Protein Science (1999), *8*:2330–2337. Cambridge University Press. Printed in the USA.

The complexed structure and antimicrobial activity of a non-β-lactam inhibitor of AmpC β-lactamase

RACHEL A. POWERS,[1] JESÚS BLÁZQUEZ,[2] G. SCOTT WESTON,[1,3]
MARÍA-ISABEL MOROSINI,[2] FERNANDO BAQUERO,[2] AND BRIAN K. SHOICHET[1]

[1]Department of Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, Illinois 60611
[2]Servicio de Microbiología, Hospital Ramón y Cajal, Ctra. Colmenar Km 9.100, 28034, Madrid, Spain

(RECEIVED June 2, 1999; ACCEPTED August 5, 1999)

Abstract

β-Lactamases are the major resistance mechanism to β-lactam antibiotics and pose a growing threat to public health. Recently, bacteria have become resistant to β-lactamase inhibitors, making this problem pressing. In an effort to overcome this resistance, non-β-lactam inhibitors of β-lactamases were investigated for complementarity to the structure of AmpC β-lactamase from *Escherichia coli*. This led to the discovery of an inhibitor, benzo(b)thiophene-2-boronic acid (BZBTH2B), which inhibited AmpC with a K_i of 27 nM. This inhibitor is chemically dissimilar to β-lactams, raising the question of what specific interactions are responsible for its activity. To answer this question, the X-ray crystallographic structure of BZBTH2B in complex with AmpC was determined to 2.25 Å resolution. The structure reveals several unexpected interactions. The inhibitor appears to complement the conserved, R1-amide binding region of AmpC, despite lacking an amide group. Interactions between one of the boronic acid oxygen atoms, Tyr150, and an ordered water molecule suggest a mechanism for acid/base catalysis and a direction for hydrolytic attack in the enzyme catalyzed reaction. To investigate how a non-β-lactam inhibitor would perform against resistant bacteria, BZBTH2B was tested in antimicrobial assays. BZBTH2B significantly potentiated the activity of a third-generation cephalosporin against AmpC-producing resistant bacteria. This inhibitor was unaffected by two common resistance mechanisms that often arise against β-lactams in conjunction with β-lactamases. Porin channel mutations did not decrease the efficacy of BZBTH2B against cells expressing AmpC. Also, this inhibitor did not induce expression of AmpC, a problem with many β-lactams. The structure of the BZBTH2B/AmpC complex provides a starting point for the structure-based elaboration of this class of non-β-lactam inhibitors.

Keywords: AmpC; antibiotic resistance; β-lactamase; non-β-lactam inhibitor of β-lactamase

The emergence of bacterial resistance to antibiotics is a major threat to public health (Neu, 1992; Davies, 1994; Baquero & Blazquez, 1997). Among the classes of antibiotics hardest hit are the β-lactams, which include the penicillins and cephalosporins. These drugs are also among the most widely prescribed.

Since the introduction of penicillin in the early 1940s, a suite of resistance mechanisms to β-lactams have emerged (Sanders, 1992). These mechanisms are ancient, probably having developed in response to β-lactams, many of which have been produced by microorganisms over evolutionary time (Bush, 1997). Human overuse of β-lactams has promoted the spread of pre-existing resistance mechanisms to many formerly sensitive bacteria. The most predominant of these mechanisms are the β-lactamases. These enzymes hydrolyze the lactam bond of the antibiotics, inactivating them.

To combat β-lactamases, compounds that resisted their action were developed (Sutherland, 1991). Inhibitors, such as clavulanic acid, and "β-lactamase stable" molecules, such as aztreonam, share the same β-lactam core found in all β-lactam antibiotics (Fig. 1A–C). This has allowed further resistance to arise through modification of pre-existing mechanisms. Point substitutions have occurred in formerly narrow spectrum β-lactamases, allowing the enzymes to hydrolyze or evade "β-lactamase stable" compounds. Porin channel mutations have arisen in Gram-negative bacteria that reduce the access of β-lactams to their cellular targets, the penicillin binding proteins (PBPs). In many bacteria, exposure to β-lactams up-regulates β-lactamase transcription (Bennett & Chopra,

Reprint requests to: Brian Shoichet, Northwestern University Medical School, Molecular Pharmacology and Biological Chemistry, Mail Code S215, 303 East Chicago Avenue, Chicago, Illinois 60611-3008; e-mail: b-shoichet@nwu.edu.
[3]Present address: Computational Design and Informatics, ArQule, Inc., 200 Boston Avenue, Medford, Massachusetts 02155.

Abbreviations: BZBTH2B, benzo(b)-thiophene-2-boronic acid; CAZ, ceftazidime; MIC, minimum inhibitory concentration; PBPs, penicillin binding proteins.

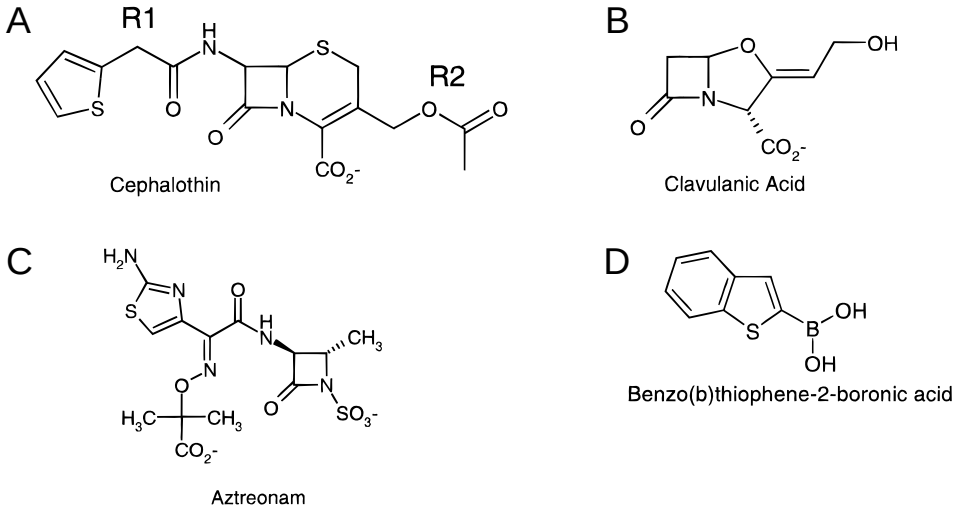

Fig. 1. Comparison of β-lactamase ligands. **A:** Cephalothin, a cephalosporin substrate. The R1 and R2 side chains are labeled. **B:** Clavulanic acid, a clinically used β-lactamase inhibitor. **C:** Aztreonam, a "β-lactamase stable" molecule. **D:** Benzo(b)thiophene-2-boronic acid.

