Crystal Structure and Activity Studies of the *Mycobacterium tuberculosis* β-Lactamase Reveal Its Critical Role in Resistance to β-Lactam Antibiotics

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β-Lactam antibiotics are extremely effective in disrupting the synthesis of the bacterial cell wall in both gram-positive and gram-negative bacteria. However, they are ineffective against *Mycobacterium tuberculosis*, due to the production of a β-lactamase enzyme encoded on the chromosome of *M. tuberculosis* that degrades these antibiotics. Indeed, recent studies have demonstrated that deletion of the *blaC* gene, the only gene encoding a β-lactamase in *M. tuberculosis*, or inhibition of the encoded enzyme resulted in significantly increased sensitivity to β-lactam antibiotics. In this paper we present a biochemical and structural characterization of *M. tuberculosis* BlaC. Recombinant BlaC shows a broad range of specificity with almost equal penicillinase and cepholothinase activity. While clavulanate is a mechanism-based inhibitor to class A β-lactamase with high potency (typically $K_i < 0.1$ μM), it is a relatively poor inhibitor of the *M. tuberculosis* BlaC ($K_i = 2.4$ μM). The crystal structure of the enzyme, determined at a resolution of 1.7 Å, shows that the overall fold of the *M. tuberculosis* enzyme is similar to other class A β-lactamases. There are, however, several distinct features of the active site, such as the amino acid substitutions N132G, R164A, R244A, and R276E, that explain the broad specificity of the enzyme, relatively low penicillinase activity, and resistance to clavulanate.

Penicillin G was the first β-lactam antibiotic discovered, and it was used extensively to treat bacterial infections from the 1940s to 1960s (5). Over the past 3 decades, many other potent β-lactam antibiotics, such as cephems, monobactams, and carbapenems, have been developed and are currently being utilized to treat a wide range of bacterial infections. In fact, the high efficacy, high specificity, and low rate of adverse reactions make β-lactam antibiotics the most prescribed antimicrobial agents worldwide (12). All β-lactam antibiotics show structural mimicry to the natural substrates of the bacterial DD-transpeptidases. DD-Transpeptidases, members of the penicillin-binding protein family, catalyze the cross-linking of two peptidoglycan strands, which is an essential step in bacterial cell wall biosynthesis. Inhibition of DD-transpeptidases by β-lactam antibiotics leads to disruption of the synthesis of the bacteria's cell wall (13).

A significant increase in the number of β-lactam-resistant strains of pathogenic bacteria has been reported over the past few years (35). Resistance to the β-lactams primarily occurs through the horizontal transfer of β-lactamase genes contained on plasmids (9). β-Lactamases inactivate β-lactam antibiotics by efficiently hydrolyzing the amide group of the β-lactam ring, which is the prevalent mechanism of bacterial resistance to β-lactam antibiotics (13). β-Lactamases are grouped into four classes, A, B, C, and D, based on their amino acid sequence homology and general enzyme catalytic properties (for a review of β-lactamases, see reference 13). Historically, class A β-lactamases were found to have much greater penicillinase

activity than cephalosporinase activity. However, several new class A β-lactamases that are active against new generations of cephalosporins and carbapenems have been discovered. They are typically grouped as extended-spectrum β-lactamases (ESBLs) to differentiate them from the non-ESBLs.

There have been few studies on the antitubercular effect of β-lactam antibiotics, probably due to the early discovery that *Mycobacterium tuberculosis* possesses intrinsic resistance to these antibiotics (25). The production of β-lactamase has been proposed to be the most significant reason for mycobacterial resistance (7, 45, 55). Other factors that were believed to contribute to the ineffectiveness of β-lactam in *M. tuberculosis* include cell envelope permeability and low penicillin-binding protein binding affinity for β-lactams (29, 53). Although *M. tuberculosis*'s outer cell wall, formed by a waxy mycolic acid layer, was thought to make a virtually impenetrable barrier, β-lactams have recently been shown to readily permeate the *M. tuberculosis* cell wall and bind the penicillin-binding proteins (7).

One approach that holds promise to counter *M. tuberculosis*'s inherent resistance is to utilize β-lactamase inhibitors in combination with β-lactam antibiotics to increase their efficacy (7). For example, class A β-lactamases are generally sensitive to serine β-lactamase inhibitors such as clavulanate and sulbactam (2, 17). These mechanism-based inhibitors covalently cross-link two serines in the active site that are essential to the hydrolysis of β-lactams and permanently inactivate β-lactamase. The combinations of β-lactam antibiotics and β-lactamase inhibitors have been used clinically to treat a wide range of bacterial infections (35). These combinations have also been shown to be somewhat effective against most strains of mycobacteria, including multidrug-resistant (MDR) strains in vitro

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(11, 48, 56). In fact, MICs of β-lactams are less than 0.5 μg/ml for MDR strains of *M. tuberculosis* when used in combination with both β-lactamase inhibitors and the antitubercular drug ethambutol (1). These results offer the possibility for treatment of tuberculosis with highly potent inhibitors in combination with β-lactam antibiotics.

blaC (Rv2068c) is the only gene in the *M. tuberculosis* genome that shows any detectable homology to any of the known β-lactamase genes. Recently, the Pavelka laboratory showed that the deletion of the *blaC* gene resulted in an increased susceptibility of *M. tuberculosis* to β-lactam antibiotics by 8- to 256-fold (14, 15), which confirmed the primary role of *blaC* in *M. tuberculosis* resistance. In previous studies, partially purified *M. tuberculosis* BlaC was classified as a class A β-lactamase type 2b based on its relatively high level of penicillinase activity (6, 46). To fully characterize *M. tuberculosis* BlaC and facilitate the design of new inhibitors, we have characterized the substrate and inhibitor profile for BlaC and determined the structure of BlaC to high resolution.

