Evolution of Broad Spectrum β-Lactam Resistance in an Engineered Metallo-β-lactamase

Song Sun, Wei Zhang, Bengt Mannervik, and Dan I. Andersson

From the Department of Medical Biochemistry and Microbiology, Uppsala University, SE-75123 Uppsala, Sweden, the Department of Chemistry-BMC, Uppsala University, SE-75123 Uppsala, Sweden, and the Department of Neurochemistry, Stockholm University SE-10961 Stockholm, Sweden

Background: Microbial multidrug resistance is a major global problem.
Results: Evolution of enhanced β-lactamase activity in engineered metalloenzyme mutants was demonstrated with seven different β-lactam antibiotics and cross-resistance to alternative antibiotics was observed.
Conclusion: Resistance against a single antibiotic can develop with collateral resistance to additional drugs.
Significance: Cataloging the substrate specificity of enzyme variants could lead to a more tailored use of antibiotics in clinical settings.

The extensive use and misuse of antibiotics during the last seven decades has led to the evolution and global spread of a variety of resistance mechanisms in bacteria. Of high medical importance are β-lactamases, a group of enzymes inactivating β-lactam antibiotics. Metallo-β-lactamases (MBLs) are particularly problematic because of their ability to act on virtually all classes of β-lactam antibiotics. An engineered MBL (evMBL9) characterized by low level activity with several β-lactam antibiotics was constructed and employed as a parental MBL in an experiment to examine how an enzyme can evolve toward increased activity with a variety of β-lactam antibiotics. We designed and synthesized a mutant library in which the substrate activity profile was varied by randomizing six active site amino acid residues. The library was expressed in Salmonella typhimurium, clones with increased resistance against seven different β-lactam antibiotics (penicillin G, amoxicillin, cephalothin, cefaclor, cefuroxime, cefoperazone, and cefotaxime) were isolated, and the MBL variants were characterized. For the majority of the mutants, bacterial resistance was significantly increased despite marked reductions in both mRNA and protein levels relative to those of parental evMBL9, indicating that the catalytic activities of these mutant MBLs were highly increased. Multivariate analysis showed that the majority of the mutant enzymes were generalists, conferring increased resistance against most of the examined β-lactams.

β-Lactam antibiotics are the clinically most important class of drugs used to treat bacterial infections (1, 2). Resistance to this class of antibiotics has increased at an alarming rate during the last decade, and high level resistance in a number of significant human and animal pathogens is today a major global problem (3–5). β-Lactam resistance can be conferred by a number of different mechanisms (6, 7), but the most common and important mechanism is the production of β-lactamases that cleave the amide bond of the characteristic four-membered β-lactam ring, rendering the drug inactive and harmless to bacteria (8–10). The β-lactamases have been divided into four classes (A–D) based on their sequence homology (11, 12). Class A, C, and D enzymes employ a catalytically active serine to attack the β-lactam bond of different β-lactam antibiotics (7, 13). These enzymes cleave the amide bond of the β-lactam ring, whereas class B metallo-β-lactamases (MBLs) catalyze the identical chemical reaction by using one or two divalent cations (Zn²⁺) coordinated with two water molecules such that one of the oxygens serves as the reactive nucleophile (14–17). MBLs, as exemplified by the New Delhi metallo-β-lactamase 1, are particularly problematic because of their ability to hydrolyze virtually all classes of β-lactam antibiotics (18).

In a protein engineering study (19), β-lactamase activity was successfully introduced into the αβα/βα metallohydrolase scaffold of human glyoxalase II (20), which has a catalytic mechanism fundamentally similar to that of MBLs. The resulting enzyme encoded by the evMBL8 gene completely lost its original glyoxalase II activity and instead was capable of catalyzing the hydrolysis of cefotaxime. In the present study, a modified version of evMBL8 (designated as evMBL9) was demonstrated to exhibit a broad substrate spectrum but conferred only a low level of resistance against the tested β-lactam antibiotics. These characteristics allowed it to be used as a starting point for experimental evolution of an engineered enzyme with a broad substrate spectrum toward increased β-lactam resistance to alternative β-lactam antibiotics. Directed evolution has proved to be a powerful tool in predicting antibiotic resistance and exploring structure-activity relationships during adaptation (21–24). Here, we designed and synthesized a focused DNA sequence library by partially randomizing six amino acid positions in the evMBL9 gene, which were predicted to be impor-

* This work was supported by grants from the Swedish Research Council (to B. M.) and by the Swedish Research Council, Vinnova, Swedish Foundation for Strategic Research, and European Union Project PAR 241476 (to D. I. A.).
† This article contains supplemental Tables S1–S4.
‡ To whom correspondence should be addressed: Dept. of Medical Biochemistry and Microbiology, Box 582, Uppsala University, SE-75123 Uppsala, Sweden. Tel.: 46184714175; E-mail: Dan.Andersson@imbim.uu.se.
tant for the substrate binding of this novel MBL based on multisequence comparisons and structural analysis. Furthermore, two random mutagenesis libraries were constructed in addition to the parental and evolved MBLS to examine contributions of residues other than the six active site residues targeted in the first library. After cloning these three libraries into the Salmonella typhimurium LT2 strain, mutants with increased resistance against β-lactam antibiotics from different groups, representing penicillinase-sensitive penicillins, penicillinase-resistant penicillins, and different generations of cephalosporin, were selected and characterized. Our results suggest that at the initial stage of MBL evolution, (i) the increased resistance is generally associated with reduction of both mRNA and protein levels, and (ii) most evolved MBLS remain “generalist” enzymes that confer increased resistance against several different classes of β-lactams. However, the most important finding was that for every β-lactam antibiotic used, MBL mutants were found that conferred a higher degree of resistance than the parental enzyme. Because some of these mutant enzymes affording enhanced bacterial resistance were expressed at significantly lower protein levels than the parental ones, the intrinsic specific activities of the enzyme were significantly enhanced.