1993; Jacobs et al., 1997). Paradoxically, β-lactam inhibitors of β-lactamases can induce the production of the enzyme they are meant to inhibit.

Class C (type I) β-lactamases like AmpC (Bush et al., 1995) are among the most difficult to counter. Compounds that are supposedly "β-lactamase stable," like aztreonam, are hydrolyzed by AmpC, and β-lactam–based inhibitors, such as clavulanic acid, do not inhibit them at clinically relevant concentrations. Presumably driven by an increased use of "β-lactamase stable" drugs and β-lactamase inhibitors that are themselves β-lactams, AmpC-based resistance to β-lactams has become increasingly prominent. The recent mobilization of these enzymes on plasmids (Gonzalez Leiza et al., 1994; Koeck et al., 1997) and the isolation of mutant enzymes in the wild (Nukaga et al., 1995) threaten further spread of these broad-spectrum β-lactamases.

We investigated non-β-lactam inhibitors of AmpC from *Escherichia coli*, reasoning that they would not be subject to the suite of existing resistance mechanisms developed against β-lactams. A non-β-lactam compound would avoid hydrolysis by mutant β-lactamases, might not be affected by porin channel mutations, and might not up-regulate β-lactamase production. Because such inhibitors would not have been in the biosphere over evolutionary time, bacteria might need to develop new resistance mechanisms, rather than simply recruiting existing ones. The high sequence and structural similarity among class C β-lactamases (Usher et al., 1998) suggests that an inhibitor of AmpC will inhibit class C enzymes from other Gram-negative pathogens.

Boronic acids inhibit β-lactamases as transition-state analogs (Beesley et al., 1983; Strynadka et al., 1996). Using structure-based methods, we identified a novel boronic acid inhibitor, benzo(b)thiophene-2-boronic acid (BZBTH2B), with a K_i of 27 nM for AmpC (Weston et al., 1998). This inhibitor is 100-fold more potent than boronic acid inhibitors previously described for this enzyme and is specific for AmpC over mechanistically related enzymes (Weston et al., 1998).

BZBTH2B does not resemble β-lactams (Fig. 1), but nevertheless inhibits AmpC β-lactamase potently and specifically. This raises several questions. To what interactions does the high affinity of this inhibitor owe? What does this inhibitor tell us about the functional group recognition in AmpC? Can such non-β-lactam inhibitors evade the classic β-lactam resistance mechanisms? To address these questions, we determined the X-ray crystallographic structure of the BZBTH2B/AmpC complex. We also investigated the actions of BZBTH2B/β-lactam combinations against pathogenic bacteria that complement AmpC expression with secondary resistance mechanisms, increasing resistance. Both the crystallographic structure and antibacterial experiments reveal surprising properties of BZBTH2B. The inhibitor makes unusual interactions with conserved, catalytic residues in the AmpC active site, and it has an antibiotic profile dramatically different from traditional anti-β-lactamase compounds, such as clavulanic acid or aztreonam (Fig. 1).

Results

Crystallographic structure of BZBTH2B/AmpC complex

The X-ray crystallographic structure of BZBTH2B bound to AmpC β-lactamase was determined to 2.25 Å resolution (Table 1). The electron density for the inhibitor in this structure was well defined (Fig. 2). Following refinement of the complexed structure, a simulated annealing omit map of the area surrounding the inhibitor was calculated and showed unambiguous positive difference density for the inhibitor when contoured at 3σ. The position of the sulfur atom of the thiophene ring of the inhibitor was verified by calculating electron density maps for models with the sulfur atom in each of two possible orientations. The two positions differed by a 180° rotation around the boron–carbon atom 1 bond of the inhibitor. $F_o - F_c$ difference electron density maps were contoured at 3σ and showed significant negative difference density surrounding the sulfur atom when in the position 180° from the one shown in Figure 2. Overall, the structure of the BZBTH2B/AmpC complex resembles the apo-enzyme, with a root-mean-square deviation (RMSD) for all atoms of 0.76 Å. The RMSD between the complexed and apo-enzymes for active site residues (Ser64, Lys67, Tyr150, Asn152, Tyr221, Lys315, and Ala318) in molecule 1 of the

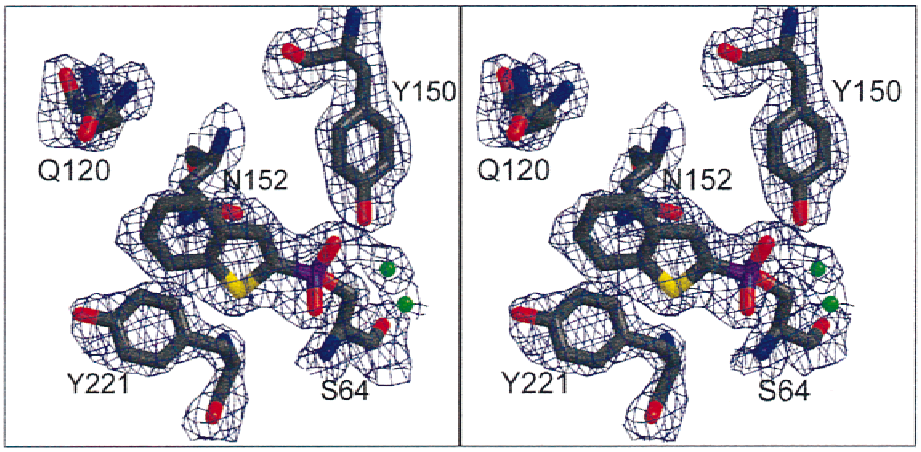

Fig. 2. $2F_o - F_c$ electron density (in stereo) of the refined model, contoured at 1σ. The area shown is the active site region with BZBTH2B covalently bound to the catalytic residue Ser64 of AmpC β-lactamase. Residues shown are within 3.8 Å of the inhibitor and are completely conserved in class C β-lactamases. Green spheres represent ordered water molecules. Carbon atoms are colored gray, oxygen atoms red, and nitrogen atoms blue. This figure was generated using the programs MOLSCRIPT (Kraulis, 1991) and Raster3D (Bacon & Anderson, 1988) and displayed using the program BOBSCRIPT (Esnouf, 1997).

asymmetric unit is 0.28 Å. This rises to 1.06 Å if Gln120 is included; this residue showed the most movement in the active site. The two monomers of AmpC in the asymmetric unit of the complex also resemble each other, differing by an RMSD of 0.84 Å for all atoms. The quality of the model was evaluated with Procheck (Laskowski et al., 1993); 90.2% of the nonglycine and nonproline residues were in the most favored regions of a Ramachandran plot (9.6% were in the additionally allowed region). The final R and R_{free} values of the refined structure were 16.7 and 22.4%, respectively. The structure has been deposited with the PDB as 1C3B.

