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ACCELERATED PUBLICATION

Identification of β-Lactamase Inhibitory Peptide Using Yeast Two-Hybrid System

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Abstract—Random oligonucleotide fragments were designed and amplified by PCR and fused with the activating domain of pGAD424 to construct a random peptide library. The DNA fragment encoding β-lactamase was fused with the binding domain of pGBT9 (+2). Subsequently, using yeast two-hybrid system we found two positive clones encoding peptides P1 and P2 that have the ability to bind β-lactamase *in vivo*. The genes encoding P1 and P2 were cloned into pGEX-4T-1. GST–peptide fusion proteins were expressed in *Escherichia coli* and isolated by glutathione-Sepharose 4B affinity chromatography. Finally, P1 and P2 were cleaved from the fusion protein with thrombin and purified by ultrafiltration. Inhibition assay of peptides with β-lactamase *in vitro* indicated that only P1 has the ability to inhibit β-lactamase.

Key words: β-lactamase, yeast two-hybrid system, β-lactamase inhibitory peptide, GST–peptide fusion system

Since the introduction of β-lactam antibiotics, bacterial resistance to these agents has become an increasing problem. The production of the enzyme β-lactamase by both Gram-positive and Gram-negative bacteria is the most common mechanism of resistance to β-lactam antibiotics [1]. β-Lactamases hydrolyze the amide bond of the β-lactam antibiotics to create an ineffective antimicrobial agent; the enzymes have been grouped into four classes (A, B, C, and D) based on amino acid sequence homology [2, 3]. The most prevalent plasmid-mediated β-lactamase in Gram-negative bacteria is the TEM-1 β-lactamase from class A [4]. Like other class A β-lactamases, TEM-1 β-lactamase is capable of hydrolyzing both penicillins and cephalosporins [2]. To overcome the drug resistance mediated by TEM-1 β-lactamase, extended-spectrum antibiotics including aztreonam, cefotaxime, and ceftazidime were developed. However, soon after their introduction, selective pressure resulted in the emergence of variant β-lactamases capable of hydrolyzing these antibiotics [5].

An additional strategy that has been employed to combat antimicrobial drug resistance is the use of β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam. Although not capable of antimicrobial activity themselves, these agents are used in conjunction with various β-lactam antibiotics to bind β-lactamase and prevent the hydrolysis of the antibiotics, thereby restoring the therapeutic value to the antimicrobial agent. Unfortunately, β-lactamase variants have been identified that are resistant to these enzyme inhibitors while still retaining the ability to hydrolyze β-lactam antibiotics [6].

Clavulanic acid was initially purified from the soil bacterium *Streptomyces clavuligerus*, which also produces a protein inhibitor of β-lactamase called β-lactamase inhibitor protein (BLIP) [7]. BLIP is a 165 amino acid protein composed of two domains of ~78 residues each and is a protein inhibitor of TEM-1 β-lactamase (K_i = 0.1-0.6 nM) [8-10]. In addition, BLIP inhibits β-lactamases from both Gram-positive and Gram-negative bacteria to varying degrees and also inhibits the cell-wall transpeptidase PBP5 from *Enterococcus faecallis* [8]. The cocrystal structure of TEM-1 β-lactamase and BLIP indicates that residues involved in binding and inhibition of β-lactamase, such as Asp49 and Phe142, lie in different domains [9], and that residues 46 to 51 (AAGDYY) of BLIP make critical interactions with the active site of TEM-1 β-lactamase [11].

These findings provide an important basis for the design and development of new drugs that could inhibit β-lactamase, which is the common target for antibiotic resistance in both Gram-positive and Gram-negative bacteria. In this paper, we report the screening of a library

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of peptide-based potential inhibitors of the TEM-1 β-lactamase by the yeast two-hybrid system. Peptides gained can provide the basis for further development of more potent peptide derivatives against β-lactamase activity.

MATERIALS AND METHODS

Strains, reagents, and plasmid constructs. All strains and plasmids utilized and constructed in this study are described in the table.

The following random oligonucleotide fragments were synthesized by Invitrogen (USA): 5′-AC**GAATTC**-ACTATCCACTGCNNYNNYNNYGCTGCAGGT-GACTACTACNNYNNYNNYNNYNNYNNYG-GCACCTCTTTCTA**GGATCC**GTGC-3′, in which N is A, T, C or G, while Y is C or G. There were *Eco*RI (5′ end) and *Bam*HI (3′ end) sites in the fragment, respectively. The fragments were amplified by PCR with the sense primer (5′-AC**GAATTC**ACTATCCACT-3′) and anti-sense primer (5′-GCAC**GGATCC**TAGAAAGA-3′) synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd (China).

Various restriction endonucleases, T4 DNA ligase, and *Taq* DNA polymerase were purchased from TakaRa Biotechnology Co., Ltd (Japan). Zymolyase-20T was purchased from the Kirin Brewery Co., Ltd (Japan). Sephadex G-75 gel-filtration chromatography columns and glutathione-Sepharose 4B affinity chromatography columns were purchased from Pharmacia (USA).