EXPERIMENTAL PROCEDURES

Construction of the S. typhimurium Expression Plasmid pUCBAD-kan:evMBL9—The expression vector used in this study was constructed from pBAD30, which is an expression vector where the expression of the cloned gene is controlled by the arabinose-inducible araBAD promoter (25). A two-step modification of the original pBAD30 resulted in the plasmid pUCBAD-kan where (i) the replication origin of pBAD30 was replaced with the pUC19 replication origin, which made pUCBAD-kan a high copy number plasmid, and (ii) the blaTEM-1 gene in pUCBAD30 was replaced with the aminoglycoside 3′-phosphotransferase (kanamycin-resistance) gene from pKD4 (26). Oligonucleotide primers used for constructing the expression vector pUCBAD-kan are listed in supplemental Table S1.

Based on the DNA sequence of the evMBL8 gene (GenBankTM accession number DQ307739.1), a 78-nucleotide sequence corresponding to the signal peptide sequence (first 25 amino acids) in Pseudomonas aeruginosa IMP-1 metallo-β-lactamase (GenBankTM accession number GU831553.1) was fused with the evMBL8 gene. Five mutations were introduced into the evMBL8 gene: D91Q, G92M, and R93T were predicted to stabilize the protein according to multisequence alignment analysis and further confirmed by CUPSAT using a modeled evMBL8 structure as the template (27), and two additional mutations (P163G and E164N) were predicted to broaden the substrate spectrum based on analysis of the original data derived from the work of Park et al. (19). These modifications in evMBL8 resulted in a new gene thereafter designated as the evMBL9 gene. A 21-nucleotide sequence containing one EcoRI restriction enzyme cutting site and one ribosome-binding site and a 8-nucleotide sequence containing a HindIII restriction enzyme cutting site were prefixed and suffixed to the evMBL9 gene (see Fig. 1). The resulting 697-nucleotide sequence, designated as EcoRI-SD-evMBL9-HindIII, was synthesized by DNA2.0. The EcoRI-SD-evMBL9-HindIII sequence was digested with EcoRI and HindIII and cloned into pUCBAD-kan. The resulting plasmid, designated as pUCBAD-kan:evMBL9, was then isolated and used to transform the S. typhimurium LT2 strain.

Library Construction—To explore the functional space of evMBL9 variants, three libraries were constructed. Library A was designed by partially randomizing six amino acid positions, Thr-34, Val-36, Phe-64, Asn-119, Lys-124, and Ala-185, in evMBL9 by using VNK, DYK, DNT, DTD, NDNT, and NNK (International Union of Biochemistry codes), respectively. The DNA for library A was synthesized by DNA2.0 in the same manner as the fragment EcoRI-SD-evMBL9-HindIII except having six degenerate amino acid positions. ~100 ng of the DNA was PCR-amplified for 10 cycles using Phusion DNA polymerase. The libraries B and C were constructed by performing error prone PCR on 30 ng of template plasmid for 25 cycles. The error prone PCR template was the pUCBAD-evMBL9 plasmid for the library B, and the template plasmid for the library C construction was prepared from a pool of 58 mutants with increased resistance isolated from the library A (supplemental Table S2). To clone the libraries, 400 ng of EcoRI + HindIII-digested PCR product was ligated to 400 ng of EcoRI + HindIII-digested pUCBAD-kan at 16°C overnight using T4 DNA ligase. The ligation mixture was purified with SureClean Kit and dissolved in 10 μl of water. The ligated DNA was then transformed in MegaX DH10B™ T1R Electroprep™ cells by electroporation. The oligonucleotide primers used for cloning the libraries and error prone PCR are listed in supplemental Table S1.

Coverage Calculation for Library A—Assuming that each sequence variant was equally represented in the library, the probability \( P(C(v)) \) of encountering a sequence variant exactly \( X \) times can be expressed by the Poisson distribution \( P(C(v) = X) = (m^X e^{-m})/X ! \), where \( m \) is the expected number of times each variant appears among the clones. In this case, \( m = v / (1.6) \) )/total

Selection and Characterization of Mutants—Approximately \( 1 \times 10^7 \), 4 \times 10^6, and 5 \times 10^6 MegaX DH10B™ T1R transformants, for the libraries A, B, and C, respectively, were propagated for 24 h at 37°C in 20 ml of LB medium with kanamycin. Plasmid DNA was prepared from the overnight culture and used to transform the S. typhimurium LT2 strain by electroporation. From each of the three libraries, ~10^7 cells were plated on five LB agar plates (~2 \times 10^6 cells on each plate) with kanamycin (50 mg/liter), arabinose (2 mM), ZnCl_2 (0.2 mM), and one at a time of the chosen β-lactam antibiotics and incubated at 37°C for 48 h. The β-lactam antibiotics used in this study were penicillin G (penicillinase-sensitive penicillin), ampicillin (penicillinase-resistant penicillin), cephalothin (first-generation cephalosporin), cefaclor (second generation cephalosporin), cefuroxime (second generation cephalosporin), cefoperazone (third generation cephalosporin), and cefotaxime (third generation cephalosporin) (28, 29). The colonies were subsequently streaked onto new plates containing the same concentrations of

Evolution of Multisubstrate β-Lactamase Activity
Evolution of Multisubstrate β-Lactamase Activity

antibiotics as used for selection with or without 2 mM arabinose, and those clones surviving only on the new plates with arabinose were selected for further characterization because the arabinose induction-dependent growth indicated that the resistance to β-lactam antibiotics was conferred by expression of the evMBL9 gene.

Determination of Minimum Inhibitory Concentrations—The minimum inhibitory concentrations (MICs) of the different β-lactam antibiotics for all tested strains in this work were measured using the E-test from AB Biomérieux as described by the manufacturer.