MATERIALS AND METHODS

Cloning, expression, and purification. *Escherichia coli* expression plasmids of the wild type and a truncated form (codons 41 to 307) of *M. tuberculosis blaC* were cloned from genomic DNA (CSU N01-AI-75320). The amplified product was inserted into pET28b (EMD Biosciences no. 69865-3) using the NdeI and HindIII restriction sites. This plasmid was subsequently transformed into *E. coli* BL21(DE3) (EMD Bioscience no. 69387-3) and cultured in Luria-Bertani–Miller media containing 50 μg/ml kanamycin at 37°C until the optical density at 600 nm reached 0.8. Expression of the *blaC* gene was induced for 20 h at 16°C by addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation and resuspended in 25 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 2 mM β-mercaptoethanol and were lysed by French press. After treatment with 1 mM DNase I, the insoluble material was removed by centrifugation. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), full-length BlaC goes into the insoluble pellet, while ~90% of truncated BlaC (41–307) remained soluble. Therefore, further purification was performed only on truncated BlaC (41–307). The supernatant containing soluble protein was applied to a Ni²⁺-loaded HiTrap chelating column (AP Biotech) using a fast protein liquid chromatography system and eluted through an imidazole gradient (0 to 0.5 M). BlaC (41–307) was eluted at an imidazole concentration of 120 mM. Fractions that contain BlaC (41–307) were pooled and dialyzed against 25 mM Tris-HCl (pH 8.0), 40 mM NaCl, and 1 mM dithiothreitol. The samples were further purified by gel filtration on a Superdex 200 column to separate the monomeric protein from aggregated material using dialysis buffer. The protein was concentrated to approximately 10 mg/ml. BlaC (41–307) was obtained at 45 mg/liter of *E. coli* culture and appeared homogeneous by SDS-PAGE and Coomassie blue staining.

Kinetic assays. Enzyme kinetic evaluation was completed for the substrates ampicillin ($\Delta\varepsilon_{235} = -820$ M⁻¹ cm⁻¹), cephalothin ($\Delta\varepsilon_{262} = -7,660$ M⁻¹ cm⁻¹), cefoxitin ($\Delta\varepsilon_{270} = -8,380$ M⁻¹ cm⁻¹), ceftazidime ($\Delta\varepsilon_{260} = -10,200$ M⁻¹ cm⁻¹), and meropenem ($\Delta\varepsilon_{299} = -2,500$ M⁻¹ cm⁻¹) (24). All assays were repeated in triplicate and carried out on a Cary 100 Bio spectrophotometer at room temperature by monitoring the UV absorbance change of β-lactams (24). The *M. tuberculosis* BlaC activity assays were performed in 100 mM phosphate buffer (pH 7.5) by adding 10 nM enzyme into substrate solution. K_m and V_{max} were determined by analyzing a Hanes plot (57). Inhibition studies of clavulanate were performed by adding enzyme into solutions containing the substrate ampicillin and inhibitor clavulanate with increasing concentrations (0.1 to 600 μM). During the measurement time, no time-dependent loss of enzyme activity could be detected. Values for K_i were determined from the *x* intercepts of a Dixon plot, assuming competitive inhibition.

Crystallization of BlaC (41–307). Crystallization of the truncated enzyme, missing the first 40 amino acids, was accomplished by the hanging-drop vapor diffusion method (33). Initial crystallization conditions were discovered by sparse matrix screen using crystal screens from Hampton Research. Hanging drops containing 2 μl of protein solution at 10 mg/ml and 2 μl of buffer (0.1 M Tris-HCl, pH 8.0, 2.0 M NH₄H₂PO₄) were equilibrated at 16°C in Linbro plates against 1 ml of the same buffer. Protein crystals appeared in 4 days as thin plate-shaped clusters and were improved to larger rod-like clusters by cocrystallization with its substrate ampicillin. Macroseeding was then used to obtain high-quality single crystals.

Data collection and processing. A nearly complete diffraction data set to 1.7-Å resolution was collected from a single crystal at 121°K using a cryoprotection solution consisting of reservoir solution (0.1 M Tris-HCl, pH 8.0, 2.0 M NH₄H₂PO₄) with the addition of 20% glycerol. Beam line 14BMC at the Advanced Photon Source, Argonne National Laboratory, was used to collect the data set using 1-degree oscillation widths over a range of 180°. The data were integrated and reduced using HKL2000 (42). The completeness of low-resolution data is slightly lower, due to the ice rings caused by the high rejection rate. Crystals of BlaC belong to the orthorhombic space group P2₁2₁2₁ with the following cell dimensions: $a = 42.74$ Å, $b = 71.28$ Å, $c = 85.17$ Å, $\alpha = 90°$, $\beta = 90°$, $\gamma = 90°$, with one molecule in each asymmetric unit.