Construction of bait plasmid and random peptide library. The β-lactamase gene was amplified from pBR322 using the sense primer with a *Bam*HI site (5′-CGGGATCCTTACCAATGCTTAAT-3′) and the anti-sense primer with an *Eco*RI site (5′-CGGAATTCGTAT-GAGTATTCA-3′). The PCR product was fused with the binding domain of pGBT9 (+2) to generate bait plasmid pYG111 (Fig. 1a).

The random oligonucleotide fragments were amplified by PCR. The PCR products were digested by *Eco*RI and *Bam*HI and cloned into the same sites of the plasmid pGAD424 to generate random peptide library (designated pYG202) (Fig. 1b). Fragments encoding random peptides were fused with the activating domain of pAGD424. pYG202 was transformed into *E. coli* DH5α, and to form the random peptide library all transformants were collected.

Strains, plasmids, and recombinant plasmid in this study

Strains/plasmids/constructs	Relevant characteristics	Source
Saccharomyces cerevisiae Y153	*MATa*, *Leu*2-3, 112, *ura*3-52, *trp*1-901, *his*3Δ200, *ade*2-101, *gal*4Δ, *gal*80Δ, *URA*3::*GAL-LacZ*, *LYS*2::*GAL-HIS*3	SIPI
E. coli DH5α	*Sup*E44, *sup*F58, *hsd*S3, *dap*D8, *Lac*Y1, *gln*V44Δ, gal-uvrB47, *tyr*T58, *gyr*A29, *ton*A53Δ, *thy*A57	SIPI
E. coli C600	*Sup*E44, *hsd*R, *thi*-1, *thr*-1, *leu*B6, *lac*Y1, *ton*A21	SIPI
pGBT9(+2)	GAL4 DNA binding domain, Ap^r and tryptophan auxotrophic marker	SIPI
pGAD424	GAL4 DNA activating domain, Ap^r and leucine auxotrophic marker	SIPI
pYG111 bait plasmid (Fig. 1a)	β-lactamase gene fused with GAL4 DNA binding domain, Ap^r and tryptophan auxotrophic marker	this work
pYG202 prey plasmid (Fig. 1b)	Random oligonucleotide fragments fused with GAL4 DNA activating domain, Ap^r and leucine auxotrophic marker	this work
pLY5	PR, PL, clts857, Amp^r, *Eco*RI and *Bam*HI sites	SIPI
pLY5BL	β-lactamase gene was subcloned into pLY5, Ap^r	this work
pGEX-4T-1	*Ptac*, *gst*, *plac*, *lac repressor*, Ap^r	SIPI
pYG205	*Ptac*, *gst-oligo*, *plac*, *lac repressor*, Ap^r	this work

Note: SIPI) Shanghai Institute of Pharmaceutical Industry; Ap^r) ampicillin resistance.

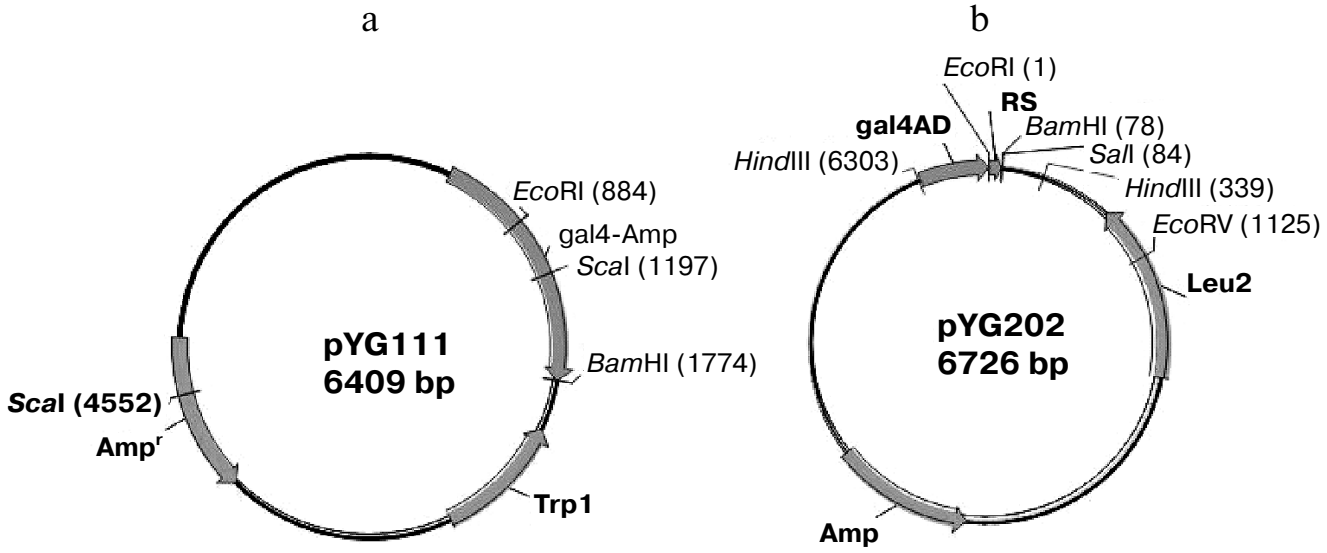

Fig. 1. Physical map of bait plasmid pYG111 (a) and prey plasmid library pYG202 (b).