Multivariate Analysis—The MIC values for the alternative antibiotics obtained with the selected MBL mutants were subjected to multivariate analysis by methods previously described (30). The values were normalized to unit variance, and the means were centered. A dendrogram tree shows the Euclidian distances among the mutants in multidimensional “resistance space,” based on hierarchical cluster analysis. The vertical distance measure (in arbitrary units) indicates the increase in variance when clusters are merged. A principal component analysis reveals the directions of variability in multidimensional space. Principal component 1 (PC1) indicates the direction of highest variability, and PC2 is the direction of the next highest variability in a direction perpendicular to PC1. The directions of the principal components are defined by the contributions of the activities with the alternative substrates. A loading plot demonstrates how the different substrates contribute to the distribution of the data points in relation to the PCs. Calculations were made with the Simca-p + 12.0 (Umetrics, Umeå, Sweden) program package.

Isolation of RNA—Total RNA was isolated using the Spin or Vacuum total RNA isolation system (part TM048) from Promega. Bacterial cell lysis was done as follows. A culture was grown overnight in LB broth containing 50 mg/liter kanamycin at 37 °C, and the following day, the culture was diluted 1:50 and grown at 37 °C in LB broth containing 50 mg/liter kanamycin and 2 mM arabinose until the A value at 600 nm was ~0.4–0.5. Two separate pellets were collected by centrifuging 2 × 1 ml of culture at 13,200 rpm. One pellet was stored at −80 °C for protein preparation, and the other was resuspended in 100 µl of freshly prepared Tris-EDTA containing 0.5 g/liter lysozyme. The subsequent steps were according to the manufacturer’s suggestions.

First Strand Synthesis of cDNA from mRNA and Quantitative Real Time PCR—The mRNA was converted to cDNA using the cDNA reverse transcription kit from Applied Biosystems (Foster City, CA) according to the manufacturer’s protocol. The quantitative real time PCR technique based on the high affinity of SYBR Green dye for double-stranded DNA was used. The fluorescence signal was monitored on-line, using the MiniOpticon real time PCR system (Bio-Rad). The PCR amplification was performed by mixing 10 µl of diluted DNA or cDNA with a 15-µl mixture of primers and SYBR Green SuperMix (Bio-Rad). The 16sRNA gene was used as an internal control for all gene copy number determinations.

Western Blot—The cells were prepared as described for RNA isolation. The frozen pellet was thawed on ice and resuspended in 250 µl of lysis buffer (0.02 M HEPES, 0.3 M KCl, 0.05% Triton X-100) supplemented with 1× complete protease inhibitor (Roche Applied Science). The cells were sonicated six times for 10 s, and protein lysates were clarified by centrifugation (15,000 × g for 10 min). Total protein concentration was determined by the Bradford assay (Bio-Rad) using BSA as a standard. Protein lysates (20 µg/lane) were subjected to SDS-PAGE followed by transfer to nitrocellulose membrane (GE Healthcare). After preblotting the membrane with 5% nonfat milk in Tris-buffer saline (pH 7.4) with 0.05% Tween 20, the membrane was probed with rabbit antibodies recognizing evMBL9. Washing with 5% nonfat milk, the membrane was incubated with goat anti-rabbit IgG (H+L)-HRP conjugate (1/3000; Bio-Rad), washed, and detected by ECL. Western blotting detection reagents (GE Healthcare). Relative optical intensities of the mature enzyme variants to that of the mature evMBL9 were calculated to allow comparison of relative protein levels.

RESULTS

Construction of evMBL9 as a Target for Evolution—In a recent study (19), using an approach designated as SIAFE (simultaneous incorporation and adjustment of functional elements), MBL activity was successfully introduced into the protein scaffold of human glyoxalase II (14). The engineered enzyme, evMBL8, obtained by structure-based design accompanied by selection, lost its original glyoxalase II activity but acquired the ability to hydrolyze cefotaxime. In the present investigation, a structural model of evMBL8 was generated with the structures of a human glyoxalase II (Protein Data Bank code 1qh5) and a metallo-β-lactamase (Protein Data Bank code 1a7t) as templates using the program Modeller (31). We further reexamined loop regions of evMBL8 and found Arg-93 in loop 4 pointing toward the interior of the macromolecule. Moreover, after homology analysis of sequences significantly similar to that of evMBL8, notably residues Gln-91, Met-92, and Thr-93, were conserved in naturally occurring proteins having the same genetic background settings in loop 4. By examining the original model published by Park et al. (19), we postulated that mutating Pro-163 and Glu-164 to Gly and Asn, respectively, would render the enzyme promiscuous. Thus, evMBL9 was constructed with these five point mutations, synthesized, and cloned in the pUCBAD-kan plasmid, and transferred into the S. typhimurium LT2 strain.

The full-length evMBL9, which consists of 222 amino acids and is 24 kDa, comprises a 25-amino acid signal peptide and a mature peptide (197 amino acids, 21 kDa) (Fig. 1). Two bands were detected by Western blotting, and the deduced molecular masses were ~21 and 24 kDa, respectively. Therefore, the signal peptide is likely to be cleaved in the secretary pathway leaving a mature protein of 21 kDa, with potential to function as a metallo-β-lactamase. MICs of 12 different β-lactam antibiotics were determined for this strain with or without 2 mM arabinose. The expression of the evMBL9 gene from the pUCBAD-kan plasmid was controlled by an arabinose-inducible araBAD promoter (25). For the evMBL9 wild type enzyme, a small but reproducibly higher MIC (1.5–4-fold) of all tested antibiotics (except for two carbapenems) was observed in the presence of arabinose (Fig. 2), showing that the increased resistance was due to the expression of the evMBL9 wild type enzyme. The broad substrate spectrum and low activity of this wild type
enzyme against different β-lactam antibiotics allowed us to use evMBL9 as the parental MBL in the evolution experiments.