Structure determination and model refinement. Initial phases were obtained by molecular replacement with the computer program MolRep, part of the CCP4 package (3), using the related structure of β-lactamase from *Streptomyces albus* G (Protein Data Bank [PDB] identification [ID] 1BSG) (10) as a search model. The best solution gave a correlation coefficient of 34.2% and a R_{factor} of 50.9% using data between 25- and 3-Å resolution. The structure from the molecular replacement solution was refined with REFMAC, again in the CCP4 suite (3). The first step in the refinement was to use rigid-body optimization, and this resulted in a slightly improved model (R_{factor}, 49.9%; R_{free}, 51.8%; 54.6 to 1.7 Å). Electron density maps were produced and manual rebuilding was carried out using XtalView (34). The model was further refined with cycles of model building and REFMAC restrained refinement (3). During the final cycles of the refinement, water molecules were added based on peaks above 3-σ in the $F_o - F_c$ electron density maps that were within hydrogen-bonding distances from the appropriate protein atom. The resulting structure had good geometry (88% of the nonglycine residues were in the most favorable conformation, and none were in a disallowed conformation) and an R_{factor} of 17.9% and R_{free} of 21.4%. PROCHECK (3) revealed no disallowed φ and ψ, and the overall structure G factor, a measure of a given stereochemical property, was consistently better than expected for this resolution.

Structure analysis. DALI (22) was used to search the PDB for proteins having folds similar to *M. tuberculosis* BlaC, in the order of structural similarity. SwissPDB viewer was used to make structural alignments (19). The model was evaluated and analyzed using computer graphic program SPOCK (8).

PDB identification. The atomic coordinates of BlaC have been deposited in the PDB with the entry code 2GDN.

RESULTS AND DISCUSSION

Sequence analysis of BlaC. *M. tuberculosis* BlaC is a 307-amino-acid protein that is predicted to have a signal peptide for translocation into the periplasm and a lipid attachment site (residues 14 to 24) using the computer program ScanProsite (18). This analysis suggests that the mature BlaC is linked via the covalently attached lipid to the outer leaflet of the inner membrane after translocation to the periplasmic space. β-Lactamase lipoproteins have been reported for several gram-positive bacteria (39, 50). However, the enzyme was also predicted using the SignalP program (4) to have a signal peptidase II cleavage site at residue 28, which would eliminate the lipid attachment site in the mature protein. It is therefore not clear if BlaC exists free or as a membrane-bound protein in the periplasm.

M. tuberculosis BlaC shares approximately 40% amino acid sequence identity with other known class A β-lactamases (Fig. 1). Interestingly, it has the highest sequence identity to the class A β-lactamase of *Streptomyces clavuligerus* (54%), the bacterium that produces the antibiotic cephamycin and the β-lactamase inhibitor clavulanate.

Three highly conserved regions of the active site, designated motifs I, II, and III, have been identified by multiple sequence alignment of class A β-lactamases from different bacteria. Motif I has a consensus sequence of Arg⁶¹-(Xaa)₂-Glu-Xaa-Phe-
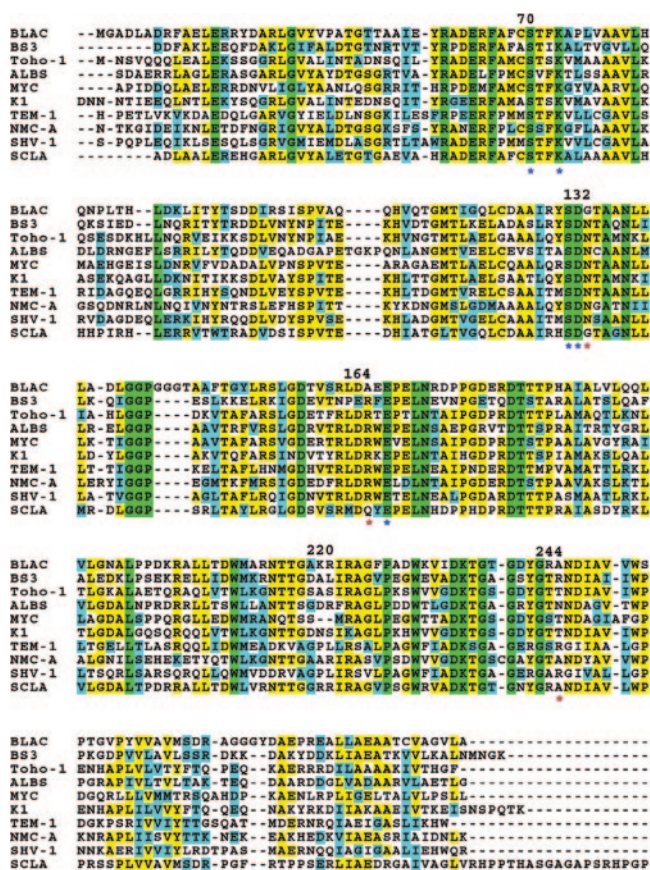
FIG. 1. Sequence alignment of β lactamases. Sequence alignment of β lactamases from *M. tuberculosis* (BLAC), *B. licheniformis* (BS3), *S. albus* (ALBS), *E. coli* (Toho-1 and TEM-1), *E. cloacae* (NMC-A), *P. vulgaris* (K1), *M. fortuitum* (MYC), *K. pneumoniae* (SHV-1). Green, completely conserved residues; yellow, identical residues; cyan, similar residues. The important residues mentioned in the context are labeled. The residues labeled by asterisks are the equivalents of those indicated significant to the activity.

$(Xaa)_3$-Ser-Xaa-Xaa-Lys^{73} (the residues of *M. tuberculosis* BlaC were numbered according to a consensus ABL numbering scheme [2]), which extends from the end of the β-strand B2 through a segment of the α-helix H2. Motif II corresponds to the sequence Ser^{130}-Asp-Asn^{132} and is located between α-helices H6 and H7. Motif III, located on β-strand B3, has the consensus sequence Asp^{233}-Lys-Thr-Gly^{236}. Additionally, all class A β-lactamases have an Ω loop that in BlaC contains residues Val^{159} to Thr^{180}. While these regions are highly conserved, BlaC from *M. tuberculosis* has several notable amino acid substitutions. The substitution of a Gly for Asn at residue 132 occurs only in class A β-lactamase from *M. tuberculosis* and its closest homolog, *S. clavuligerus* β-lactamase. The substitutions R244A and R169A in *M. tuberculosis* β-lactamase are also rare in class A β-lactamases. Another important difference identified from amino acid sequence alignment is the four-residue insertion Gly^{145A} to Ala^{150} and the two-residue insertion Gly^{271} and Tyr^{272} in the *M. tuberculosis* BlaC sequence.