Screening of yeast two-hybrid system. For the two-hybrid screening, *S. cerevisiae* Y153 (100 ml, A_{600} = 0.68) was transformed with 100 μg of pYG111 and 100 μg of pYG202. Then we made two dilutions of transformation reactions (100 μl of 1 : 100, 1 : 1000) and spread on SD-Leu-Trp-His plates to determine the total number of transformants. To identify the optimal spreading density for His^+ transformants, aliquots of the cell suspension (25, 50, and 100 μl) were spread on SD-Leu-Trp-His contained 25 mM 3-AT (60 plates in total). The plates were incubated at 30°C for six days. The remainder of the transformation solution was kept at 4°C for five days. Once it was established that 50 μl was the optimal spreading density, 50 μl aliquots were spread on 40 SD-Leu-Trp-His plates containing 25 mM 3-AT. These plates were incubated at 30°C for six days.

β-Galactosidase assays. His^+ transformants were screened out by the expression of β-galactosidase using the colony lift assay with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as the substrate or the liquid assay with *o*-nitrophenyl-β-D-galactoside (ONPG) as the substrate (CLONTECH Yeast Protocols Handbook). The liquid assay gives a quantitative measure of β-galactosidase expression in Miller units: Miller units = $1000A_{420}/(VtA_{600})$, where V is the volume of cell suspension, and t is the time for color development (CLONTECH Yeast Protocols Handbook).

Segregation of peptide-containing plasmids from *S. cerevisiae* transformants by shuttling through *E. coli* C600. His^+ $LacZ^+$ transformants were grown in 5 ml of the appropriate media for 1-2 days at 30°C with shaking. The cells were pelleted and resuspended in 200 μl of yeast zymolyase buffer (containing 30 mM DTT) placed for about 15 min at room temperature. The cells were pelleted and resuspended in 500 μl of yeast zymolyase buffer, with 1.5 mg zymolyase T-20 added. Then the cells were placed for 2 h in a 37°C constant temperature water bath and agitated gently. Protoplasts were prepared through enzyme lysing of the yeast cell wall. Plasmid DNA was extracted by alkaline lysis, then the plasmid DNA was transformed to competent cell of *E. coli* C600 (CLONTECH Yeast Protocols Handbook). *E. coli* transformants were selected on M_9 + Pro + Trp-Leu plates containing 100 μg/ml ampicillin. Plasmid was prepared from ampicillin resistant transformants using the high pure plasmid isolation kit from TakaRa.

Peptide purification. Peptides were purified using the protein fusion and purification system according to the manufacture's instructions. *Eco*RI-*Bam*HI fragments containing the peptide genes were obtained by PCR in pYG202 and subcloned into the same sites in pGEX-4T-1, then expressed as a fusion protein with the glutathione-S-transferase protein (GST) in *E. coli* DH5α. The fusion protein was purified on a glutathione-Sepharose 4B affinity chromatography column by eluting with 10 mM glutathione, and the peptide was subsequently released from GST by cleavage with thrombin. The peptides were separated from GST by filtration through Microcon Ultracel-YM 5000 membrane (Millipore).

Expression and purification of β-lactamase. The β-lactamase gene was subcloned into plasmid pLY5 to construct plasmid pLY5BL. The β-lactamase was expressed in the form of inclusion bodies. The inclusion bodies were washed, dissolved, and refolded. Finally, β-lactamase was purified to ~90% homogeneity with Sephadex G-75 gel-filtration chromatography (data not shown).

