ISSN 0006-2979, Biochemistry (Moscow), 2010, Vol. 75, No. 3, pp. 336-341. © Pleiades Publishing, Ltd., 2010.
Published in Russian in Biokhimiya, 2010, Vol. 75, No. 3, pp. 411-417.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM09-258, February 14, 2010.

Studies on Amino Acid Replacement and Inhibitory Activity of a β-Lactamase Inhibitory Peptide

Liping Xie, Mingfei Xu, Tao Yang, Chunbao Zhu, Baoquan Zhu, and Youjia Hu*

State Key Laboratory of New Drug and Pharmaceutical Process, Shanghai Institute of Pharmaceutical Industry, 1111 Zhongshan Road (North No. 1), Shanghai 200437, China; fax: 86-21-3505-2484; E-mail: bebydou@hotmail.com

Received August 31, 2009
Revision received September 28, 2009

Abstract—An SHV β-lactamase gene was amplified from a β-lactam resistant *Klebsiella pneumoniae* K-71 genomic DNA. After expression and purification, we demonstrated that peptide P1 could inhibit the hydrolysis activity of both TEM-1 and SHV β-lactamase *in vitro*. Three mutations were introduced into P1 in which the first residue S was replaced by F, the 18th residue V was mutated to Y, and the 15th residue Y was substituted with A, C, G, and R to obtain the mutants of P1-A, P1-C, P1-G, and P1-R, respectively. The mutant peptides were purified and their inhibitory constants against TEM-1 and SHV β-lactamase were determined. All these β-lactamase inhibitory peptides could inhibit the activity of both β-lactamases, while the mutant peptides showed stronger inhibitory activities against TEM-1 β-lactamase than against SHV β-lactamase. Inhibition data suggested that P1-A improved the β-lactamase inhibitory activity by over 3-fold compare to P1. When P1-A was incubated with *K. pneumoniae* K-71 in Luria–Bertani medium containing ampicillin, it showed a much stronger growth of inhibition ratio over P1. This study gives us a good candidate for development of novel β-lactamase inhibitors.

DOI: 10.1134/S0006297910030107

Key words: β-lactamase inhibitory peptide, β-lactamase, amino acid replacement, *Klebsiella pneumoniae*, inhibitory constant

β-Lactam antibiotics such as penicillins and cephalosporins constitute a major class of therapeutic agents that are currently employed in the treatment of infectious diseases. But abuse usage of β-lactam antibiotics in therapy has led to the emergence of resistant strains including methicillin-resistant *Staphylococcus aureus*. The most important mechanism of β-lactam resistance is mediated by β-lactamase. Strategies to overcome the resistant strain bearing β-lactamase have been and still continue to be developed. These include development of β-lactam antibiotic analogs resistant to β-lactamase, such as carbapenems [1] and monobactams [2], and production of β-lactamase inhibitors, which could be used together with the β-lactam antibiotics in clinic, such as clavulanic acid [3], sulbactam [4], and tazobactam [5]. Efforts have been made toward the development of potent inhibitors of β-lactamase to extend the lifecycle and spectrum of β-lactam antibiotics.

β-Lactamase inhibitory protein (BLIP) [6], which inhibits the β-lactam degradation activity of class A β-lactamase, is a 165-amino-acid protein produced by the Gram-positive bacterium *Streptomyces clavuligerus*. BLIP consists of two tandemly linked 76-amino-acid domains. BLIP–β-lactamase complex has become a protein–protein interaction model system with increasingly more detailed information on structure–activity relationships. Recent advances in structural biology have revealed that residues 46-51 (AAGDYY) of BLIP are crucial for the interaction between BLIP and TEM-1 β-lactamase [7], a major type of class A β-lactamase.

We previously reported a 24-amino-acid peptide P1 that contains the core sequence of BLIP (46-51 residues) that has TEM-1 β-lactamase inhibitory activity [8]. There are also reports on alanine-scanning mutagenesis of residues on the BLIP binding surface revealing that there are two hotspots of binding energy [9], and one mutation (Y50A) actually increases binding affinity for TEM-1 β-lactamase by 50-fold [10].