The inhibitor appears to form quadrupole–quadrupole interactions with Tyr221 and quadrupole–dipole interactions with Asn152 (Fig. 3). The distance between ring carbon atom 7 of the inhibitor and the aromatic ring centroid of Tyr221 is 4.0 Å, and the distance between the centroid of the second ring of BZBTH2B and the amide nitrogen of Asn152 is 3.0 Å (Table 2). The angle between the rings of the inhibitor and Tyr221 is 47°, and the amide nitrogen of Asn152 is perpendicular to the ring plane of the inhibitor. These distances and angles are consistent with quadrupolar interactions observed in other protein structures (Thornton et al., 1988).

An observed interaction with mechanistic implications is the hydrogen bond between the boronic acid O2 atom and Tyr150 (2.8 Å, Fig. 3, Table 2). In forming this new interaction, the catalytic site has rearranged subtly. Lys67 and Tyr150, which usually form a hydrogen bond (Oefner et al., 1990; Lobkovsky et al., 1993, 1994; Usher et al., 1998), have moved by ~0.7 and 0.4 Å, respectively. Consequently, the interaction between them is lost; Lys67 now forms a hydrogen bond with the main-chain oxygen of Ala220 (not shown) while Tyr150 interacts with the boronic acid O2 atom (Fig. 3).

Table 1. *Data collection and refinement statistics*

Cell constants (Å; deg)	a = 118.86, b = 78.01, c = 98.96; β = 116.07
Resolution (Å)	2.25
Unique reflections	33,738
R_{merge}	9.4 (13.7)[a]
Completeness (%)	86.6 (76.4)[a]
$\langle I \rangle / \langle \sigma_I \rangle$	11.4
Resolution range for refinement (Å)	20–2.25 (2.37–2.25 Å)[a]
Number of water molecules	94
RMSD bond lengths (Å)	0.012
RMSD bond angles (deg)	1.732
R-factor (%)	16.7
R_{free} (%)	22.4[b]
Average B-factor, protein (Å^2)	28.63[c]
Average B-factor, inhibitor (Å^2)	25.42[c]

[a] Values in parentheses are for the highest resolution shell used in refinement.
[b] R_{free} was calculated with 10% of reflections set aside randomly.
[c] Values cited were calculated for both molecules in the asymmetric unit.

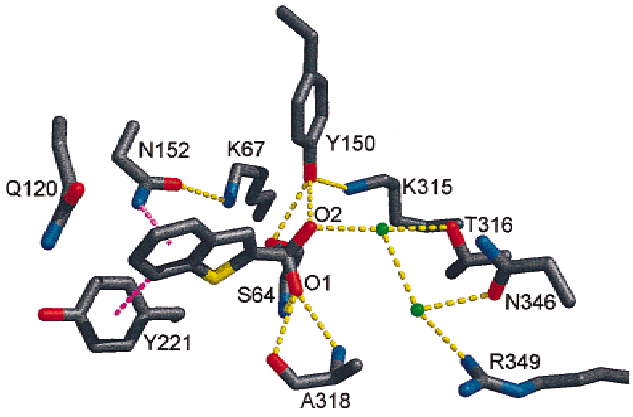

Fig. 3. Key interactions (Table 2) observed in the structure of BZBTH2B in complex with AmpC. Dashed lines indicate hydrogen bonding (yellow) or quadrupolar interactions (magenta). Green spheres represent ordered water molecules. The atoms are colored as in Figure 2. The figure was generated with MidasPlus (Ferrin et al., 1988).

Table 2. *Interactions in complexed and native AmpC β-lactamase*

Interaction	Distance (Å)[a]	
	Complex	Native
Y150OH–K315Nζ	2.89	2.54
Y150OH–O2	2.74	NP[b]
Y150OH–S64Oγ	2.91	3.21
Y150OH–K67Nζ	3.51	3.06
K67Nζ–A220O	2.96	3.47
S64N–O1	3.01	NP[b]
A318N–O1	2.84	NP[b]
A318O–O1	2.84	NP[b]
O2–H_2O	2.61	NP[b]
H_2O–T316Oγ1	3.03	NP[b]
H_2O–H_2O	3.50	NP[b]
H_2O–N346Oδ1	2.79	NP[b]
H_2O–R349Nη1	3.12	NP[b]
N152Oδ1–K67Nζ	2.65	2.67
K67Nζ–S64Oγ	2.60	3.49
N152Nδ2–Q120Oε1	6.11	3.05
N152Nδ2–centroid BZBTH2B aryl ring	3.07	NP[b]
CP7 BZBTH2B–centroid Y221	3.98	NP[b]
Centroid BZBTH2B aryl ring-centroid Y221	5.34	NP[b]

[a]All distances are for molecule 2 of the asymmetric unit.
[b]Not present in native structure.