**Mutant Library Design**—Molecular modeling of evMBL8 identified candidate residues participating in substrate binding (19), which was the basis for the library A design in this study (Fig. 3). Four residues Thr-10, Val-12, Trp-15, and Val-18 in loop 1 are part of the binding site. Trp-15 and Val-18 are largely conserved among IMP-1 homologs, so only Thr-10 and Val-12 were randomized. Phe-40 in loop 2, Asn-95 and Lys-100 in loop 4, and Ala-161 in loop 6 were also chosen for mutagenesis, because they are also quite variable in nature. In general, to reduce the selection/screening burden and circumvent practical problems in gene synthesis, reduced amino acid alphabets were used so that the natural diversity in each position mentioned above was largely taken into consideration and incorporated. The benefit of such a strategy has also been shown in previous studies (32, 33). Thus, library A was designed by par-
**Evolution of Multisubstrate β-Lactamase Activity**

**TABLE 1**

Frequency of resistant evMBL9 variants isolated from libraries A and B

| β-Lactam antibiotics | No arabinose | 2 mM arabinose | Weighted frequency | B/A | D/C |
|-----------------------|--------------|----------------|-------------------|-----|-----|
| **Library A**         |              |                |                   |     |     |
| Penicillin G (8)      | $5.7 \times 10^{-5}$ | $8.0 \times 10^{-5}$ | $4.5 \times 10^{-5}$ | 18/24 | 9/12 |
| Penicillin G (12)     | $1 \times 10^{-5}$ | $2.3 \times 10^{-5}$ | $1.0 \times 10^{-5}$ | 27/56 | 18/19 |
| Ampicillin (3)        | $8.5 \times 10^{-6}$ | $2.2 \times 10^{-5}$ | $7.2 \times 10^{-6}$ | 25/32 | 6/14 |
| Ampicillin (4)        | $<5 \times 10^{-7}$ | $3.6 \times 10^{-6}$ | $2.5 \times 10^{-7}$ | 11/32 | 1/5 |
| Cephalothin (4)       | $2.2 \times 10^{-5}$ | $4.1 \times 10^{-5}$ | $5.1 \times 10^{-6}$ | 16/32 | 2/8 |
| Cefalothin (5)        | $7.0 \times 10^{-6}$ | $1.9 \times 10^{-5}$ | $1.9 \times 10^{-6}$ | 23/56 | 4/17 |
| Cefadroxil (2)        | $5.0 \times 10^{-7}$ | $1.1 \times 10^{-6}$ | $3.0 \times 10^{-7}$ | 6/11 | 4/8 |
| Cefadroxil (3)        | $<5 \times 10^{-7}$ | $5.0 \times 10^{-7}$ | $0$ | 0/5 | 0/0 |
| Cefuroxime (8)        | $1.0 \times 10^{-6}$ | $9.5 \times 10^{-6}$ | $2.1 \times 10^{-6}$ | 22/24 | 4/17 |
| Cefuroxime (10)       | $<5 \times 10^{-7}$ | $5.0 \times 10^{-7}$ | $1.0 \times 10^{-7}$ | 4/5 | 1/4 |
| Cefoperazone (0.4)    | $8.0 \times 10^{-6}$ | $8.8 \times 10^{-6}$ | $2.4 \times 10^{-6}$ | 20/56 | 6/8 |
| Cefoperazone (0.5)    | $1.0 \times 10^{-6}$ | $1.0 \times 10^{-6}$ | $0$ | 0/10 | 0/0 |
| Cefotaxime (0.4)      | $5.0 \times 10^{-7}$ | $1.2 \times 10^{-6}$ | $1.2 \times 10^{-7}$ | 9/32 | 4/11 |
| Cefotaxime (0.17)     | $<5 \times 10^{-7}$ | $1.0 \times 10^{-6}$ | $0$ | 0/8 | 0/0 |
| **Library B**         |              |                |                   |     |     |
| Penicillin G (12)     | $1.3 \times 10^{-6}$ | $2.4 \times 10^{-6}$ | $7.5 \times 10^{-7}$ | 20/64 | 9/9 |
| Ampicillin (3)        | $<5 \times 10^{-8}$ | $9.0 \times 10^{-7}$ | $8.4 \times 10^{-8}$ | 15/40 | 2/8 |
| Cephalothin (5)       | $1.9 \times 10^{-5}$ | $7.0 \times 10^{-6}$ | $4.3 \times 10^{-7}$ | 6/54 | 1/2 |
| Cefadroxil (3)        | $<5 \times 10^{-8}$ | $3.0 \times 10^{-7}$ | $0$ | 2/7 | 0/1 |
| Cefuroxime (10)       | $7.5 \times 10^{-7}$ | $1.3 \times 10^{-6}$ | $7.6 \times 10^{-8}$ | 7/40 | 1/3 |
| Cefoperazone (0.4)    | $1.3 \times 10^{-6}$ | $9.5 \times 10^{-7}$ | $1.1 \times 10^{-7}$ | 3/27 | 1/1 |
| Cefotaxime (0.14)     | $<5 \times 10^{-8}$ | $8.5 \times 10^{-7}$ | $0$ | 4/17 | 0/2 |

*The concentrations of β-lactam antibiotics (mg/liter) used for selection are indicated in parentheses.

*Weighted frequency indicates the frequency of mutations that conferred resistance by overexpression of evMBL9 variants.

Positionally randomizing six residues (Thr-34, Val-36, Phe-64, Asn-119, Lys-124, and Ala-185) in evMBL9, which resulted in $1.6 \times 10^7$ sequence variants. The DNA pool was constructed by direct synthesis and cloned into the pUCBAD-kan vector. Library A contained $1 \times 10^7$ transformants, which leads to an estimation of ~46% library coverage, assuming that each sequence variant was equally represented in the library. Library B was constructed by using a random mutagenesis strategy. The coding region for the mature protein (amino acid residues 26–222) was amplified by error prone PCR at a 0.2% mutagenesis frequency. The amplified material was cloned into the pUCBAD-kan vector, and the resulting library contained $4 \times 10^6$ transformants. Interrogation of library B allowed us to examine the potential of improving catalytic activity for all residues in the functional parental enzyme. To combine site-directed mutagenesis and random mutagenesis, library C was constructed by performing error prone PCR on the pooled variants that were isolated from library A showing increased MIC values compared with wild type evMBL9 strain. The mutagenesis rate of the error prone PCR for library C construction was estimated to be 0.2%.