On the basis of this information, amino acid replacements in P1 by point mutations and the inhibitory potency of different mutants against TEM-1 and SHV β-lacta-

Abbreviations: BLIP, β-lactamase inhibitory peptide; IPTG, isopropyl-β-D-thiogalactopyranoside; MIC, minimal inhibitory concentration.

* To whom correspondence should be addressed.

mases, another major type of class A β-lactamases, are reported in this paper.

MATERIALS AND METHODS

Strains and plasmids. *Escherichia coli* DH5α is used for gene cloning and SHV β-lactamase expression. *Escherichia coli* BL21(DE3) is used for expression of different P1 mutants. *Klebsiella pneumoniae* K-71 is a β-lactam antibiotics resistant strain, and it is used for SHV β-lactamase gene isolation and β-lactamase inhibitory activity test of different mutants. Plasmids used in this study are: pET-32a(+) for construction of plasmids for expression of different mutants of P1; pLY-5 with P_RP_L promoter, for construction of a plasmid for expression of SHV β-lactamase; pUC18 for construction of a plasmid to determine the *in vivo* activity of SHV β-lactamase.

Gene cloning and plasmid construction. To obtain different mutants of the P1 gene, several oligonucleotides were designed and synthesized by Invitrogen (China). Attached restriction sites are underlined: oligo 1, 5′-TTTACCATTCACTGTTCTGTTACCGCAGCTGGC-3′; oligo 2A, 5′-ACCGTGGTAGCAGTAAGCATCG-CCAGCTGCGGTAACA-3′; oligo 2G, 5′-ACCGTG-GTAGCAGTAACCATCGCCAGCTGCGGTAACA-3′; oligo 2C, 5′-ACCGTGGTAGCAGTAACAATCGCCA-GCTGCGGTAACA-3′; oligo 2R, 5′-ACCGTGGTA-GCAGTATCGATCGCCAGCTGCGGTAACA-3′; oligo 3, 5′-GTAGAATTCATGTTCCGCTTTACCATT-CACTGTTCT-3′; oligo 4, 5′-ATAGTCGACTAGAAG-GAAGTACCGTTCGCACCGTGGTAGCAGTA-3′.

The P1-A mutant gene was obtained by mixing oligo 1, 2A, 3, and 4 followed by PCR amplification. The resulting gene encodes 24-amino-acid peptide P1-A in which the residues 1, 15, and 18 of P1 (S, Y, and V) have been mutated to F, A, and Y, respectively. This PCR product was digested with *Eco*RI and *Sal*I and inserted into pET-32a(+) to obtain the P1-A expression plasmid pYG565. Following the same protocol, pYG566, pYG567, and pYG568 for P1-G, P1-C, and P1-R expression were constructed by using appropriate oligo 2 in PCR, respectively.

The SHV β-lactamase gene (*shv*) was amplified using *K. pneumoniae* K-71 genomic DNA isolated following the protocol described elsewhere [11] as template. Two degenerated primers were designed according to the published sequences of SHV β-lactamase in GenBank (AY790341, AY677211, DQ174306, AF677211, EF373969, EF035565, DQ013287). The sequences of two primers were as follows: primer 1, 5′-ATA-GAATTCATGCGTTATDTTCNCCTGTGTAT-TATCTCCCYGTTA-3′; primer 2, 5′-TTAGGATCCT-TAGCGTTGCCAGTGCYCGATCAGCGCGC-3′. The PCR product (~800 bp) was digested with *Eco*RI and *Bam*HI and inserted into pLY-5 to obtain the SHV β-lactamase expression plasmid pYG561 in which *shv* was transcribed under the control of P_RP_L, a temperature-induced promoter.

To determine the *in vivo* activity of this recombinant SHV β-lactamase, plasmid pYG562 was constructed based on pUC18 in which the *bla* gene (contribute to ampicillin resistance) was substituted with the *shv* gene. To obtain pYG562, four primers were designed to amplify *shv* and pUC18 backbone without *bla* that both have the compatible cohesive ends. The sequences of these four primers were as follows: P1S/SHV, 5′-CTAACTAG-TATGCACCATCATCATCATCATCGTTAT-3′; P2B/SHV, 5′-TTAGGATCCTTAGCGTTGCCAGTG-CYCGATCAGCGCGC-3′; P1B/pUC, 5′-GGAA-GATCTTAACTGTCAGACCAAGTTTACTCAT-3′; P2S/pUC, 5′-GCACTAGTACTCTTCCTTTTTCAA-TATTATTGAAGC-3′. The PCR product of *shv* was digested with *Spe*I and *Bam*HI and ligated with the pUC18 PCR product digested with *Spe*I and *Bgl*II to obtain pYG562.