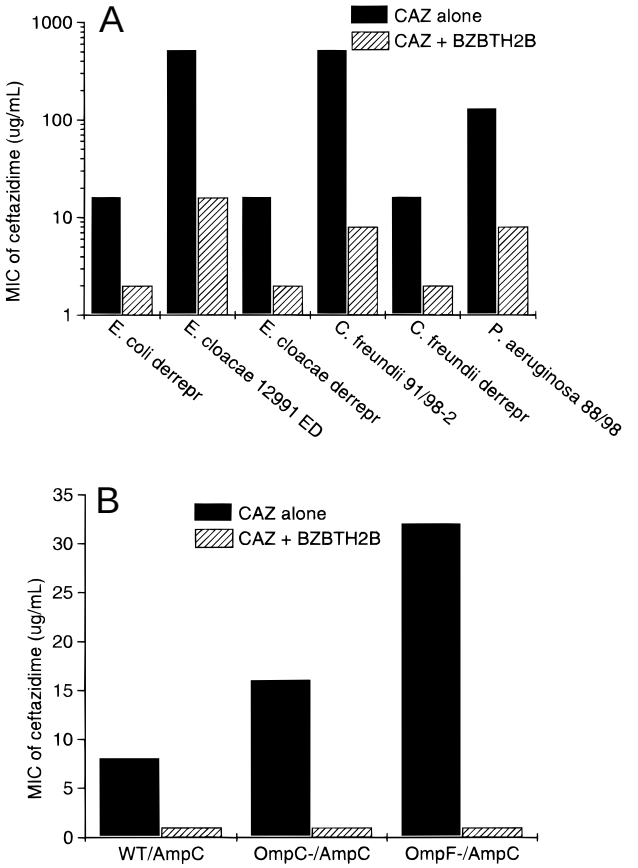

Fig. 4. A: Potentiation of the activity of the β-lactam ceftazidime (CAZ) by the inhibitor BZBTH2B against several resistant clinical isolates exhibiting derepressed production of chromosomal β-lactamase. **B:** Potentiation of the activity of CAZ by BZBTH2B against *E. coli* expressing AmpC, *E. coli* lacking OmpC but expressing AmpC, or *E. coli* lacking OmpF but expressing AmpC.

Two well-ordered water molecules appear in the active site, making extensive interactions that involve the inhibitor and the shifted Tyr150. The first water hydrogen bonds with the boronic acid O2 atom and with the active site residue Thr316 (Fig. 3; Table 2). The second water forms a hydrogen bond with the first water molecule. This second water also interacts with catalytic residues Asn346 and Arg349 (Fig. 3; Table 2). Additionally, the boronic acid O1 atom hydrogen bonds with the backbone nitrogens of Ser64 (2.9 Å) and Ala318 (2.7 Å), and also with the carbonyl oxygen of Ala318 (2.9 Å) (Table 2).

Antimicrobial activity of BZBTH2B

The high affinity of BZBTH2B led us to investigate its ability to potentiate the activity of β-lactams against resistant bacteria. BZBTH2B increased the activity of the widely used third-generation cephalosporin ceftazidime (CAZ) for all bacterial strains overexpressing AmpC β-lactamase against which it was tested (Fig. 4A). These included common hospital-acquired pathogens such as *E. coli*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, and *Citrobacter freundii*, which are often resistant to most β-lactams. BZBTH2B increased the efficacy of CAZ up to 64-fold and reduced (improved) its minimum inhibitory concentration (MIC) to as low as 2 μg/mL for some pathogens. BZBTH2B alone had no measurable antibiotic activity at these concentrations.

Because BZBTH2B is not a β-lactam, we hypothesized that it would be unaffected by traditional β-lactam resistance mechanisms. To test this, we investigated how the inhibitor was affected by porin channel mutants that are known to further increase resistance to β-lactams in conjunction with β-lactamases. *E. coli* with mutations in either of the main porin channels, OmpC or OmpF, were more resistant to CAZ compared with the parent strain, as expected. Conversely, BZBTH2B activity was unaffected by these mutants, potentiating the effect of CAZ to the same levels in the porin-mutant strains as in the parent wild-type strain (Fig. 4B).

Another traditional β-lactam resistance mechanism is the upregulation of β-lactamase transcription caused by β-lactams; β-lactams, including β-lactamase inhibitors such clavulanic acid and tazobactam (Kadima & Weiner, 1997), can induce expression of the enzyme that inactivates them. To test the role of BZBTH2B on the induction of AmpC, we investigated its ability to potentiate the action of the β-lactams CAZ and piperacillin. We compared the potentiation effect of BZBTH2B with that of cefoxitin (a cephalosporin) and clavulanic acid (a β-lactam-based inhibitor of β-lactamases) (Fig. 5).

In these experiments, two disks soaked with a known amount of a primary β-lactam, CAZ (top disk) and piperacillin (bottom disk), are placed on an agar plate that has been inoculated with a bacterium in which AmpC expression is inducible. A third disk soaked with a different compound, either cefoxitin, clavulanic acid or BZBTH2B, is placed on the agar plate between the two primary β-lactam disks. As the β-lactams diffuse from the primary disks into the agar, a clear zone (or halo) is created around the disk, indicating where bacteria are unable to grow. The shape and size of

this halo indicate the effect of the compound on the center disk on the induction of AmpC. The results of this experiment show that the circular inhibition halos normally surrounding CAZ and piperacillin are significantly diminished in the regions nearest to the cefoxitin disk (Fig. 5A) and the clavulanic acid disk (Fig. 5B). In contrast, the inhibition halos of CAZ and piperacillin are dramatically increased in the region near the BZBTH2B disk (Fig. 5C).

Discussion

BZBTH2B does not resemble β-lactams (Fig. 1), and yet it binds to AmpC tightly (27 nM). Our first interest in undertaking these studies was to determine the interactions responsible for this affinity.

Perhaps the most surprising interactions are those observed to occur in the region of the β-lactamase that is thought to be specific for the amide group found in the R1-side chain of β-lactams (Fig. 1). The R1-amide group is ubiquitous among penicillins and cephalosporins. Similarly, the amide recognition residue Asn152 is completely conserved among class C β-lactamases, and the analogous Asn132 and Asn161 are highly conserved among class A β-lactamases and some classes of PBPs, respectively (Massova & Mobashery, 1998). In complexes with serine β-lactamases and PBPs, the R1-amide oxygen hydrogen bonds with the asparagine