Kinetic study. Initial attempts to express the full-length protein were unsuccessful. However, by truncating the codons for the first 40 amino acids in our expression construct, we could

produce large quantities of active enzyme. This truncation was not predicted to interfere with the enzyme's catalytic function based on what was known about homologous enzymes. The molecular weight of the truncated protein was confirmed by matrix-assisted laser desorption ionization (actual mass, 30,629 Da; calculated mass, 30,666 Da).

Kinetic measurements of the hydrolysis of the substrates ampicillin, cephalothin, cefoxitin, ceftazidime, and meropenem, as well as the inhibition of the enzyme activity with clavulanate, were carried out in order to compare the enzyme activity to other class A β-lactamases (Table 1). Overall, the results show that *M. tuberculosis* BlaC has a broad-spectrum substrate profile compared to other class A β-lactamases. While the penicillinase activity of BlaC ($k_{cat}/K_m = 293$ mM^{-1} s^{-1}) is about 10-fold lower than most class A β-lactamases, the enzyme showed relatively good activity against cephalothin ($k_{cat}/K_m = 103$ mM^{-1} s^{-1}) in contrast to previous studies where *M. tuberculosis* was shown to possess only minor cephalothin activity (59). Indeed, we found that the cephalosporinase activity of *M. tuberculosis* BlaC is comparable to its penicillinase activity. Furthermore, BlaC shows modest activity ($k_{cat}/K_m \sim 10$ mM^{-1} s^{-1}) against cefoxitin, ceftazidime, and meropenem. Inhibition studies indicated that clavulanate, a very potent inhibitor of most of class A β-lactamases ($K_i < 0.1$ μM), is a relatively poor inhibitor of *M. tuberculosis* BlaC ($K_i = 2.4$ μM). The substrate profile of BlaC is quite similar to that of *Mycobacterium fortuitum* β-lactamase, which also has relatively high cephalosporinase activity (45).

Overall structure. The crystal structure of BlaC was solved using molecular replacement and refined to 1.7-Å resolution. The final R_{factor} and R_{free} values for all reflections were 0.18 and 0.21, respectively (Table 2). BlaC crystals are orthorhombic, belonging to space group P2$_1$2$_1$2$_1$, with one molecule in the asymmetric unit. The refined structure of BlaC includes 265 of the 267 residues in the truncated form of BlaC (41–307) and 248 ordered water molecules. The structure of the N-terminal six-histidine tag along with the first two residues, Gly-Ala, was not defined due to disorder of this region in the electron density map. Like other class A β-lactamases, the BlaC monomer contains an α domain and an α/β domain. The α domain consists of six long α helices (H2, H5, H6, H7, H9, and H10) and three short α helices (H3, H4, and H8). The α/β domain includes five antiparallel β-strands (B1 to B5), the N- and C-terminal α helices (H1 and H12), and one short α helix, H11 (Fig. 2). The overall structure of BlaC is very similar to other class A β-lactamase structures. The root mean square differences (RMSD) of the C$_α$ atoms of several class A β-lactamases range between 0.95 to 3.66 Å and are listed in Table 3. The C$_α$

TABLE 1. Kinetic parameters of *M. tuberculosis* class A β-lactamase with different antibiotics as substrates

Class	Antibiotic	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})	K_i (μM)
Penicillin	Ampicillin	18.5 ± 0.9	63 ± 7	293	
Cephalosporin	Cephalothin	12.1 ± 1.1	117 ± 14	103	
	Cefoxitin	1.1 ± 0.3	195 ± 15	5.6	
	Ceftazidime	0.2 ± 0.1	593 ± 29	0.4	
Carbapenem	Meropenem	0.9 ± 0.3	279 ± 23	3.2	
inhibitor	Clavulanate				2.4 ± 1.1

TABLE 2. Data collection, processing, and refinement statistics

Parameter	Value[b] for BlaC
Data collection	
Maximum resolution (Å)	1.72
Space group	$P2_12_12_1$
a (Å)	42.7
b (Å)	71.3
c (Å)	85.2
Unique reflections	25,908 (2,808)
R_{sym}[a] (%)	11.4 (30.6)
Completeness (%)	91.1 (100)
Redundancy	6.8 (6.9)
I/σ	23.2 (6.8)
Refinement statistics	
Resolution range (Å)	54.64–1.72
Number of reflections	24,570
Number of atoms/subunit	
Protein	2,230
Solvent	248
R_{cryst} (%)	17.9
R_{free} (%)	21.4
Avg B factors (Å^2)	
Protein	20.7
Solvent	32.8
RMSD from ideal values	
Bonds (Å)	0.011
Angles (°)	1.44

[a] $R_{sym} = \Sigma_{hkl} \Sigma_i I_i(hkl) - \langle I(hkl) \rangle / \Sigma_{hkl} \Sigma_i I_i(hkl)$, where I_i is the intensity of observation and $\langle I \rangle$ is the mean intensity of reflection.

[b] The numbers given in parentheses denote the respective values of the highest-resolution shell.