Recombinant protein expression and purification. pYG565, pYG566, pYG567, and pYG568 together with the original P1 expression plasmid pET132 (P1 gene cloned into pET-32a(+), same structure with pYG56x) were transformed into *E. coli* BL21(DE3). *Escherichia coli* culture (250 ml) was grown to log phase (A_{600} ~ 0.5) and induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 37°C. Cells were pelleted by centrifugation and disrupted by ultrasonication. The lysate was loaded onto a Ni-NTA affinity column (Qiagen, USA) and purified following the manufacture's manual. The eluted protein was then dialyzed overnight against 150 mM NaCl, 20 mM Tris-HCl, pH 6.5, containing 10% glycerol. After dialysis, the fusion protein was digested with recombinant enterokinase (Zhiyuan Biochemical Co., China) and separated by ultrafiltration (Millipore, USA). All procedures were conducted at 4°C.

Expression and purification of TEM-1 and SHV β-lactamase followed the protocol we described previously [8].

***In vitro* inhibition assay.** The *in vitro* degradation activity of SHV β-lactamase on β-lactam antibiotics and the inhibitory activity of inhibitory peptide P1 were assayed as described previously [12].

Determination of the minimal inhibitory concentration (MIC) of ampicillin against bacteria. Bacteria were allowed to grow to log phase (A_{600} ~ 0.5) and inoculated into fresh Luria–Bertani (LB) broth containing various concentration of ampicillin ranging from 10 to 140 μg/ml. MIC was determined from the concentration of antibiotic at which no growth was observed.

Assay for inhibitory constant (K_i) of β-lactamase inhibitory peptide. This was carried out similarly with the *in vitro* inhibition assay. Ampicillin as the substrate was used at concentrations of 25 and 50 μg/ml. All inhibitory peptides were tested at concentrations ranging from 0.5 to

16 μM. The K_i values were determined by linear regression of data from Dixon plots [13, 14].

Determination of inhibitory activity of β-lactamase inhibitory peptides against *K. pneumoniae*. *Klebsiella pneumoniae* cultures were allowed to grow to log phase (A_{600} ~ 0.5) and divided into five groups. Ampicillin was added to final concentration of 50 μg/ml. Then 20 μl of β-lactamase inhibitory peptide (2.23 μM) before or after enterokinase digestion was added to make the total volume up to 2.3 ml. Phosphate buffered saline (peptide dissolving solution, 20 μl) was used as a control. After incubation at 37°C for 2 h, appropriately diluted broth was plated on LB agar containing ampicillin. The colony-forming units were counted after overnight incubation to determine the inhibitory activity of β-lactamase inhibitory peptides against *K. pneumoniae*. This test was performed in triplicate.

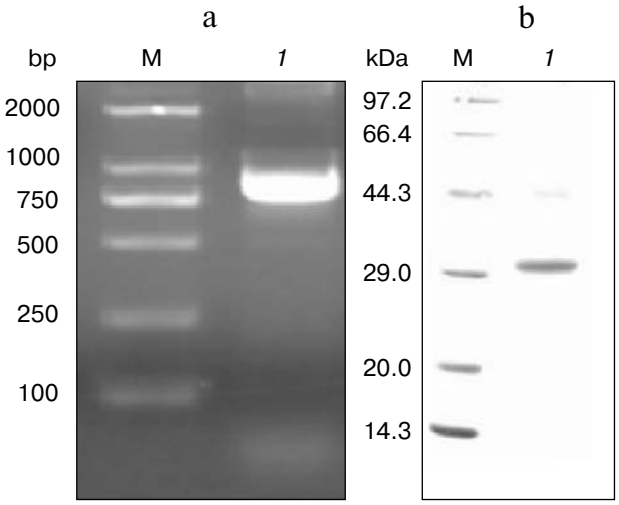

Fig. 1. a) Agarose gel electrophoresis of SHV β-lactamase gene (*shv*). An ~800-bp PCR product was gel purified for further cloning. M, standard markers (Takara, China); *1*) gene *shv* DNA. b) SDS-PAGE of purified SHV β-lactamase. M, standard markers; *1*) SHV β-lactamase.