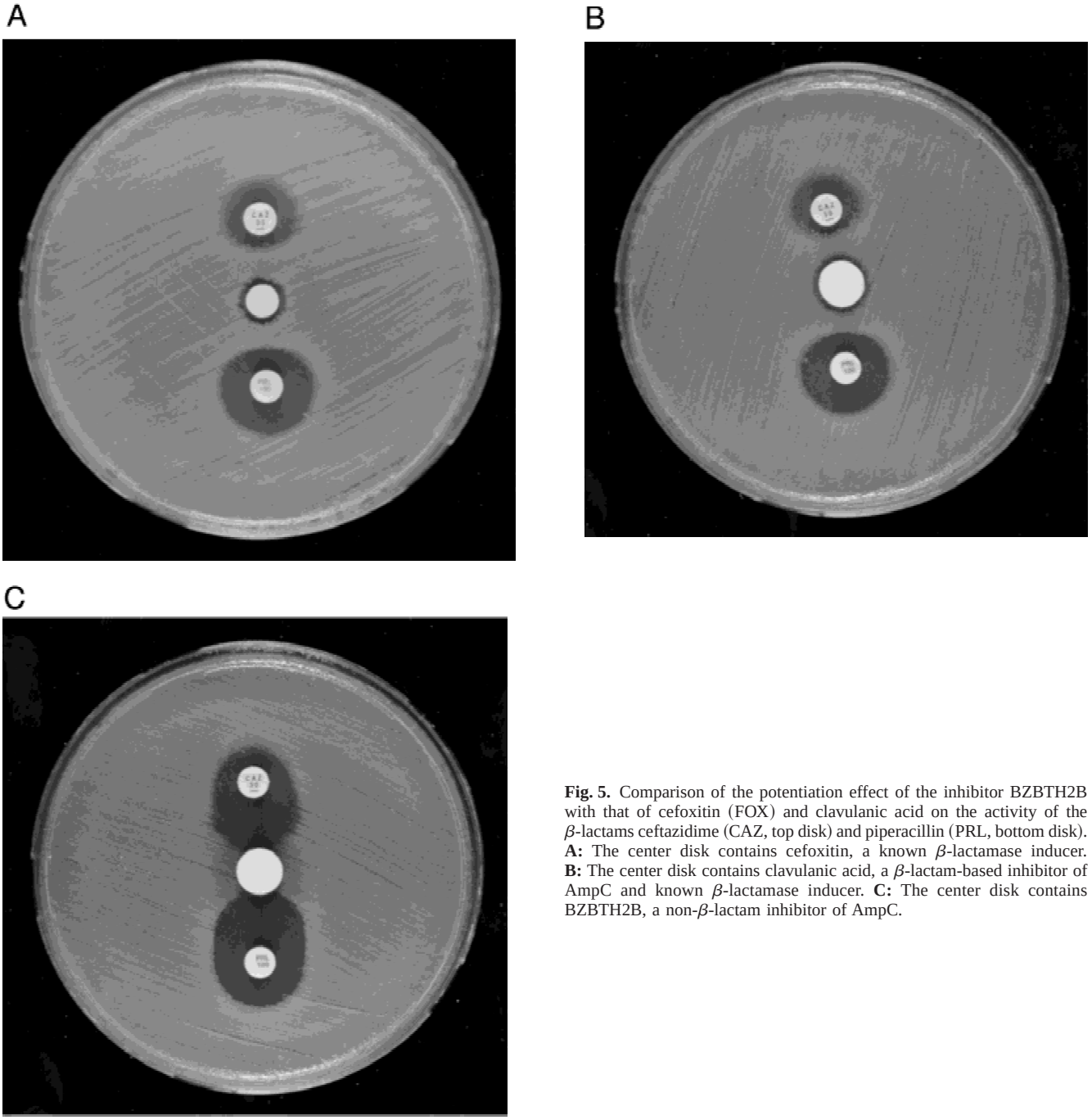

Fig. 5. Comparison of the potentiation effect of the inhibitor BZBTH2B with that of cefoxitin (FOX) and clavulanic acid on the activity of the β-lactams ceftazidime (CAZ, top disk) and piperacillin (PRL, bottom disk). **A:** The center disk contains cefoxitin, a known β-lactamase inducer. **B:** The center disk contains clavulanic acid, a β-lactam-based inhibitor of AmpC and known β-lactamase inducer. **C:** The center disk contains BZBTH2B, a non-β-lactam inhibitor of AmpC.

side-chain nitrogen of the enzyme (Oefner et al., 1990; Strynadka et al., 1992; Chen et al., 1993; Lobkovsky et al., 1994; Kuzin et al., 1995) (Fig. 6A). In the BZBTH2B/AmpC complex, the benzothiophene ring of the inhibitor has replaced the amide group of the β-lactam side chain; the inhibitor side chain overlays well with the amide group of cognate ligands (Fig. 6B). The quadrupolar interactions appear to be critical for the binding of BZBTH2B to AmpC; related inhibitors that lack the second aryl ring of BZBTH2B have affinities that are 100 to 1,000-fold lower for AmpC (Weston et al., 1998). Benzothiophene is not widely considered an amide analog; nevertheless, this electron-rich ring appears to be key to the recognition of BZBTH2B by the R1-amide recognition site of AmpC (Weston et al., 1998).

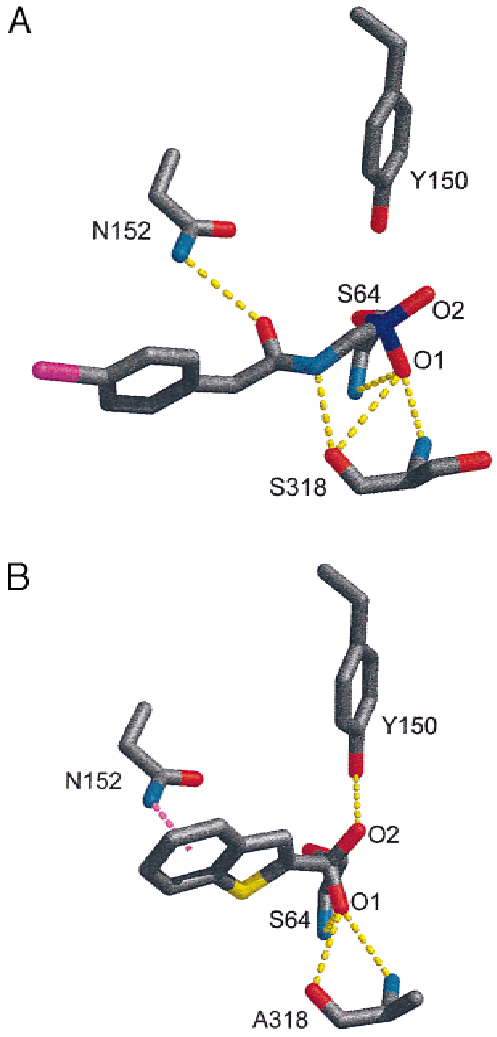

Fig. 6. Comparison of interactions in the amide recognition site between a ligand with an amide side chain and BZBTH2B. Dashed lines indicate hydrogen bonding (yellow) or quadrupolar (magenta) interactions between 2.6 and 3.2 Å. The figure was generated with MidasPlus. **A:** Phosphonate monoester inhibitor, m-carboxyphenyl[[N-[(p-iodophenyl)acetyl]amino] methyl]phosphonate, in complex with the AmpC homolog from *E. cloacae* (Lobkovsky et al., 1994). The conserved residues believed to make up the R1-amide site (Asn152 and Ser/Ala318) recognize the amide group of the inhibitor. Asn152 forms a hydrogen bond with the carbonyl oxygen of the amide group of the inhibitor. **B:** In the structure of BZBTH2B, the aryl rings of the inhibitor are observed to bind in the R1-amide site. Asn152 makes quadrupole–dipole interactions with the second aryl ring of BZBTH2B.

