **E^{123}**	Y-G-TD	C^{155}	FIKA**CVLGNVLDA**
evMBL1.11	**KRVVGWGKVPSNG**	TPFTDD**E**TEKL **P^{59}**	C^{71} **R^{86}**	ELAK**DVGI**	Y-G-TD	C^{155}	FIKA**CVLGNVLDA**
evMBL1.12	**RRVVGWGSVPSNE** **A^{36}**	TPS**T**DH**D**TEKL **$Q^{55}R^{58}$** **A^{70}**	C^{71} **G^{90}**	ELAK**EHGS** **$T^{109}P^{115}D^{121}$**	Y-G-TD **A^{151}**	C^{155}	FIKA**CVLGNVLDA**

Fig. S3. Sequences of 13 positive mutants generated by the SIAFE process. Critical functional residues (catalytic and substrate-binding elements) are typically shown. Random mutations introduced by mutagenic PCR are represented with their sequence numbers and marked in bold type.

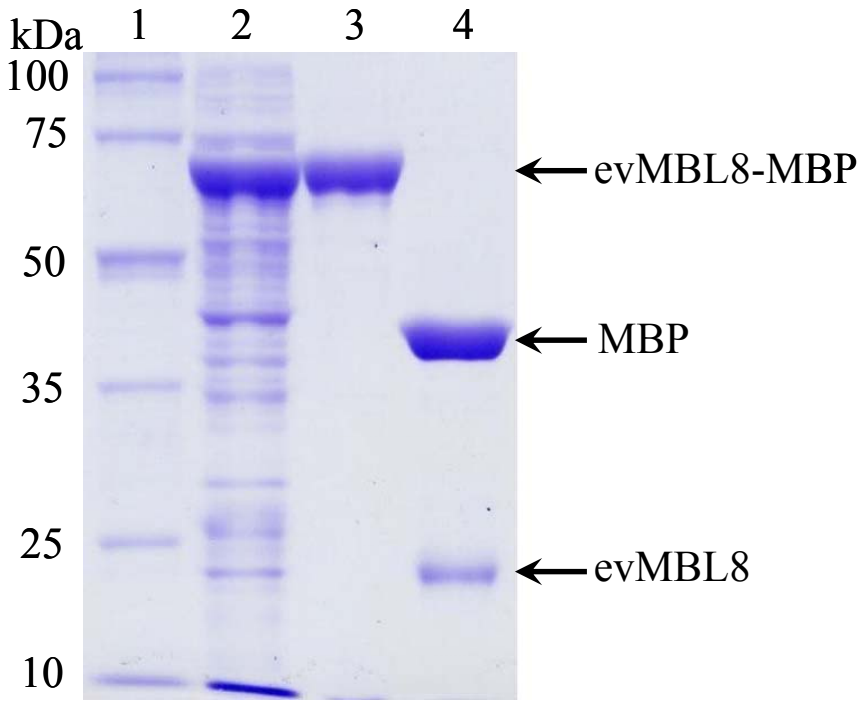


Fig. S4. SDS-PAGE of evMBL8. Lane 1, molecular weight marker; lane 2, crude extract after overexpression, lane 3, evMBL8-MBP after affinity purification; lane 4, evMBL8 and MBP after Factor Xa cleavage.