RESULTS

Expression and purification of SHV β-lactamase. The SHV β-lactamase gene (*shv*) was amplified from the genomic DNA of *K. pneumoniae* K-71. The PCR product was ~800 bp (Fig. 1a), corresponding to the sequences published in GenBank (700-950 bp). The sequencing result showed that this cloned *shv* shared 97.6-99.0% identity with published sequences. Residue 4 was F instead of the consensus I, and residue 199 was P rather than the consensus S. The recombinant SHV β-lactamase was expressed and purified to homogeneity according to SDS-PAGE (Fig. 1b). Its purity was over 93% (estimated by densitometry) and the concentration was 3.15 mg/ml by the Bradford assay.

Degradation activity of recombinant SHV β-lactamase against β-lactam antibiotics. *Klebsiella pneumoniae* K-71 is an ampicillin resistant strain. The MIC of ampicillin against K-71 is 120 μg/ml (Table 1). To determine if this cloned *shv* gene contributed to ampicillin resistance, two tests were designed and studied.

Plasmid pYG562 was constructed by replacing the *bla* gene with the *shv* gene in pUC18. Plasmid pYG562 was transformed into DH5α. The MIC of ampicillin against the pYG562/DH5α transformant and DH5α as a control were tested. DH5α cannot grow in LB even with 10 μg/ml ampicillin. But pYG562/DH5α can tolerate 120 μg/ml ampicillin (Table 1). This result showed that this cloned *shv* gene contributed to the ampicillin resistance *in vivo*.

Further, the purified recombinant SHV β-lactamase was used to assay its hydrolysis activity against ampicillin *in vitro*. The concentration of ampicillin in the reaction mixture was monitored by absorbance at 235 nm. SHV β-lactamase could quickly degrade ampicillin after mixing (Fig. 2), with activity similar to that of TEM-1 β-lactamase reported previously [8].

Inhibitory activity of P1 against SHV β-lactamase. Compared with SHV β-lactamase alone, preincubation with P1 significantly reduced the activity of SHV β-lactamase for hydrolysis of ampicillin *in vitro* (Fig. 2). This suggested that P1 peptide also has inhibitory activity against SHV β-lactamase comparable with that against TEM-1 β-lactamase reported previously [8].

Expression and purification of P1 mutant peptides. By using PCR and four primers, three mutations were intro-

Table 1. MIC (μg/ml) of ampicillin against bacteria

Bacteria	10	20	50	60	70	80	90	100	110	120	130	140
K. pneumoniae	+	+	+	+	+	+	+	+	+	–	–	–
E. coli DH5α	–	–	–	–	–	–	–	–	–	–	–	–
E. coli pYG562/DH5α	+	+	+	+	+	+	+	+	+	+	–	–

Note: +, growth observed; –, growth not observed.

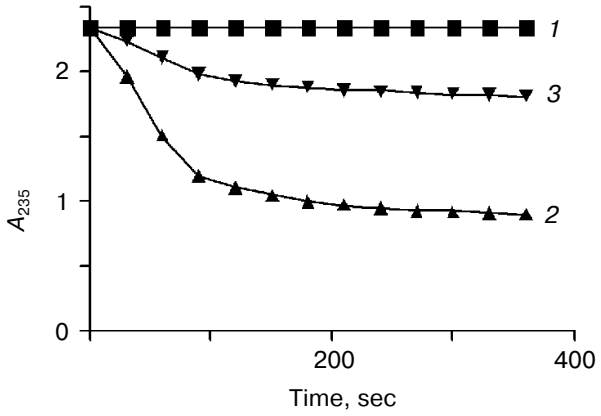

Fig. 2. Inhibitory activity of P1 against SHV β-lactamase *in vitro*: *1*) ampicillin; *2*) ampicillin + SHV lactamase; *3*) ampicillin + SHV lactamase + P1.