Despite its peculiar side chain, BZBTH2B is a transition-state analog and makes several interactions with AmpC that are mechanistically intriguing. Prominent among them is the hydrogen bond between the boronic acid O2 atom and the hydroxyl of Tyr150 (2.8 Å, Fig. 3). Several groups have proposed that Tyr150 is the general base for β-lactam hydrolysis (Oefner et al., 1990; Lobkovsky et al., 1994; Dubus et al., 1996); other groups have argued that it may not have a central role (Dubus et al., 1994). Indeed, substrate modification studies (Bulychev et al., 1997) have suggested that the catalytic base may be contributed at least partly by the substrate itself. Tyr150 has not previously been observed to interact directly with a ligand in any of the class C β-lactamase crystal structures, which has contributed to this controversy. The observation of a well-formed hydrogen bond with the O2 atom of the transition-state analog oxygen of BZBTH2B is consistent with its role as a catalytic base; the participation of other groups cannot be excluded.

An appropriate question is which transition state is the BZBTH2B/AmpC complex mimicking? Like serine proteases, the serine β-lactamase mechanism may be understood as proceeding through two acylation and two deacylation transition states (Lobkovsky et al., 1994), and Tyr150 could be involved in both. A clue to the identity of the mimicked transition state comes from the bound water molecule hydrogen bonding with the O2 atom of BZBTH2B and a second water molecule hydrogen bonding with the first. These two water molecules are part of an extensive hydrogen-bonding network with conserved residues Tyr150, Thr316, Asn346, and Arg349, and with the O2 atom of BZBTH2B (Fig. 3). They are located above the plane of where the β-lactam system would be expected to lie, as is the O2 atom of BZBTH2B itself. This is consistent with the suggestion that in class C β-lactamases the hydrolytic water attacks from the β-face of the β-lactam ring system (Bulychev et al., 1997). The O2 oxygen of BZBTH2B may represent the deacylating water following hydrolytic attack on the acyl-enzyme intermediate; the two water molecules to which it hydrogen bonds suggest a direction from which the attack takes place. These observations suggest that the BZBTH2B/AmpC complex mimics one of the deacylation transition states in β-lactam hydrolysis. The interactions of the boronic acid O2 atom with the waters and Tyr150 distinguish the BZBTH2B complex from that of a phosphonate transition-state analog complex with the class C β-lactamase from *E. cloacae*. If the phosphonate complex represents the later deacylation transition state associated with the departure of the leaving group Ser64 (Lobkovsky et al., 1994), the BZBTH2B/AmpC complex might represent the earlier deacylation transition state associated with hydrolytic attack.

As an aside, the O1 atom of the boronic acid forms hydrogen bonds with the main-chain nitrogens of Ser64 and Ala318. In this, it resembles "oxyanion" hole interactions observed in transition-state analog complexes of other β-lactamases and serine proteases. Unlike serine proteases, the O1 boronic acid oxygen is 2.84 Å from a main-chain carbonyl oxygen, that of Ala318. The O1 is almost certainly protonated and donates a hydrogen bond to the carbonyl of Ala318. As was described for another complex (Usher et al., 1998), the interaction of a ligand oxygen with the carbonyl oxygen of residue 318, or its equivalent, is ubiquitous among class A and class C β-lactamase and PBP complexes and may distinguish them mechanistically from serine proteases.

Based on the high affinity of this novel inhibitor for AmpC, we investigated the effect that BZBTH2B had on resistant bacteria when given in combination with a β-lactam antibiotic. BZBTH2B potentiated the activity of ceftazidime (CAZ) by up to 64-fold against several resistant strains of bacteria (Fig. 4A).

BZBTH2B does not resemble β-lactams (Fig. 1), and we reasoned that it might evade classic resistance mechanisms that have evolved against β-lactams. To test this hypothesis, we investigated the effect of some of these resistance mechanisms on the action of BZBTH2B. Porin channels are the major routes of entry for β-lactams into the cell. Mutations in these porin channels often increase resistance in conjunction with β-lactamases by reducing the access of β-lactams to PBPs. Because BZBTH2B is not a β-lactam, it might have other routes of diffusion through the outer membrane. Consistent with this view, the efficacy of the BZBTH2B/CAZ combination was undiminished in mutant strains of *E. coli* that lack two of these porin channels, compared to the wild-type strain (Fig. 4B).

Exposure to β-lactams, including β-lactam-based inhibitors of β-lactamases, often results in the up-regulation of β-lactamase transcription (Bennett & Chopra, 1993; Jacobs et al., 1997). An example of this inductive effect may be seen in the disk diffusion experiments (Fig. 5). The reduced size and flattened shape of the CAZ and piperacillin inhibition halos are consistent with the roles of cefoxitin (Fig. 5A) and clavulanic acid (Fig. 5B) as inducers of AmpC expression, as expected (Kadima & Weiner, 1997; Sanders et al., 1997). We reasoned that BZBTH2B, as a non-β-lactam, might not act as an inducer of β-lactamase expression. Consistent with this view, the inhibition halos of CAZ and piperacillin are dramatically increased in the region near the BZBTH2B disk (Fig. 5C). Rather than reducing the inhibition zone of CAZ and piperacillin, BZBTH2B increases them dramatically. Unlike β-lactams, BZBTH2B does not appear to induce β-lactamase expression; it simply inhibits the enzyme.

The overuse of antibiotics in the last half-century has led to the mobilization and propagation of pre-evolved resistance mechanisms, such as β-lactamases. Many of these resistance mechanisms, like the antibiotics to which they respond, are ancient. By targeting the three-dimensional structures of bacterial proteins, we have the opportunity to develop compounds that are new to microbial evolution. Such compounds may evade these ancient resistance mechanisms. BZBTH2B is an example of one such novel inhibitor. Although its chemistry differs from that of β-lactams, it binds to AmpC tightly; because its chemistry differs from β-lactams, it evades at least some of the classic β-lactam resistance mechanisms. The structure of the complex between BZBTH2B and AmpC reveals its mechanism of action and provides a template for further chemical elaboration.