TABLE 3. Structure comparison of class A β-lactamases from different species

Enzyme	Source	PDB ID	Sequence identity (%)	RMSD (Å)
BlaC	*M. tuberculosis*			
BS3	*B. licheniformis*	1I2S	47.3	1.57
ALBS	*S. albus*	1BSG	45.0	3.66
Toho-1	*E. coli*	1IYS	46.5	1.87
SME-1	*S. marcescens*	1DY6	42.5	0.97
NMC-A	*E. cloacae*	1BUE	41.1	1.00
K1	*P. vulgaris*	1HZO	42.9	0.95
MYC	*M. fortuitum*	1MFO	40.7	1.54
SHV-1	*K. pneumoniae*	1SHV	40.0	2.74
TEM-1	*E. coli*	1BT5	38.0	2.85

compared to SHV-1 and TEM-1 (RMSD > 2.5 Å) (enzymes from *Klebsiella pneumoniae* and *E. coli*, respectively). ALBS (PDB ID 1BSG) was used as a search model because it displayed significant sequence homology to BlaC. Surprisingly, it showed a fairly large RMSD value (3.66 Å) compared to BlaC. Indeed, several other class A β-lactamases with lower sequence homology are structurally more similar to BlaC.

Enzyme active-site structure. In all class A β-lactamases, the hydrolysis of the β-lactam substrates is accomplished by a nucleophilic attack initiated by active-site serine residue Ser[70]. It has been proposed that Glu[166], a general base in the active site, is the serine70-activating residue based on the ultra-high-resolution crystal structure (0.91 Å for SHV-2 and 0.85 Å for TEM-1) studies (37, 41). However, others believe that Lys[73] is involved in the activation (49). Although the mechanism of Ser[70] activation is contentious (36), the deacylation mechanism has been established. Glu[166] has been demonstrated to be critical for the deacylation of the acyl-enzyme intermediate based on site-directed mutagenesis studies (23). The enzyme active site is located at the interface between the α and the α/β domains (Fig. 2). Ser[70] and Lys[73], located on α-helix H2 in the center of the active site (Fig. 3), are completely conserved in all class A β-lactamases and are surrounded by other key residues on β-strand B3 (Lys[234], Thr[235], and Thr[237]) and the loop region between H5 and H6 (Ser[130] and Gly[132]), as well as the Ω loop (Glu[166]). These eight residues are all involved in direct hydrogen bonding interactions with β-lactam substrates. Two bound water molecules, WAT[36] and WAT[65], are also highly conserved in the structures of all class A β-lactamases determined to date. WAT[65] is part of a hydrogen bond network with the side chain oxygen of Ser[70], as well as the side chain oxygen of Glu[166], both at a distance of 2.5 Å. This hydrolytic water functions to deacylate Ser[70] from the product after hydrolysis of β-lactam substrates (21, 31, 40). WAT[36] is in the oxyanion hole, forming hydrogen bonds with the backbone nitrogen of Ser[70] and the backbone nitrogen of Thr[237] at distances of 2.6 Å and 2.7 Å, respectively. Although ampicillin improved the diffraction quality of the crystals, neither the substrate nor any products could be identified in the active site.

Although the overall structure of *M. tuberculosis* BlaC is similar to other class A β-lactamases, some differences are clear, the most obvious being the size of the active site (Fig. 4). The active site of *M. tuberculosis* BlaC is significantly larger than the other class A β-lactamases with known structures. Three factors that contribute to the increased size of the active

alignments of BlaC with other class A β-lactamases of known structure indicate that *M. tuberculosis* BlaC is more similar to K1 (RMSD = 0.95 Å) and NMC-A (RMSD = 1 Å) (enzymes from *Proteus vulgaris* and *Enterobacter cloacae*, respectively)
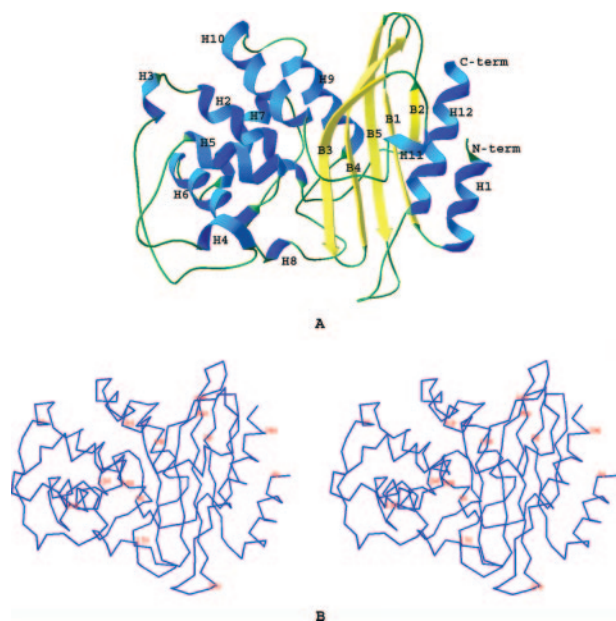

FIG. 2. A. BlaC structure and its secondary structures. The helices are represented as H1 to H12 and the strands as B1 to B5. B. Stereoscopic view of C_α of BlaC labeled every 20 residues.