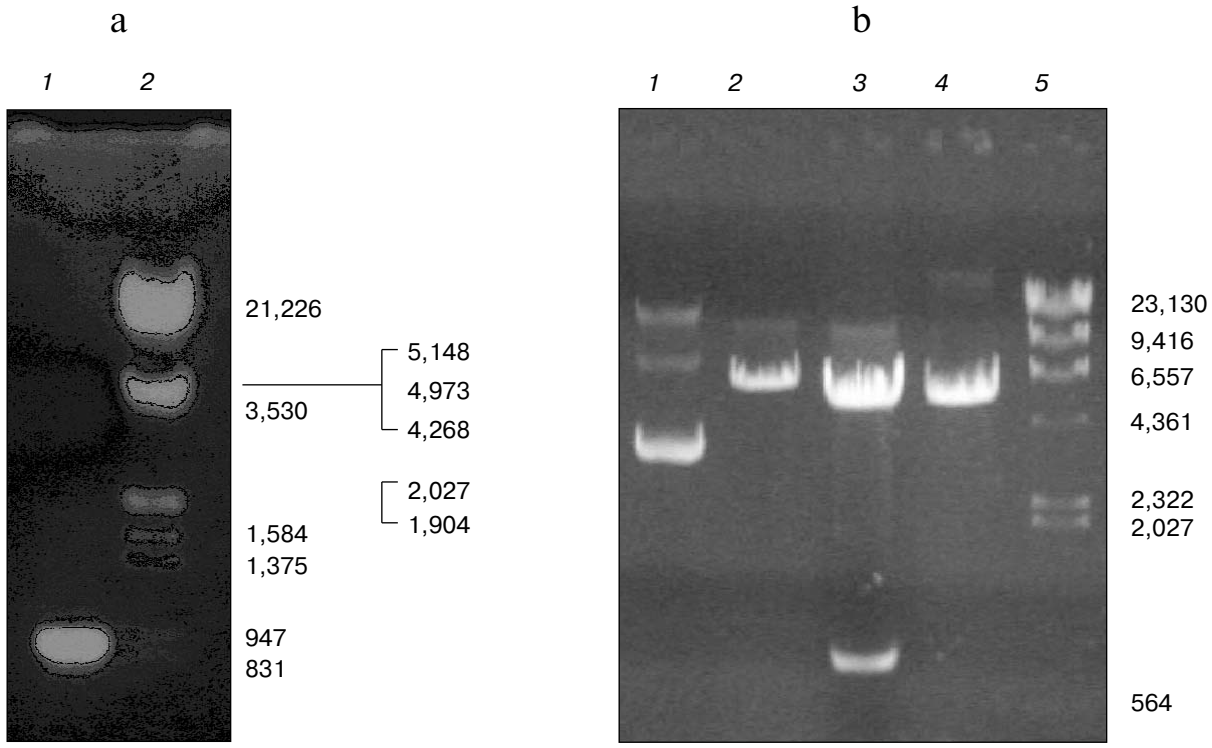

Fig. 2. a) Amplification of β-lactamase gene using PCR: *1*) PCR product; *2*) λ DNA/*Hin*dIII + *Eco*RI marker. b) Electrophoresis analysis of pYG111: *1*) pYG111 (no digest); *2*) pYG111/*Eco*RI; *3*) pYG111/*Eco*RI + *Bam*HI; *4*) pGBT9(+2)/*Eco*RI + *Bam*HI; *5*) λ DNA/*Hin*dIII + *Eco*RI marker.

***In vitro* inhibition assay.** Inhibition assays were performed as described previously [9]. Briefly, various concentrations of peptides or control peptide (insulin) were incubated with TEM-1 β-lactamase for 2 h at 25°C to establish equilibrium. Enzyme assays were performed in 0.05 M phosphate buffer (pH 7.0) containing 1 mg/ml BSA. After incubation, 100 μl of ampicillin solution (10 mg/ml) was added, and hydrolysis of the substrate was monitored at 235 nm. The final volume of the reaction was 3 ml.

RESULTS

Construction of bait plasmid and prey plasmid library. The TEM-1 β-lactamase gene was amplified by PCR with primers containing *Eco*RI and *Bam*HI sites, respectively. The size of PCR products (Fig. 2a) and sequencing result of the gene both matches the expectation very well. Then the TEM-1 β-lactamase gene was fused with the binding domain of pGBT9 (+2) to generate bait plasmid, pYG111, the structure of which was tested by digestion with restriction endonucleases *Eco*RI and *Bam*HI (Fig. 2b).

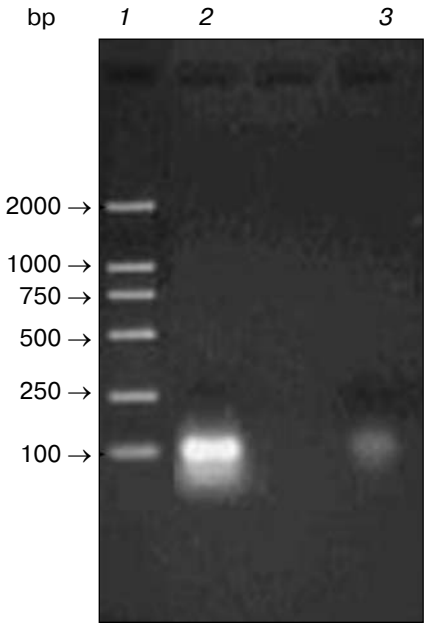

Fig. 3. Amplification of single oligonucleotide chain by PCR: *1*) marker; *2*) amplified oligonucleotides; *3*) negative control (without templates).

Random oligonucleotides were PCR amplified. PCR products were analyzed by agarose gel electrophoresis (Fig. 3). Then PCR products were subcloned into pGAD424 to construct prey plasmid library, designated pYG202. Finally, *E. coli* DH5α was transformed with pYG202, and transformation mixtures were spread on 100 plates of LB (containing 100 μg/ml ampicillin) and incubated for 16 h at 37°C. With amount of 10^7, all transformants were collected.

Screening of random peptide library. Bait plasmid pYG111 and random peptide library (plasmid pYG202) were co-transformed into *S. cerevisiae* Y153, and the transformants were screened for *HIS3* expression. More than 10 million transformants were screened, and about 6000 His^+ transformants were obtained. In addition to *HIS3*, Y153 has a second reporter gene, *lac*Z. When the His^+ transformants were screened for *lac*Z expression, twenty transformants were His^+ $LacZ^+$ phenotype in the primary screening.