Materials and methods

Enzyme preparation and crystal growth

AmpC from *E. coli* was expressed and purified to homogeneity as described (Usher et al., 1998). BZBTH2B was obtained from Lancaster Synthesis (Windham, New Hampshire) and was used without further purification. Cocrystals of BZBTH2B/AmpC were grown by vapor diffusion in hanging drops over 1.7 M potassium phosphate buffer (pH 8.7) using microseeding techniques. The initial concentration of protein in the drop was 100 μM, and the concentration of inhibitor was 360 μM. The inhibitor was added to the crystallizing drop in a 2% dimethylsulfoxide (DMSO) solution, 1.7 M potassium phosphate buffer (pH 8.7). Crystals appeared within three to five days after equilibration at 23 °C.

Data collection and refinement

The protein crystal was mounted in a silanized glass capillary and allowed to equilibrate overnight before data collection. Data were collected on an R-Axis-IIC image plate system at room temperature. Data were from a single crystal that showed significant decay by the end of the data collection.

Reflections were indexed, integrated, and scaled using the Denzo/Scalepack program suite (Otwinowski & Minor, 1997) (Table 1). The space group was C2 with two AmpC molecules in the asymmetric unit. Each AmpC molecule contained 358 residues. An initial model was built using molecular replacement with the native structure (Usher et al., 1998). Phases were calculated in X-PLOR (Brünger, 1992). The model was refined using rigid body and positional refinement techniques. Electron density maps were calculated in X-PLOR, and model building was done in the program O (Jones et al., 1991). The inhibitor was built into the observed difference density, and the structure of the complex was further refined in X-PLOR.

Antimicrobial experiments

Susceptibility testing was performed and interpreted following the guidelines of the National Committee for Clinical Laboratory Standards (1997). To test the inhibitory activity of BZBTH2B, the compound was dissolved in 50% DMSO, and dilutions were performed using growth medium. An adequate final concentration in which to determine the minimum inhibitory concentration (MIC) was obtained where the concentration of DMSO was maintained below 5%. The MIC of the β-lactam ceftazidime (CAZ), in the presence and absence of BZBTH2B, was determined against several resistant clinical isolates (*E. coli* derrepr, *E. cloacae* 12991 ED, *E. cloacae* derrepr, *C. freundii* 91/98-2, *C. freundii* derrepr, and *P. aeruginosa* 88/98) that show an AmpC-derepressed phenotype. The construction of the plasmid pBGMHN, which contains the AmpC gene from *E. cloacae* MHN-1, has been described (Morosini et al., 1998). Plasmid pBGMHN was introduced by transformation into the different strains of *E. coli* K-12. The ratio of CAZ to BZBTH2B used was 1:1 (w/w). Ceftazidime was kindly provided by Glaxo (Glaxo, Spain).

For the porin channel mutant experiments, the MICs of CAZ against *E. coli* lacking either OmpC or OmpF in the presence and absence of BZBTH2B were determined. These assays were performed in liquid media following the guidelines of the National Committee for Clinical Laboratory Standards (1997). The ratio of CAZ to BZBTH2B used was 1:1 (w/w). The *E. coli* K-12 strains used in this work were: MC4100 (F-Δ(*argF-lac*)*U169 araD139 deoC1 flbB5301 ptsF25 relA1 thiA rpsL150*) (Casadaban, 1976), MH621 (MC4100 *ompF*::*lacZ*) (Hall & Silhavy, 1981), MH221 (MC4100 *ompC*::*lacZ*) (Hall & Silhavy, 1979).

For the β-lactamase induction experiments, plates of Mueller Hinton agar were inoculated with a clinical strain of *E. cloacae* in which production of AmpC is inducible by β-lactam antibiotics. Inhibitors were added to blank disks, and the final content of inhibitor per disk was 64 μg of BZBTH2B and 32 μg clavulanic acid. Disks of ceftazidime, cefoxitin, and piperacillin contained 30, 30, and 100 μg, respectively.

Acknowledgments

This work was partly supported by NSF MCB 9734484 and NIH GM59957 (both to B.K.S.). RAP was partly supported by NIH Training Grant T32 ES07284. We thank J. Widom, D. Freymann, L. Van Eldik, and B. Beadle for reading this manuscript and Dr. L. Martinez for some clinical strains of bacteria.

References

Bacon DJ, Anderson WF. 1988. A fast algorithm for rendering space-filling molecule pictures. *J Mol Graph 6*:219–220.

Baquero F, Blazquez J. 1997. Evolution of antibiotic resistance. *Trends Ecol Evol 11*:482–487.

Beesley T, Gascoyne N, Knott-Hunziker V, Petursson S, Waley SG, Jaurin B, Grundstrom T. 1983. The inhibition of class C beta-lactamases by boronic acids. *Biochem J 209*:229–233.

Bennett PM, Chopra I. 1993. Molecular basis of beta-lactamase induction in bacteria. *Antimicrobiol Agents Chemother 37*:153–158.

Brünger AT. 1992. *X-PLOR version 3.1: A system for X-ray crystallography and NMR*. New Haven, Connecticut: Yale University Press.

Bulychev A, Massova I, Miyashita K, Mobashery S. 1997. Nuances of mechanisms and their implications for evolution of the versatile beta-lactamase activity: From biosynthetic enzymes to drug resistance factors. *J Am Chem Soc 119*:7619–7625.

Bush K. 1997. The evolution of beta-lactamases. *Ciba Found Symp 207*:152–163.

Bush K, Jacoby GA, Medeiros AA. 1995. A functional classification scheme for beta-lactamases and its correlation with molecular structure. *Antimicrobiol Agents Chemother 39*:1211–1233.

Casadaban MJ. 1976. Transposition and fusion of the lac genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. *J Mol Biol 104*:541–555.

Chen CC, Rahil J, Pratt RF, Herzberg O. 1993. Structure of a phosphonate-inhibited beta-lactamase. An analog of the tetrahedral transition state/intermediate of beta-lactam hydrolysis. *J Mol Biol 234*:165–178.

Davies J. 1994. Inactivation of antibiotics and the dissemination of resistance genes. *Science 264*:375–382.

Dubus A, Ledent P, Lamotte-Brasseur J, Frere JM. 1996. The roles of residues Tyr150, Glu272, and His314 in class C beta-lactamases. *Proteins 25*:473–485.