FIG. 3. Stereo view of the substrate-binding site of *M. tuberculosis* BlaC.

site are (i) amino acid substitution of several key residues, (ii) rearrangement of conserved side chains, and (iii) a four-residue insertion in the active-site region. The β-lactam-binding motif Ser-Asp-Asn is highly conserved among the class A β-lactamases. In *M. tuberculosis* BlaC, glycine occupies the position of asparagine. In the class A β-lactamases with structures determined thus far, the side chain of this asparagine points toward the center of the active site, forming a hydrogen bond with the side chain nitrogen of Lys[73]. The carbamide group of the asparagine is in close contact with the ester carbonyl groups of penicillin and cephems in the complex structures published (47, 49). The 6α-1*R*-hydroxyl group of imi-

FIG. 4. Stereo view of active sites in class A β-lactamases. A. Superimposition of active sites of BlaC (green) and NMC-A (red). B. Superimposition of active sites of BlaC (green) and Toho-1 (red).

penem was also observed to hydrogen bond with the side chain oxygen of Asn[132] in the structure of *E. coli* TEM-1 (PDB ID: 1BT5) (32) and *E. cloacae* NMC-A (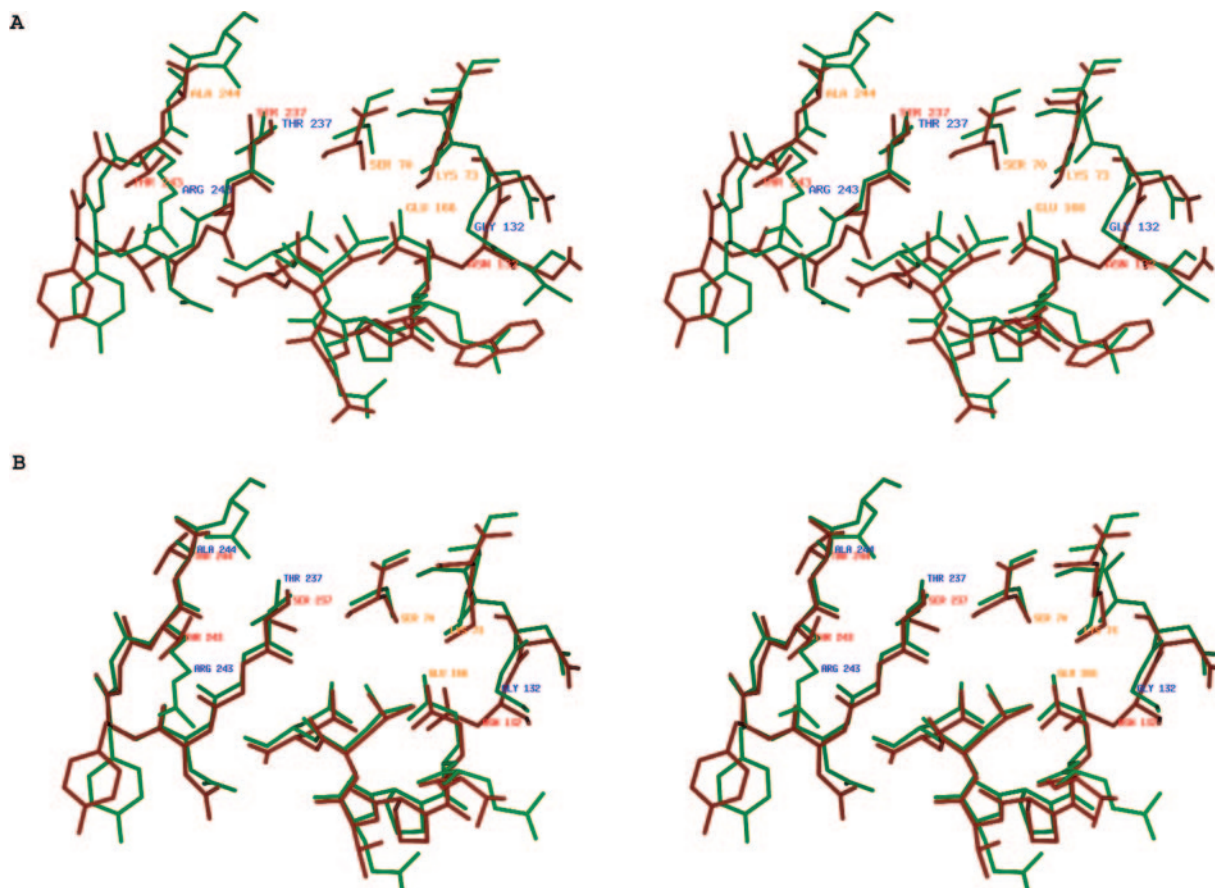PDB ID: 1BUE) (51). It was proposed that the extra space at this location, created by about a 1-Å shift of Asn[132] (equivalent to Gly[132] in *M. tuberculosis* BlaC), and that its side chain pointing away from catalytic center would accommodate the substituents at the R6 position of carbapenem and therefore increase carbapenemase activity (38, 51). In fact, this is true for all the carbapenemases of known structures. Having a glycine instead of asparagine in this position makes the BlaC substrate-binding pocket wider and likely more flexible. Therefore, this substitution in BlaC offers an alternative approach to enlarge the active site and could allow *M. tuberculosis* BlaC to specifically bind carbapenems or cephems with large α substituents. Furthermore, on the same side of the substrate-binding site as Gly[132], the side chain of Ser[104] is unusual in that it is extended away from the substrate-binding site. In other class A β-lactamases, this position is typically an asparagine or an aspartate and the side chain points into the active site. Like the Asn[132]-to-Gly[132] substitution, the conformation of the Ser[104] side chain creates more space in the active site and reduces the potential hydrogen bonding interactions with the substrate. On β-strand B3 of BlaC, Thr[237] is