The GAL4AD-peptide fusion plasmids from the His^+ $LacZ^+$ transformants were segregated by shuttling through *E. coli* C600. Then these plasmids were transformed back into Y153 containing pYG111. Transformed cells were plated onto SD-Trp-Leu as well as SD-Trp-Leu-His + 3-AT media. Growth on SD-Trp-Leu confirms the presence of both the BD (bait) and AD (peptide) plasmids. Colony formation on SD-Trp-Leu-His + 3-AT demonstrates activation of the *HIS3* reporter gene. These His^+ colonies were tested for activation of *lac*Z reporter again. Through re-screening, two positive clones were obtained finally (Fig. 4). Single and co-transformants were respectively plated on nonselective (YPD) synthetic dextrose medium lacking tryptophan and histidine (SD-Trp-His), or leucine and histidine (SD-Leu-

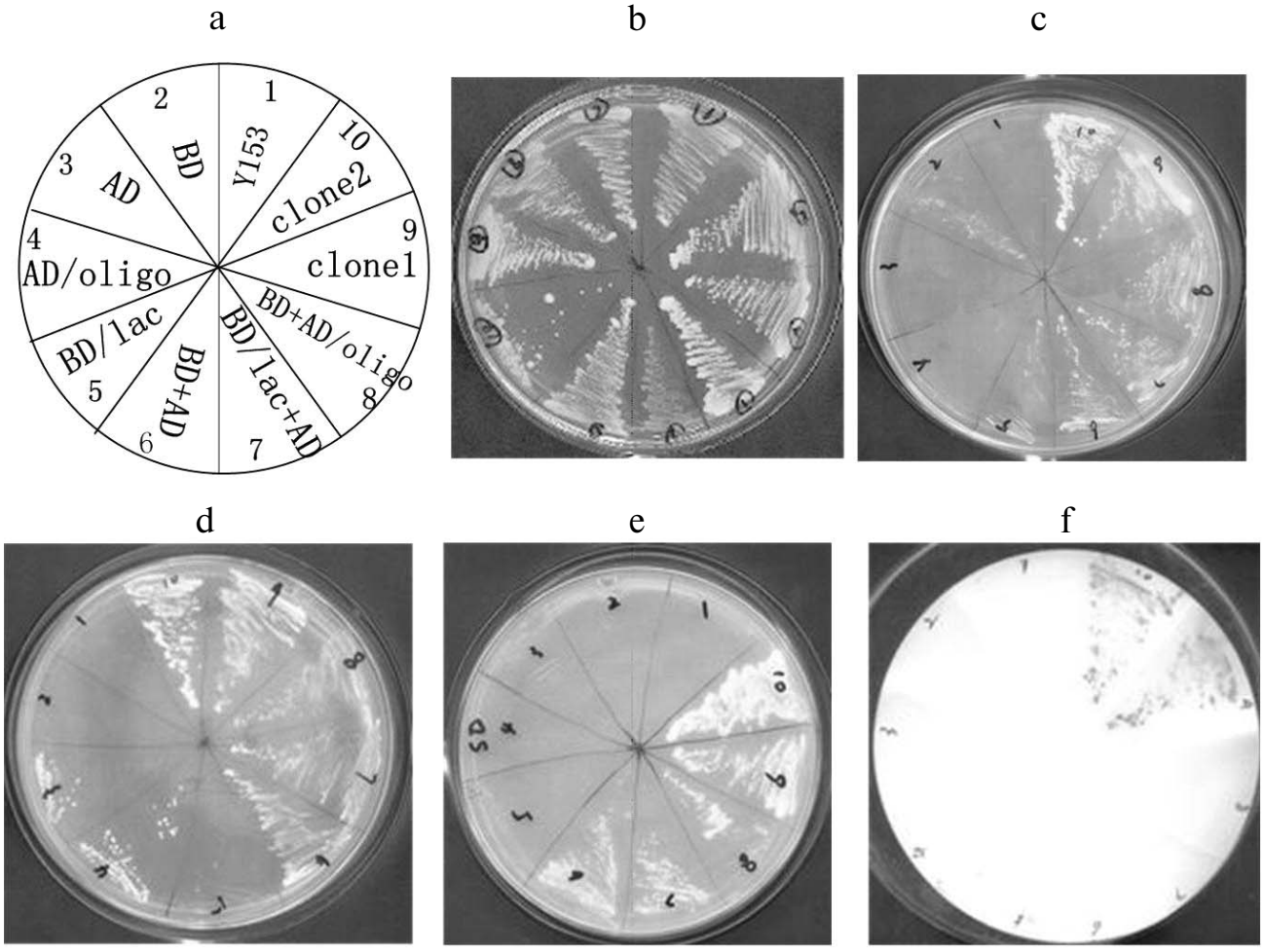

Fig. 4. Two-hybrid results showing interactions of peptide and β-lactamase. a) Template for (b) through (f); b-e) growth on YPD, SD-Trp-His, SD-Leu-His, and SD-Trp-Leu-His + 3-AT, respectively; f) β-galactosidase filter assay. BD, binding domain; BD/lac, β-lactamase fused to binding domain; AD, activating domain; AD/oligo, random peptide fused to activating domain.