Isolation and Characterization of Mutants—Variants with increased resistance against each of seven β-lactam antibiotics (penicillin G, ampicillin, cephalothin, cefadroxil, cefuroxime, cefoperazone, and cefotaxime) were selected from the libraries A and B on LA plates with a single antibiotic and 2 mM arabinose. To determine whether the expression of the evMBL9 variant was responsible for the increased resistance, resistant colonies were restreaked on new LA plates containing the same concentrations of antibiotics used for selection with or without 2 mM arabinose. Only for clones where growth was dependent on the presence of arabinose, the plasmids were transformed to new cells followed by determination of the MICs against the same antibiotics as those used in the selection (supplemental Table S2). The mutation frequency was calculated as the number of survivors divided by the number of plated cells (Table 1). Two ratios were also determined for each β-lactam antibiotic selection: one was the ratio between the number of arabinose-dependent mutants and the number of tested mutants (B/A in Table 1), and the other was between the number of mutants with evMBL9 variants that conferred resistance in wild type background and the number of tested arabinose-dependent mutants (D/C in Table 1). The frequency of mutations that conferred resistance by overexpression of evMBL9 variants was calculated as the frequency of all types of mutations with arabinose multiplied by the two determined ratios (weighted frequency in Table 1). Based on the frequency of mutations that increased resistance against each of the seven β-lactam antibiotics, it appears to be most easy for evMBL9 to evolve resistance against penicillin G but relatively difficult to increase resistance toward cefadroxil and cefotaxime. Library C, which was constructed based on the pooled evMBL9 variants isolated from library A, was also screened for resistant mutants against seven β-lactam antibiotics. The relative MICs of β-lactam antibiotics for transformants with increased resistance were determined for the three libraries A, B, and C (Fig. 4). Even though relatively few resistant mutants were successfully isolated from library B, a similar pattern was observed for all three libraries: the relative MIC to penicillin G was increased most (up to more than 6- and 10-fold in the libraries A and C, respectively), and the relative MIC to cephalothin, a first generation cephalosporin, was also highly increased (up to more than 4- and 10-fold in libraries A and C, respectively).
formants isolated from libraries A, B, and C. No mutations were found in the promoter region or ribosome-binding site of the evMBL9 variants isolated from libraries A or C, but some appeared in several transformants from library B (supplemental Table S3). The expected and observed amino acid substitutions in the six randomized positions for 33 resistant transformants isolated from library A are summarized in Table 2, and the observed substitutions were ranked at each position according to their frequency of appearance. The statistical significance of the observed frequency of appearance was tested for each of the expected substitutions using the binomial test. For residue 34, amino acids Gly, Asp, and Asn (p = 0.0002, p = 0.002, and p = 0.047, respectively) were over-represented; for residue 36, none of the observed substitutions were either over- or under-represented, and Thr was expected to occur at this position (p = 0.004) but was not observed in the 33 transformants; for residue 64, Phe, as the wild type amino acid at this position, occurred in a frequency (21 of 33) significantly higher than expected (p = 2.8 × 10^{-15}), which indicated that Phe-64 experienced strong purifying selection; for residue 119, amino acids Gly and Asp were over-represented (p = 0.005 and p = 0.005, respectively); for residue 124, none of the expected substitutions were either over- or under-represented; for residue 185, only Stop codon was over-represented (p = 1.7 × 10^{-19}) and occurred at a high frequency (12 of 33) that indicated that evMBL9 tended to remove the C-terminal amino acids following residue 185 under the antibiotic selection. To identify a potential signature of the amino acid substitutions at these six positions in terms of their physicochemical properties, we categorized the 20 amino acids based on their side chain charge (positive, negative, and neutral), side chain polarity (polar and nonpolar), and molecular mass (high, >130; and low, ≤130) and subsequently tested the statistical significance of the observed frequency of appearance for each of the categories using the binomial test. Polar amino acids were under-represented (p = 0.009) at residue 36, which was due to the lack of Thr at this position. Nonpolar amino acids and high molecular mass amino acids were over-represented (p = 0.02) at residue 64 because of the frequent appearance of Phe at this position. Negatively charged amino acids were over-represented (p = 0.005) at residue 119 because of an over-representation of Asp at this position. Negatively charged residues were slightly over-represented (p = 0.04) at residue 185.

All of the amino acid substitutions identified more than once in individual resistant transformants isolated from the three libraries are listed for the positions other than the six randomized ones (Table 3). These substitutions are also likely to contribute to the increased resistance. It is noteworthy that the Stop codon mutation at positions following Ala-185 occurred frequently, which is consistent with the previous observation that Stop codon occurred in high frequency at position 185A. In addition, four lysine to glutamate mutations at residue 132 and two lysine to glutamate mutations at residue 147 were identified in individually isolated mutants indicating that a negative charge at these two positions is favorable. Nevertheless, a defined structure of the evMBL9 protein, which is currently unavailable, is required to elucidate the functionality of these substitutions.