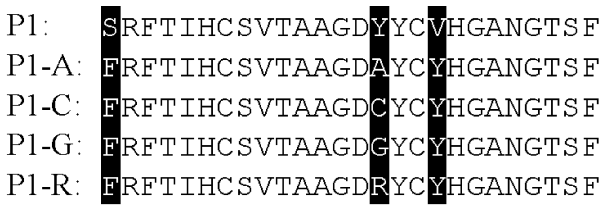

Fig. 3. Amino acid sequence alignment of P1 and four mutants. P1, the original sequence published previously [8]; P1-A, three substitutions made at residues 1 (S→F), 15 (Y→A), and 18 (V→Y); P1-C, P1-G, and P1-R, residue 15 of P1-A substituted with C, G, or R, respectively.

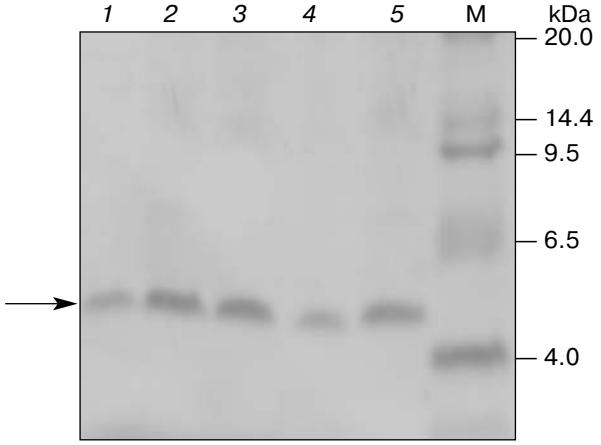

Fig. 4. SDS-PAGE of purified β-lactamase inhibitory peptides: *1*) P1-A; *2*) P1-G; *3*) P1-C; *4*) P1-R; *5*) P1. M, molecular weight standards.

duced into the original P1. Residue 1 (S) was mutated to F, and residue 18 (V) was replaced by Y. This was designed according to the structure–activity relationship of BLIP published elsewhere [10]. Moreover, residue 15 (Y), a key residue in the binding model of BLIP and β-lactamase, was designed to be substituted with A, C, G, or R. The sequences of the four mutant peptides are listed in Fig. 3.

All the transformants bearing pET132 and pYG565~568 were induced by IPTG and the recombinant protein was expressed as a fusion protein. The fusion protein was purified on a Ni^{+}-affinity column, and the peptide was subsequently released from the fused part by cleavage with enterokinase. The purity and concentration of the peptides were tested by SDS-PAGE (Fig. 4). The final peptide we obtained has 39 amino acid residues, including the residues encoded by the introduced restriction sites, and carried the enterokinase recognition site of pET-32a(+). Thus, the molecular weight of the recombinant peptide was about 4.3 kDa. The purity of the recombinant peptide was over 95%.

Kinetic analysis of β-lactamase inhibitory peptides. Inhibitory constants against TEM-1 and SHV β-lactamase of all the peptides are shown in Table 2. From the plot, we know that these β-lactamase inhibitory peptides are competitive inhibitors of both TEM-1 and SHV β-lactamase. Substitution of Y15 with A or G improves the inhibitory activity, while substitution by C or R reduces the activity. We also noted inhibitory activity differences between mutant peptides against TEM-1 and SHV β-lactamase. Obviously, the peptides are more active against TEM-1 β-lactamase than SHV β-lactamase. The Y15A mutation has more significant improvement on β-lactamase inhibitory activity.

Inhibitory activity of β-lactamase inhibitory peptides *in vivo*. We proved that P1 and its analogs have inhibitory activity against SHV β-lactamase originating from K-71 *in vitro*. In this experiment, P1 (with and without the fusion protein) and P1-A (showed the strongest inhibitory activity *in vitro*, with and without the fusion protein) were used to test their inhibitory activity *in vivo*. The results showed that both P1 and P1-A inhibit the growth of *K. pneumoniae* in LB containing ampicillin. Nevertheless, the peptide without the fusion protein

Table 2. Inhibition constants of β-lactamase inhibitory peptides against TEM-1 and SHV β-lactamase