unusual for class A β-lactamases. This location is most often occupied by an alanine, as found in non-ESBLs, or a serine in the ESBLs. The hydroxyl group of Thr[237] points to the same direction as the comparable serine in the ESBLs. Ser[237] (equivalent to Thr[237] in *M. tuberculosis* BlaC) is believed to play a critical role in the extended-spectrum activity of K1 (*P. vulgaris*) β-lactamase. Superimposition of BlaC structure with substrate-bound complex forms of Bs3 from *Bacillus licheniformis* (PDB ID: 1I2W) and a mutant of the RTEM-1 from *E. coli* (PDB ID: 1FQG) (16, 49) indicates that Thr[237] potentially forms three hydrogen bonding interactions with the substrate: backbone oxygen to carbamide nitrogen of the α substituent of β-lactam (~3.5 Å), backbone nitrogen to ester carbonyl oxygen of β-lactam (~2.8 Å), and side chain oxygen to carboxyl oxygen of the β substituent of β-lactam (~3 Å). The side chain of Arg[220] extends into the top part of the active site, as illustrated in Fig. 5, and forms hydrogen bonds with the side chain oxygen of Thr[237], the backbone oxygen of Gly[236], and the ester carboxyl oxygen of bound β-lactam. A similar hydrogen bond is seen only in the carbapenemase NCM-A (*E. cloacae*). The position of Arg[220] is usually occupied by a serine in ESBL type enzymes. The hydrogen bonding between Thr[237] and Arg[220] of *M. tuberculosis* BlaC pulls the side chain of Thr[237] about 2.5 Å away from the active site. This conformation helps to contain the relatively large substituents, such as the 6α-1R-hydroxyl group of carbapenems, which could be critical for their binding. On the other side of the β sheets, Arg[243] is unusual for class A β-lactamases. This position is typically occupied by a threonine. The two nitrogens of the guanidinyl group of Arg[243] form hydrogen bonds with the backbone CO group of Asp[172] and Asn[170] at distances of 2.8 Å and 3.1 Å, respectively, and serve to link B4 with the Ω loop. Another observed difference of potential importance is that Arg[171] in *M. tuberculosis* BlaC is usually either a threonine or a serine in other class A β-lactamases. The long side chain of Arg[171] extends toward the side of the entrance to the active site. On the opposite site of the Ω loop, the region Ile[102] to
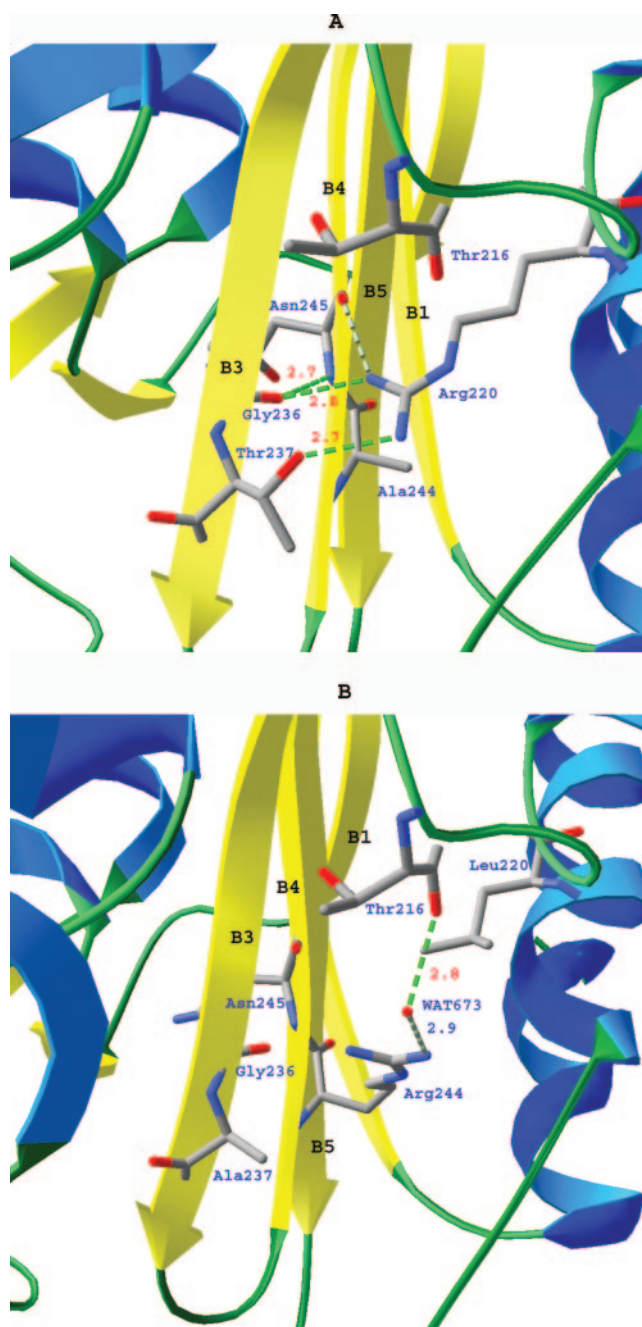
FIG. 5. Comparison of hydrogen bond pattern involving residues 244 and 220 in *M. tuberculosis* BlaC (A) and class A β-lactamase of *Bacillus licheniformis* 749/C (B).