His), or tryptophan, leucine, and histidine (SD-Trp-Leu-His), but containing 25 mM 3-AT to select for clones in which the *HIS3* gene was activated (Fig. 4). Two positive clones were transferred to nitrocellulose filter for the β-galactosidase filter assay, on which they turned blue.

Alignment of sequences and assay of binding activity *in vivo*. DNA sequencing of these two clones revealed that they correspond to two unique peptide-encoding genes (Fig. 5a). They were designated P1 and P2, respectively. Sequence alignment (Fig. 5b) indicated that the sequences of P1 and P2 had a great deal of difference in the random region of the designed fragment.

In *S. cerevisiae* Y153, the interaction of bait and prey leads to the expression of β-galactosidase, which is used to measure the strength and stability of the bait–prey interaction. To ascertain the strength of the interaction between the peptides and β-lactamase, the activity of β-galactosidase in cell extract prepared from the various strains was measured by monitoring the formation of *o*-nitrophenol at 420 nm. P1 demonstrated stronger inter-

a

P1: 5'-TTCACTATCCACTGCAGTGTCACTGCTGCAGGTGACTACTACTGTGTTCATGGCGCTAA-

TGGCACCTCTTTCTAG-3'

P2: 5'-TTCACTATCCACTGCCGTTTCCCTGCTGCAGGTGACTACTACGATTCTCGTTACTTTCTT-

GGCACCTCTTTCTAG-3'

b

P1 (1) FTIHCSVTAAGDYYCVHGANGTSF

P2 (1) FTIHCRFPAAGDYYDSRYFLGTSF

c

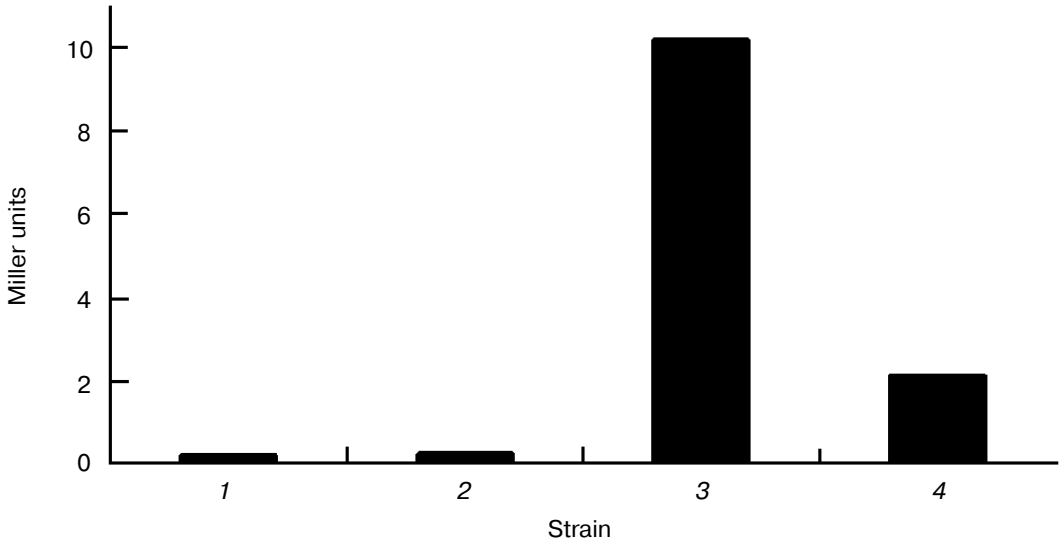

Fig. 5. a) DNA sequences of the peptides that interact with β-lactamase; b) alignment of P1 and P2; c) the strength of the β-lactamase–peptide interaction: *1*) Y153; *2*) BD + AD/Y153; *3*) BD-lac + AD-P1/Y153; *4*) BD-lac + AD-P2/Y153. (The sequence of P1 is pending a patent of China.)

action with β-lactamase with 10.2 Miller units, while P2 was approximately fivefold less with 2.1 Miller units. The control indicated that there was no interaction in yeast (Fig. 5c). The results can be interpreted as showing the effect of some amino acid residues in peptides on its interaction with β-lactamase.

Expression and purification of peptides, and assay of inhibitory activity *in vitro*. To investigate whether the peptides inhibited the activity of β-lactamase *in vitro*, they were expressed as GST fusion protein in *E. coli* and purified by affinity chromatography (Fig. 6a). The peptides were purified and separated from GST by cleavage with thrombin and ultrafiltration (Fig. 6b). The yields of purified peptides were 710 μg of P1 and 750 μg of P2.

The peptides were assayed for their ability to inhibit the activity of β-lactamase as described above. The results of this assay indicated that P1 can inhibit the activity of β-lactamase, but P2 cannot do so (Figs. 7 and 8). In addition, P1 inhibits the activity of β-lactamase in a dose-dependent manner.