Peptide	K_i, nM	
	TEM-1	SHV
P1	379	1160
P1-A	108	637
P1-G	307	785
P1-C	1650	1330
P1-R	2510	3550

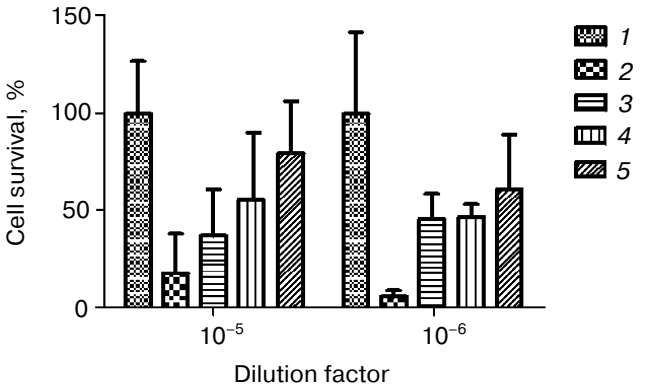

Fig. 5. Effect of β-lactamase inhibitory peptides on survival of *K. pneumoniae* cells in presence of ampicillin: *1*) control (phosphate buffered saline); *2*, *3*) P1-A after and before enterokinase treatment, respectively; *4*, *5*) P1 after and before enterokinase treatment, respectively. Control was normalized to 100%. Results reflect the mean data from triplicates.

showed a stronger activity than its fusion counterpart. Accordingly, P1-A also showed a much stronger inhibitory activity against *K. pneumoniae in vivo* than P1. Under 10^{-5} or 10^{-6} dilution, more than 85% of the resistant bacteria were inhibited by prior incubation with P1-A (Fig. 5).

DISCUSSION

β-Lactamase inhibitory protein was separated from the soil bacterium *Streptomyces clavuligerus*. Several reports [15-17] have pointed out that five residues in domain 1 (F36, H41, D49, Y53, and W150) play critical roles in BLIP–β-lactamase binding. W112, H148, R160, and W162, located in domain 2, are also important for binding. In addition, E73 and K74, within the linkage of these two domains, could cause salt bridge formation between Y50 and the active pocket of β-lactamase [18]. This would stabilize the BLIP–β-lactamase conformation to improve binding affinity. In addition, Y50 forms a large stacking structure with Y51, F36, H41, and Y53 [19].

In the previous report using a yeast two-hybrid system [8], we obtained P1 peptide containing the core sequence of BLIP that can inhibit the activity of TEM-1 β-lactamase *in vitro*. In this paper, we proved that P1 is also active against SHV β-lactamase *in vitro*. By introducing various point mutations into P1, we found that residue 15, equivalent to Y50 in BLIP, is very important for the binding of β-lactamase. Since an A or G mutation has a positive effect whereas C or R mutation has a negative effect, we suggested that a nonpolar residue at position 15 favors binding of β-lactamase to the β-lactamase inhibitory peptide. The relatively short nonpolar side chain of A might promote the interaction between H41 and Y51 of BLIP and TEM-1 β-lactamase. This might result from nonpolar side chain of Y50 favoring insertion of BLIP into the active pocket formed by P107, M129, and V216 of β-lactamase [20-23].

Comparing the inhibitory constant (K_i) of the wild type BLIP, the small peptides showed a much lower affinity than BLIP (108 vs. 0.5 nM). This is probably because P1 and its analogs contain only part of the core sequences of BLIP but lack other binding motifs.

Within the class A β-lactamases, SHV is most prevalent among Gram-negative bacteria. A concern was expressed that its broad specificity allows it to hydrolyze a wide variety of β-lactam antibiotics [24].

The ampicillin resistant *K. pneumoniae* has emerged as a common cause of some serious epidemic and nosocomial infections in hospitals, resulting in high morbidity and mortality [25]. *Klebsiella pneumoniae* is resistant to penicillins and expanded-spectrum cephalosporins through the production of β-lactamases. In this work, P1-A peptide showed inhibitory activity against *K. pneumoniae in vivo*.

Better understanding of the structure–activity relationship in the BLIP–β-lactamase interface can facilitate the design of other new inhibitors against this type of β-lactamase. We anticipate that P1-A could be a good candidate for development of novel β-lactamase inhibitors.