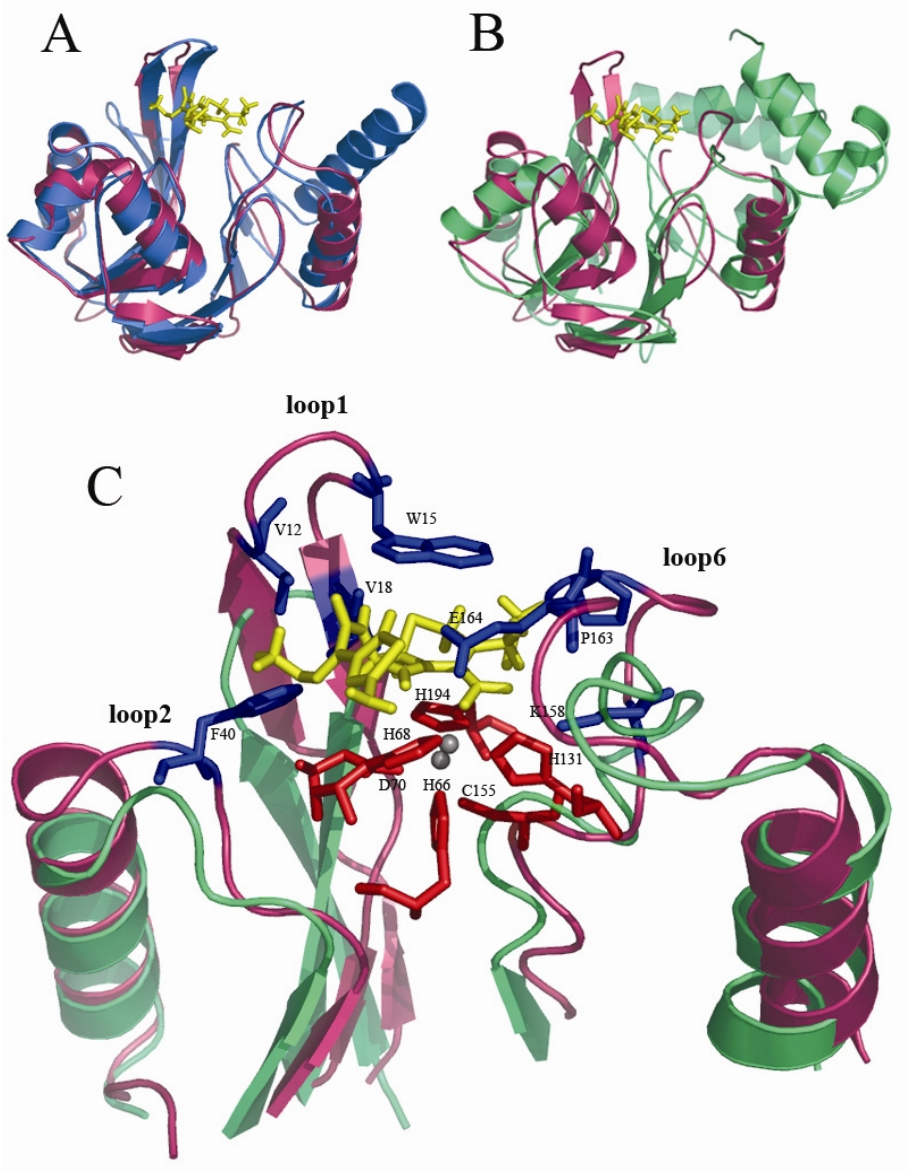


Fig. S5. Molecular modeling of evMBL8. (A) Superposition of the modeled structure of evMBL8 (pink ribbon) with the structure of IMP-1 (blue ribbon). (B) Superposition of the modeled structure of evMBL8 (pink ribbon) with the structure of GlyII (green ribbon). (C) The active site structure of evMBL8 (pink ribbon) aligned with that of GlyII (green ribbon). Residues participating in substrate binding are shown in blue, and ligands for metal coordination are in red. Cefotaxime is shown in yellow.

Table S1. Evolutionary process for generation of β-lactamase activity

	Evolution process							
	SIAFE	shuffling	shuffling	shuffling	shuffling ep-PCR[c]	shuffling	shuffling	shuffling
Cef. (mg/L)[a]	0.2	0.5	0.9	1.3	2.0	2.8	3.6	4.5
Library size	1.5×10^8	7.1×10^7	5.0×10^7	3.5×10^7	2.5×10^7	3.4×10^7	6.5×10^7	7.0×10^7
Positive[b]	312	260	173	236	244	147	129	15

[a] Cefotaxime concentration on selective plates
[b] Number of confirmed positive clones
[c] Additional error-prone PCR was used

Table S2. Relative activities of IMP-1 and evMBL8 for various substrates

Substrate	Relative activity (%)[a]	
	IMP-1	evMBL8
Cefotaxime	100	100
Cephalothin	643	ND[b]
Benzylpenicillin	792	ND
Ampicillin	274	ND

[a] Expressed as percentage of the value of cefotaxime in duplicate experiments.

[b] ND, not detected under specified experimental conditions.

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References