**Multivariate Analysis of Resistance Profile**—Cross-resistance to six alternative β-lactam antibiotics was determined for 52 transformants selected on the basis of increased resistance against each of the particular antibiotics used for selection; 32, 14, and 6 mutants were selected from the libraries A, B, and C, respectively (supplemental Table S4). The MIC values for the seven alternative antibiotics represent the resistance profile for each mutant, which can be regarded as a point in seven-dimensional “drug resistance space.” Multivariate analysis was performed on the cross-resistance data to analyze the Euclidian distances among the points in resistance space, and a dendrogram based on hierarchical clustering demonstrated that the 52 selected mutants could be divided into three major clusters based on the MIC values (Fig. 5A). The multivariate data were further investigated by PC analysis, which is a powerful tool to
Evolution of Multisubstrate β-Lactamase Activity

TABLE 2
The expected and observed amino acid (AA) substitutions in the six randomized positions for the 33 resistant transformants isolated from the library A

| Thr-34 | Val-36 | Phe-64 | Asn-119 | Lys-124 | Ala-185 |
|--------|--------|--------|---------|---------|---------|
| VNK    | DYK    | NDT    | NDT     | NDT     | NNK     |

* The AA substitutions at each of the six positions were ranked according to their frequency of appearance. The number of appearances is indicated in the parentheses for each AA substitution.

TABLE 3
Amino acids substitutions in positions other than the six randomized ones that appeared more than once in the resistant clones isolated from the three libraries

| Amino acid substitutions | Library A | Library B | Library C | Total |
|--------------------------|-----------|-----------|-----------|-------|
| M11                      | 3         | 0         | 1         | 4     |
| T62I                     | 4         | 0         | 1         | 5     |
| G80W                     | 3         | 0         | 0         | 3     |
| R127(M/G)                | 1         | 2         | 0         | 3     |
| K132E                    | 2         | 0         | 2         | 4     |
| K147E                    | 0         | 2         | 0         | 2     |
| E196Stop                 | 0         | 0         | 4         | 4     |
| E205Stop                 | 1         | 1         | 0         | 2     |

identify patterns in the seven dimensions represented by the different β-lactam antibiotics used for selection. The position of a given point in the PC diagram is determined by loadings based on the activities with the alternative substrates. These loadings can be plotted together with the PC scores in a biplot, demonstrating the interdependence of the loadings as well as their correlation with the scores. The two first principal components, PC1 and PC2, accounted for 81% of the variance of the data, indicating that the resistance space can be approximated in two dimensions (Fig. 5B). In the biplot, the points have been colored based on the three groups of the dendrogram. The red cluster represents mutants with generally high MIC values, and it is noteworthy that five of the seven mutants in this cluster derive from library C. In Fig. 5B, the principal component scores are also shown with the loading provided by the different antibiotics in the multivariate analysis. Mutant points close to the loading of a particular β-lactam antibiotic have a strong correlation with that drug. Loadings close to one another indicate that resistances to the corresponding antibiotics are highly correlated. Cefaclor and cefoperazone clearly show the lowest correlation among the antibiotics (Fig. 5B). Finally, the mutants located close to the horizontal PC1 axis lack a strong bias for particular β-lactam antibiotics, indicating that most mutant enzymes selected were generalists in terms of β-lactamase activity.

mRNA and Protein Levels Were Reduced in the Majority of the Evolved evMBL9 Gene Mutants—We measured the relative protein levels for 22 resistant transformants, among which 16, 4, and 2 were randomly selected from libraries A, B, and C, respectively. To examine the correlation between resistance and protein level, the relative protein level was plotted against the relative MIC value corresponding to each of the seven β-lactam antibiotics for the 22 selected resistant transformants (Fig. 6, A–G). The results showed that for most transformants, as the resistance level increased, the protein levels were significantly decreased (up to more than 50-fold) (Fig. 7A). To examine the contribution of reduced mRNA level to the protein reduction, we determined the mRNA levels for the 22 transformants, and for 21 of them the relative mRNA level was reduced up to 200-fold, which indicated that mRNA was destabilized at the initial stage of the MBL evolution (Fig. 7B). To dissect the influence of mutations on the mRNA and protein levels, the relative mRNA level was plotted against the relative protein level for the 22 transformants (Fig. 6H). In this plot, the mRNA and protein level for the wild type copy of evMBL9 (DA16022) is set to 1.0. For 10 of 22 tested transformants, the protein/mRNA ratio was decreased up to 10-fold, and an increased protein/mRNA ratio up to 6-fold was observed for the other 12 transformants. One should note that for six transformants (DA17130, DA17349, DA18365, DA18387, DA19009, and DA19011), the protein/mRNA ratio could be even lower because the determined protein level was below the detection limit of Western blotting, and an estimated relative protein level 2% was assigned to these six transformants. This result suggests that protein stability was decreased by some mutations introduced. Because MIC values were increased for these mutants, this indicates that compared with the wild type
evMBL9 strain, catalytic activity of the evolved enzymes was increased. For certain mutant enzymes, the MIC value/relative protein level ratio was increased up to 400-fold as compared with the parental enzyme, indicating a great improvement in enzyme specific activity (Fig. 8).

**DISCUSSION**

In this study we engineered a novel metallo-\(\beta\)-lactamase (evMBL9) to increase its resistance against each of seven different \(\beta\)-lactam antibiotics. The parental evMBL9 was shown to have modest activity against most of the tested \(\beta\)-lactam antibiotics and therefore was employed as a starting point in the adaptive molecular evolution. To study how the broad spectrum evMBL9 could evolve toward increased resistance, three libraries were designed and constructed. We first explored the functional space of evMBL9 variants by the focused library A targeting six residues that were predicted to be involved in substrate binding. Library B was constructed by using a random mutagenesis approach on evMBL9, whereas library C was constructed by using a similar strategy as for library B, but instead of using the parental evMBL9 gene, a pool of resistant evMBL9 variants isolated from the library A was subjected to random mutagenesis. In this sense library C can be considered to consist of second generation mutants. Multiple \(\beta\)-lactam-resistant clones could be selected from the three different libraries, and multivariate analysis of resistance profiles against the seven \(\beta\)-lactam antibiotics showed that most mutant enzymes were generalists, which indicated that at the early stage of MBL evo-
Evolution of Multisubstrate β-Lactamase Activity

The improvement of the catalytic activity for one specific substrate did not sacrifice activities with other substrates. On the contrary, most of the mutants developed significant collateral activity with other β-lactam substrates and showed broad spectrum properties. This finding is in line with the observation in a directed evolution study carried out with a natural MBL that was evolved toward increased resistance against one cephalosporin without sacrificing a broad substrate profile (34).