This work was supported by the Science and Technology Commission of Shanghai Municipality (Grant Nos. 08DZ2230600, 08DZ2270700, 07DZ22002).

REFERENCES

1. Rasmussen, B. A., and Bush, K. (1997) *Antimicrob. Agents Chemother.*, **41**, 223-232.
2. Sykes, R. B., Bonner, D. P., Bush, K., and Georgopapadakou, N. H. (1982) *Antimicrob. Agents Chemother.*, **21**, 85-92.
3. Fisher, J., Charnas, R. L., and Knowles, J. R. (1978) *Biochemistry*, **17**, 2180-2184.
4. English, A. R., Retsema, J. A., Girard, A. E., Lynch, J. E., and Barth, W. E. (1978) *Antimicrob. Agents Chemother.*, **14**, 414-419.
5. Micetich, R. G., Maiti, S. N., Spevak, P., Hall, T. W., Yamabe, S., Ishida, N., Tanaka, M., Yamazaki, T., Nakai, A., and Ogawa, K. (1987) *J. Med. Chem.*, **30**, 1469-1474.
6. Doran, J. L., Leskiw, B. K., Aippersbach, S., and Jensen, S. E. (1990) *J. Bacteriol.*, **172**, 4909-4918.
7. Rudgers, G. W., Huang, W., and Palzkill, T. (2001) *Antimicrob. Agents Chemother.*, **45**, 3279-3286.
8. Sun, W., Hu, Y., Gong, J., Zhu, C., and Zhu, B. (2005) *Biochemistry (Moscow)*, **70**, 753-760.
9. Wang, J., Palzkill, T., and Chow, D. C. (2009) *J. Biol. Chem.*, **284**, 595-609.
10. Zhang, Z., and Palzkill, T. (2003) *J. Biol. Chem.*, **278**, 45706-45712.

11. Pospiech, A., and Neumann, B. (1995) *Trends Genet.*, **11**, 217-218.
12. Petrosino, J., Rudgers, G., Gilbert, H., and Palzkill, T. (1999) *J. Biol. Chem.*, **274**, 2394-2400.
13. Cornish-Bowden, A. (1974) *Biochem. J.*, **137**, 143-144.
14. Dixon, M. (1953) *Biochem. J.*, **55**, 170-171.
15. Ness, S., Martin, R., Kindler, A. M., Paetzel, M., Gold, M., Jensen, S. E., Jones, J. B., and Strynadka, N. C. (2000) *Biochemistry*, **39**, 5312-5321.
16. Reichmann, D., Rahat, O., Albeck, S., Meged, R., Dym, O., and Schreiber, G. (2005) *Proc. Natl. Acad. Sci. USA*, **102**, 57-62.
17. Thai, W., Paradkar, A. S., and Jensen, S. E. (2001) *Microbiology*, **147**, 325-335.
18. Strynadka, N. C., Jensen, S. E., Alzari, P. M., and James, M. N. (1996) *Nature Struct. Biol.*, **3**, 290-297.
19. Zhang, Z., and Palzkill, T. (2004) *J. Biol. Chem.*, **279**, 42860-42866.
20. Albeck, S., and Schreiber, G. (1999) *Biochemistry*, **38**, 11-21.
21. Reynolds, K. A., Thomson, J. M., Corbett, K. D., Bethel, C. R., Berger, J. M., Kirsch, J. F., Bonomo, R. A., and Handel, T. M. (2006) *J. Biol. Chem.*, **281**, 26745-267453.
22. Selzer, T., Albeck, S., and Schreiber, G. (2000) *Nature Struct. Biol.*, **7**, 537-541.
23. Yao, J., and Asakura, T. (2003) *J. Biochem.*, **133**, 147-154.
24. Rice, L. B., Carias, L. L., Hujer, A. M., Bonafede, M., Hutton, R., Hoyen, C., and Bonomo, R. A. (2000) *Antimicrob. Agents Chemother.*, **44**, 362-367.
25. DiPersio, J. R., Deshpande, L. M., Biedenbach, D. J., Toleman, M. A., Walsh, T. R., and Jones, R. N. (2005) *Diagn. Microbiol. Infect. Dis.*, **51**, 1-7.