DISCUSSION

Protein–protein interactions have a key role in most biological processes and are important targets for drug design [12]. Over the past decade, there has been increased interest in the design of small peptide molecules that mimic functional epitopes of various proteins. The yeast two-hybrid system is one of the effective methods for analysis of large randomized libraries of specific proteins or peptides that bind a target protein with high affinity and sensitivity. The yeast two-hybrid system was originally devised by Fields and Song [13] as a genetic method for detecting interactions between proteins *in vivo*. The basis of this system is that certain eukaryotic transcriptional activators, which are composed of a DNA-binding domain and an activating domain, can be reconstructed *in vivo* from their separately expressed domains [14]. Thus, if a "bait" protein expressed as a fusion with the DNA-binding domain interacts with a "prey" protein expressed as a fusion with the activating domain, a functional transcriptional activator is generated that initiates expression of a "reporter" gene. It has been subsequently improved to allow screening of cDNA fusion libraries or random peptide libraries to identify partners of specific baits.

In this work, the yeast two-hybrid system was used to identify peptides that can interact with β-lactamase in a random library. There are nine NNY (Y stands for C or T) in the random oligonucleotide fragment. The aim of design is that amino acids encoded by NNY distribute

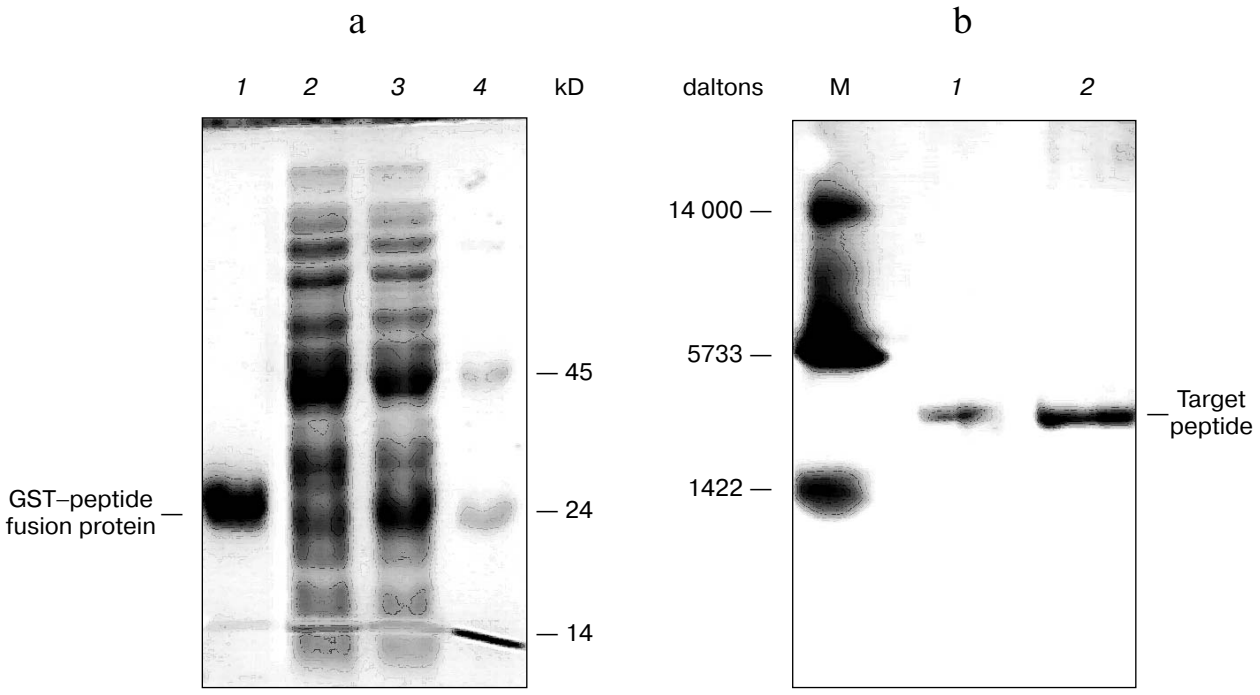

Fig. 6. SDS-PAGE map of GST fusion protein and target peptide. a) Lanes: *1*) elution; *2*) flow through; *3*) cytoplasmic extracts of *E. coli* expression GST fusion protein; *4*) protein molecular weight marker. b) Lanes: M) protein marker (composed of cytochrome *c*, 13,000 daltons; insulin, 5733 daltons; and bacitracin, 1422 daltons); *1*) P1; *2*) P2.

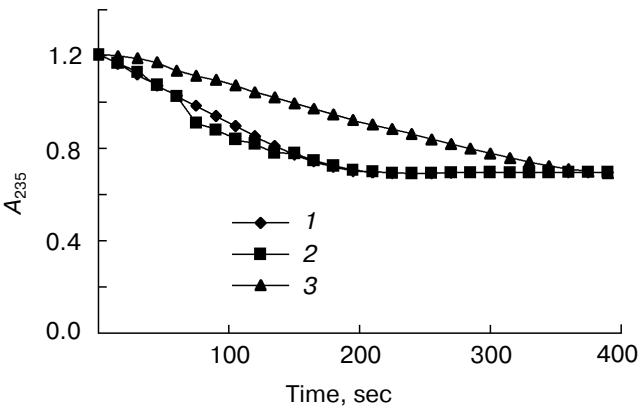

Fig. 7. Effects of P1 and P2 on the time-courses of β-lactamase reaction: *1*) without peptides; *2*) P1 (50 μM); *3*) P3 (50 μM).