For most mutants the evMBL9 mRNA and protein levels were reduced, despite providing significantly increased resis-

![Figure 6](image-url)
tance in bacteria, suggesting that the catalytic activities of these mutants were increased by several orders of magnitude (Fig. 8). However, we were unable to purify the wild type evMBL9 protein, although several attempts were made using different strategies, indicating a high instability of this engineered protein. Directed evolution studies carried out on serine-\(\beta\)-lactamases have shown that resistance mutations compromise the protein stability and are usually followed by compensatory mutations that restore the stability (35–38). Instead of directly measuring the thermal stability of the evMBL9 variants, the protein/mRNA ratios were determined for the resistant transformants to infer the protein stability. Although the wild type evMBL9 is already highly unstable, for 10 of 22 transformants, the protein/mRNA ratios were decreased up to 10-fold, which implies that the improved \(\beta\)-lactamase activity in these novel MBLs was acquired at the cost of decreased protein stability. The fact that the protein/mRNA ratio was increased up to 6-fold in the additional 12 transformants suggests that stabilizing mutations have also occurred in some mutants.

Finally, it is unclear why for 21 of the 22 examined mutants the mRNA level was reduced (in several cases by 1 to 2 orders of magnitude). One possibility for libraries A (16 of 22 mutants) and C (2 of 22 mutants) is that one or several of the six residues (Thr-34, Val-36, Phe-64, Asn-119, Lys-124, and Ala-185) targeted for mutagenesis are located in a region of the mRNA that is particularly important for its stability. However, this explanation cannot account for the effects seen with mutants from library B (4 of 22 mutants), because the library was generated by random mutagenesis, and at present we cannot explain the reduced mRNA levels in these mutants.

It has been argued that evolution of drug resistance can follow only a limited number of trajectories in sequence space (39). However, we have previously demonstrated that the evolution of intrinsically promiscuous enzymes can circumvent unfavorable mutations by enhancing activities with alternative substrates (40). The results of the present investigation illustrate this notion in the case of an MBL with activity against several different \(\beta\)-lactam antibiotics of clinical relevance. From the clinical perspective, it would appear that acquisition of enhanced resistance against one particular antibiotic may be accompanied by collateral resistance against alternative drugs. Such evolution of multidrug resistance could jeopardize chemotherapy. However, by means of multivariate analysis of resistance data obtained with different drugs, it is possible to distinguish antibiotics that can be expected to impart the least

---

**FIGURE 7.** The relative mRNA protein levels of resistant transformants. A, the relative protein fold change was calculated as the protein expression level of the resistant transformants divided by that of the parental strain. (Values marked with asterisks are below the 2% detection limit.) B, the relative mRNA fold change was calculated as the mRNA expression level of the resistant transformants divided by that of the parental strain. The letters in the parenthesis following the strain number indicate which antibiotic was used to select the mutant: PG, penicillin G; AMP, ampicillin; CE, cephalothin; CF, cefaclor; XM, cefuroxime; CPS, cefoperazone; CT, cefotaxime.

**FIGURE 8.** Histogram of MICs normalized to evMBL9 protein expression levels. The normalized MIC value was calculated as the relative MIC value divided by the relative protein level for the resistant transformant. The values are plotted on a logarithmic scale. The letters in parentheses following the strain number indicate which antibiotic was used to select the mutant: PG, penicillin G; AMP, ampicillin; CE, cephalothin; CF, cefaclor; XM, cefuroxime; CPS, cefoperazone; CT, cefotaxime.
Evolution of Multisubstrate β-Lactamase Activity

covariation of resistance. In addition, by cataloging the substrate specificities of resistance enzymes, as outlined in this study, this type of knowledge could be used in the clinic to tailor antibiotic use and thereby more effectively treat infections caused by resistant pathogens.