Dubus A, Normark S, Kania M, Page MG. 1994. The role of tyrosine 150 in catalysis of beta-lactam hydrolysis by AmpC beta-lactamase from *Escherichia coli* investigated by site-directed mutagenesis. *Biochemistry 33*:8577–8586.

Esnouf RM. 1997. An extensively modified version of MolScript that includes greatly enhanced coloring capabilities. *J Mol Graph Model 15*:132–134, 112–113.

Ferrin TE, Huang CC, Jarvis LE, Langridge R. 1988. The MIDAS display system. *J Mol Graph 6*:13–27.

Gonzalez Leiza M, Perez-Diaz JC, Ayala J, Casellas JM, Martinez-Beltran J, Bush K, Baquero F. 1994. Gene sequence and biochemical characterization of FOX-1 from Klebsiella pneumoniae, a new AmpC-type plasmid-mediated beta-lactamase with two molecular variants. *Antimicrobiol Agents Chemother 38*:2150–2157.

Hall MN, Silhavy TJ. 1979. Transcriptional regulation of *Escherichia coli* K-12 major outer membrane protein 1b. *J Bacteriol 140*:342–350.

Hall MN, Silhavy TJ. 1981. The ompB locus and the regulation of the major outer membrane porin proteins of *Escherichia coli* K12. *J Mol Biol 146*:23–43.

Jacobs C, Frere JM, Normark S. 1997. Cytosolic intermediates for cell wall biosynthesis and degradation control inducible beta-lactam resistance in Gram-negative bacteria. *Cell 88*:823–832.

Jones TA, Zou JY, Cowan SW, Kjeldgaard. 1991. Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr A 47*:110–119.

Kadima TA, Weiner JH. 1997. Mechanism of suppression of piperacillin resistance in enterobacteria by tazobactam. *Antimicrobiol Agents Chemother 41*:2177–2183.

Koeck JL, Arlet G, Philippon A, Basmaciogullari S, Thien HV, Buisson Y, Cavallo JD. 1997. A plasmid-mediated CMY-2 beta-lactamase from an Algerian clinical isolate of *Salmonella senftenberg*. *FEMS Microbiol Lett 152*:255–260.

Kraulis PJ. 1991. MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. *J Appl Crystallogr 24*:946–950.

Kuzin AP, Liu H, Kelly JA, Knox JR. 1995. Binding of cephalothin and cefotaxime to D-Ala-D-Ala-peptidase reveals a functional basis of a natural mutation in a low-affinity penicillin-binding protein and in extended-spectrum beta-lactamases. *Biochemistry 34*:9532–9540.

Laskowski RA, MacArthur MW, Moss DS, Thornton JM. 1993. PROCHECK: A program to check the stereochemical quality of protein structures. *J Appl Crystallogr 26*:283–291.

Lobkovsky E, Billings EM, Moews PC, Rahil J, Pratt RF, Knox JR. 1994. Crystallographic structure of a phosphonate derivative of the Enterobacter cloacae P99 cephalosporinase: Mechanistic interpretation of a beta-lactamase transition-state analog. *Biochemistry 33*:6762–6772.

Lobkovsky E, Moews PC, Liu H, Zhao H, Frere JM, Knox JR. 1993. Evolution of an enzyme activity: crystallographic structure at 2-A resolution of cephalosporinase from the ampC gene of Enterobacter cloacae P99 and comparison with a class A penicillinase. *Proc Natl Acad Sci USA 90*:11257–11261.

Massova I, Mobashery S. 1998. Kinship and diversification of bacterial penicillin-binding proteins and beta-lactamases. *Antimicrobiol Agents Chemother 42*:1–17.

Morosini MI, Negri MC, Shoichet B, Baquero MR, Baquero F, Blazquez J. 1998. An extended-spectrum AmpC-type beta-lactamase obtained by in vitro antibiotic selection. *FEMS Microbiol Lett 165*:85–90.

National Committee for Clinical Laboratory Standards. 1997. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A4*, Vol. 17. Villanova, Pennsylvania: University of Villanova.

Neu HC. 1992. The crisis in antibiotic resistance. *Science 257*:1064–1073.

Nukaga M, Haruta S, Tanimoto K, Kogure K, Taniguchi K, Tamaki M, Sawai T. 1995. Molecular evolution of a class C beta-lactamase extending its substrate specificity. *J Biol Chem 270*:5729–5735.

Oefner C, D'Arcy A, Daly JJ, Gubernator K, Charnas RL, Heinze I, Hubschwerlen C, Winkler FK. 1990. Refined crystal structure of beta-lactamase from *Citrobacter freundii* indicates a mechanism for beta-lactam hydrolysis. *Nature 343*:284–288.

Otwinowski Z, Minor W. 1997. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol 276*:307–326.

Sanders CC. 1992. Beta-lactamases of Gram-negative bacteria: New challenges for new drugs. *Clin Infect Dis 14*:1089–1099.

Sanders CC, Bradford PA, Ehrhardt AF, Bush K, Young KD, Henderson TA, Sanders WE Jr. 1997. Penicillin-binding proteins and induction of AmpC beta-lactamase. *Antimicrobiol Agents Chemother 41*:2013–2015.

Strynadka NC, Adachi H, Jensen SE, Johns K, Sielecki A, Betzel C, Sutoh K, James MN. 1992. Molecular structure of the acyl-enzyme intermediate in beta-lactam hydrolysis at 1.7 A resolution. *Nature 359*:700–705.

Strynadka NC, Martin R, Jensen SE, Gold M, Jones JB. 1996. Structure-based design of a potent transition state analogue for TEM-1 beta-lactamase. *Nat Struct Biol 3*:688–695.

Sutherland R. 1991. Beta-lactamase inhibitors and reversal of antibiotic resistance. *Trends Pharmacol Sci 12*:227–232.

Thornton JM, Singh J, Campbell S, Blundell TL. 1988. Protein–protein recognition via side-chain interactions. *Biochem Soc Trans 16*:927–930.

Usher KC, Blaszczak LC, Weston GS, Shoichet BK, Remington SJ. 1998. Three-dimensional structure of AmpC beta-lactamase from *Escherichia coli* bound to a transition-state analogue: Possible implications for the oxyanion hypothesis and for inhibitor design. *Biochemistry 37*:16082–16092.

Weston GS, Blazquez J, Baquero F, Shoichet BK. 1998. Structure-based enhancement of boronic acid-based inhibitors of AmpC beta-Lactamase. *J Med Chem 41*:4577–4586.