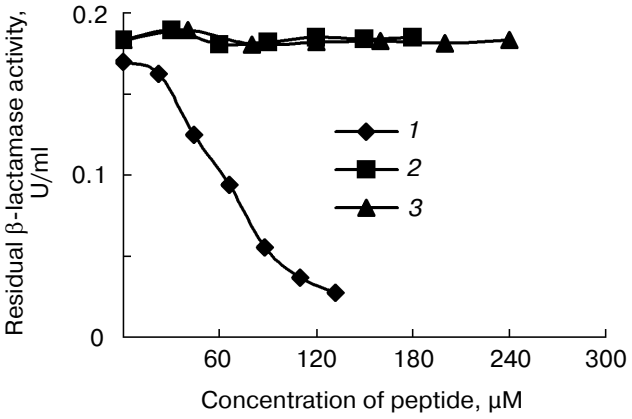

Fig. 8. Inhibitory effect of peptides on β-lactamase: *1*) P1; *2*) P2; *3*) insulin.

evenly in this fragment. Additionally, gene sequence (GCTGCAGGTGACTACTAC) was also designed into the random sequence as core sequence, because amino acids (AAGDYY) encoded by this gene sequence make critical interactions with the active site of TEM-1 β-lactamase [11]. This design may improve inhibitory activity of peptides and facilitates screening. Theoretically, adequate screening would require a minimum of 10^{10} transformants. However, *E. coli* and yeast transformation efficiency could hardly exceed 10^{10}, thus limiting the integrality of the library two-hybrid screening. This limitation caused by the transformation efficiency may be partially overcome by screening the peptide library repeatedly.

Both P1 and P2 can interact with β-lactamase *in vivo*, but the inhibition assay *in vitro* indicated that only P1 can inhibit β-lactamase. Although the core sequence of amino acids (AAGDYY) makes critical interactions with the active site of TEM-1 β-lactamase [11], our work indicates that certain amino acids in both flanks of the core sequence affect peptide interacting with the active site of TEM-1 β-lactamase. We presume that these amino acids control peptide conformation, with some conformations unable to fit the active site of TEM-1 β-lactamase. More work is needed to understand the inhibition mechanism of P1. We also expect stronger inhibitory peptides to be found by some other techniques, such as point mutation.

Peptide inhibitors have been used to prevent the function of proteins associated with diseases [15]. Recently, Thompson et al. obtained two peptides that inhibited activity of exotoxin A from *Pseudomonas aeruginosa*, using the yeast two-hybrid system [16]. So this strategy could be used to screen peptide inhibitors of TEM-1 β-lactamase, which is the target of bacterial resistance.

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REFERENCES

1. Livermore, D. M. (1995) *Clin. Microbiol. Rev.*, **8**, 557-584.
2. Bush, K., Jacoby, G. A., and Medeiros, A. A. (1995) *Antimicrob. Agents Chemother.*, **39**, 1211-1233.
3. Ghuysen, J. M. (1991) *Annu. Rev. Microbiol.*, **45**, 37-67.
4. Wiedemann, B., Kliebe, C., and Kresken, M. (1989) *J. Antimicrob. Chemother.*, **24** (Suppl. B), 1-22.
5. Petrosino, J., Cantu, C., III, and Palzkill, T. (1998) *Trends Microbiol.*, **6**, 323-327.
6. Bush, K. (2002) *Curr. Opin. Invest. Drugs*, **3**, 1284-1290.
7. Doran, J. L., Leskiw, B. K., Aippersbach, S., and Jensen, S. E. (1990) *J. Bacteriol.*, **172**, 4909-4918.
8. Strynadka, N. C., Jensen, S. E., Johns, K., Blanchard, H., Page, M., Matagne, A., Frere, J. M., and James, M. N. (1994) *Nature*, **368**, 657-660.
9. Petrosino, J., Rudgers, G., Gillbert, H., and Palzkill, T. (1999) *J. Biol. Chem.*, **274**, 2394-2400.
10. Rudgers, G. W., and Palzkill, T. (1999) *J. Biol. Chem.*, **274**, 6963-6971.
11. Rudgers, G. W., Huang, W., and Palzkill, T. (2001) *Antimicrob. Agents Chemother.*, **45**, 3279-3286.
12. Vidal, M., and Endoh, H. (1999) *Trends Biotechnol.*, **17**, 374-381.
13. Fields, S., and Song, O. (1989) *Nature*, **340**, 245-246.
14. Brent, R., and Ptashne, M. (1985) *Cell*, **43**, 729-736.
15. Ferrer, M., and Harrison, S. C. (1999) *J. Virol.*, **73**, 579-582.
16. Thompson, C., Merrill, A. R., and Mangroo, D. (2003) *FEMS Microbiol. Lett.*, **218**, 85-92.