REFERENCES

1. Walsh, C. (2003) Where will new antibiotics come from? Nat. Rev. Microbiol. 1, 65–70
2. Elander, R. P. (2003) Industrial production of β-lactam antibiotics. Appl. Microbiol. Biotechnol. 61, 385–392
3. Thomson, J. M., and Bonomo, R. A. (2005) The threat of antibiotic resistance in Gram-negative pathogenic bacteria. β-Lactams in peril. Curr. Opin. Microbiol. 8, 518–524
4. Rice, L. B. (2006) Antimicrobial resistance in gram-positive bacteria. Annu. Rev. Med. 57, 111–119; discussion 562–570
5. Rice, L. B. (2012) Mechanisms of resistance and clinical relevance of resistance to β-lactams, glycopeptides, and fluoroquinolones. May Clin. Proc. 87, 198–208
6. Wilke, M. S., Lovering, A. L., and Strynadka, N. C. (2005) β-Lactam antibiotic resistance. A current structural perspective. Curr. Opin. Microbiol. 8, 525–533
7. Fisher, J. F., Meroueh, S. O., and Mobashery, S. (2005) Bacterial resistance to β-lactam antibiotics. Compelling opportunism, compelling opportunity. Chem. Rev. 105, 395–424
8. Medeiros, A. A. (1984) β-Lactamases. Br. Med. Bull. 40, 18–27
9. Livermore, D. M. (1995) β-Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. 8, 557–584
10. Poole, K. (2004) Resistance to β-lactam antibiotics. Cell Mol. Life Sci. 61, 2200–2223
11. Amblé, R. P. (1980) The structure of β-lactamases. Philos. Trans. R. Soc. Lond. B Biol. Sci. 289, 321–331
12. Bush, K., Jacoby, G. A., and Medeiros, A. A. (1995) A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. 39, 1211–1233
13. Helfand, M. S., and Bonomo, R. A. (2003) β-Lactamases. A survey of protein diversity. Curr. Drug Targets Infect. Disord. 3, 9–23
14. Wang, Z., Fast, W., Valentine, A. M., and Benkovic, S. J. (1999) Metallo-β-lactamase. Structure and mechanism. Curr. Opin. Chem. Biol. 3, 614–622
15. Wang, Z., Fast, W., and Benkovic, S. J. (1999) On the mechanism of the metallo-β-lactamase from Bacteroides fragilis. Biochemistry 38, 10013–10023
16. Garau, G., Bebrone, C., Anne, C., Galleni, M., Frère, J. M., and Dideberg, O. (2005) A metallo-β-lactamase enzyme in action. Crystal structures of the monozinc carbapenemase CphA and its complex with biapenem. J. Mol. Biol. 345, 785–795
17. Ullah, I. H., Walsh, T. R., Taylor, I. A., Emery, D. C., Verma, C. S., Gamblin, S. I., and Spencer, J. (1998) The crystal structure of the L1 metallo-β-lactamase from Stenotrophomonas maltophilia at 1.7 A resolution. J. Mol. Biol. 284, 125–136
18. Walsh, T. R., Tolken, M. A., Poirel, L., and Nordmann, P. (2005) Metallo-β-lactamases. The quiet before the storm? Clin. Microbiol. Rev. 18, 306–325
19. Park, H. S., Nam, S. H., Lee, J. K., Yoon, C. N., Mannervik, B., Benkovic, S. J., and Kim, H. S. (2006) Design and evolution of new catalytic activity with an existing protein scaffold. Science 311, 535–538
20. Cameron, A. D., Riddrström, M., Olin, B., and Mannervik, B. (1999) Crystal structure of human glyoxalase II and its complex with a glutathione-cholesterol substrate analogue. Structure 7, 1067–1078
21. Stemmer, W. P. (1994) Rapid evolution of a protein in vitro by DNA shuffling. Nature 370, 389–391
22. Cramer, A., Raillard, S. A., Bermudez, E., and Stemmer, W. P. (1998) DNA shuffling of a family of genes from diverse species accelerates directed evolution. Nature 391, 288–291
23. Vakulenko, S., and Golemi, D. (2002) Mutant TEM β-lactamase producing resistance to ceftazidime, ampicillin, and β-lactamase inhibitors. Antimicrob. Agents Chemother. 46, 646–653
24. Peimbert, M., and Segovia, L. (2003) Evolutionary engineering of a β-Lactamase activity on a β-Ala β-Ala transpeptidase fold. Protein Eng. 16, 27–35
25. Guzman, L. M., Belin, D., Carson, M. J., and Beckwith, J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J. Bacteriol. 177, 4121–4130
26. Datsenko, K. A., and Wanner, B. L. (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc. Natl. Acad. Sci. U.S.A. 97, 6640–6645
27. Parthiban, V., Gromiha, M. M., and Schomburg, D. (2006) CUPSAT. Prediction of protein stability upon point mutations. Nucleic Acids Res. 34, W239–W242
28. Wright, A. J. (1999) The penicillins. Mayo Clin. Proc. 74, 290–307
29. Demain, A. L., and Elander, R. P. (1999) The β-lactam antibiotics. Past, present, and future. Anticancer Res. 19, 351–359
30. Kurtovic, S., and Mannervik, B. (2009) Identification of emerging quinolone-resistant species in directed enzyme evolution. Biochemistry 48, 9330–9339
31. Eswar, N., Webb, B., Marti-Renom, M. A., Madhusudhan, M. S., Eramian, D., Shen, M. Y., Pieper, U., and Sali, A. (2006) Comparative protein structure modeling using Modeller. Curr. Proteo. Bioinformatics, Chapter 5, Unit 5.6, 15:5.6.1–5.6.30
32. Alcolombri, U., Elias, M., and Tawfik, D. S. (2011) Directed evolution of sulfotransferases and paraoxonases by ancestral libraries. J. Mol. Biol. 411, 837–853
33. Jochens, H., and Bornscheuer, U. T. (2010) Natural diversity to guide focused directed evolution. Chembiochem 11, 1861–1866
34. Tomatis, P. E., Rasia, R. M., Segovia, L., and Vila, A. J. (2005) Mimicking natural evolution in metallo-β-lactamases through second-shell ligand mutations. Proc. Natl. Acad. Sci. 102, 13761–13766
35. Wang, X., Minasov, G., and Shoichet, B. K. (2002) Evolution of an antibiotic resistance enzyme constrained by stability and activity trade-offs. J. Mol. Biol. 320, 85–95
36. Beadle, B. M., and Shoichet, B. K. (2002) Structural bases of stability-function tradeoffs in enzymes. J. Mol. Biol. 321, 285–296
37. Sideraki, V., Huang, W., Palzkil, T., and Gilbert, H. F. (2001) A secondary drug resistance mutation of TEM-1 β-lactamase that suppresses misfolding and aggregation. Proc. Natl. Acad. Sci. U.S.A. 98, 283–288
38. Thomas, V. L., McReynolds, A. C., and Shoichet, B. K. (2010) Structural bases for stability-function tradeoffs in antibiotic resistance. J. Mol. Biol. 396, 47–59
39. Weintreich, D. M., Delaney, N. F., Depristo, M. A., and Hartl, D. L. (2006) Darwinian evolution can follow only very few mutational paths to fitter proteins. Science 312, 111–114
40. Zhang, W., Dourado, D. F., Fernandes, P. A., Ramos, M. J., and Mannervik, B. (2012) Multidimensional epistasis and fitness landscapes in enzyme function tradeoffs in enzymes. Nature 500, 55–59