Ile[105] of BlaC is quite different from other class A β-lactamases. While all other class A β-lactamases have either tyrosine or histidine with their aromatic side chains covering the entrance, Ile[105] at the same position makes BlaC's active site ~3 Å wider than other class A β-lactamases. A four-residue insertion, consisting of Gly[145A] to Thr[145D], at the beginning of α helix H7 makes H7 ~6 Å longer than that of other class A β-lactamases.

Structural basis for broad substrate profile. *M. tuberculosis* BlaC has a broad-spectrum substrate profile. The penicillinase
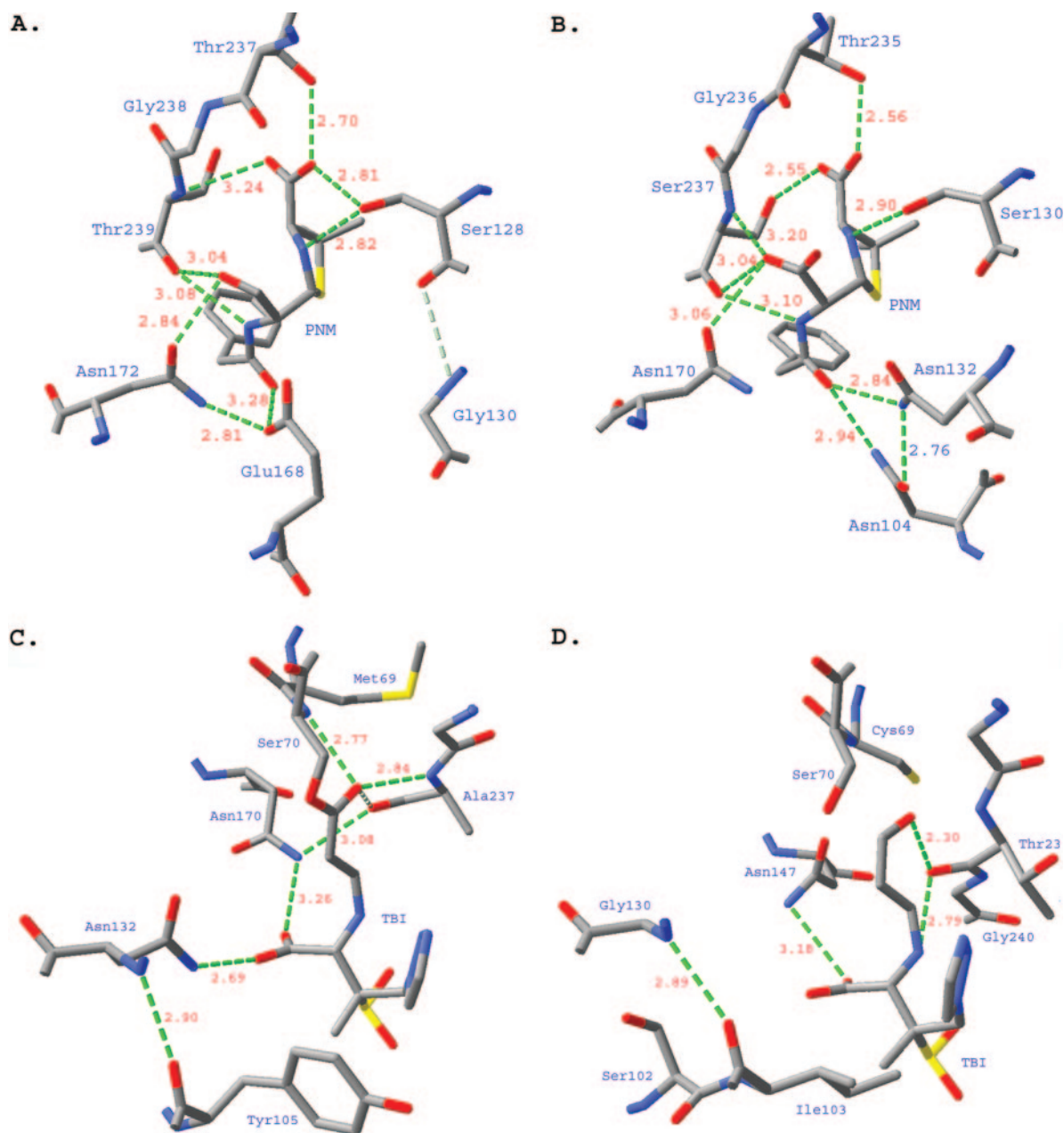
FIG. 6. A. Active site of Toho-1 in complex with benzylpenicillin. B. Active site of *M. tuberculosis* BlaC with a docked ligand benzylpenicillin, using SwissPDB. C. Active site of E166A mutant SHV-1 in complex with the *trans*-enamine intermediate of tazobactam. D. Active site of *M. tuberculosis* BlaC with a docked intermediate of tazobactam, using SwissPDB.

activity of BlaC is about 10-fold lower than most of class A β-lactamases. However, the enzyme showed moderate activity against cephalothin and some activity against cefoxitin, ceftazidime, and meropenem. The broad substrate profile is due to the enzyme's large and flexible substrate-binding site, which allows it to accommodate different types of substrates. While a large active site can provide broader substrate specificity, it can also lead to weak enzyme-ligand interactions. This could explain the generally low activity of *M. tuberculosis* BlaC in hydrolyzing substrates and inhibitors. The substrate profile of *M. tuberculosis* BlaC is quite similar to the β-lactamase of *S.*

clavuligerus; however, the *M. tuberculosis* enzyme has better activity against all β-lactams we have tested. The structure suggests that the low penicillinase activity is in part due to the Asn-to-Gly substitution at position 132. As a conserved residue in other class A β-lactamases, the nitrogen atom of the Asn[132] side chain forms a hydrogen bond with the carbonyl oxygen of the α substituent of β-lactam substrates. Glycine at this position would result in a loss of hydrogen bonding to the substrate, as shown by the superimposition of Toho-1–benzylpenicillin (47) and *M. tuberculosis* BlaC structures (Fig. 6A and B). The hydrogen bond is apparently important for stabilizing the

substrate in the active site. Therefore, the loss of hydrogen bonding interactions could explain the low activity of *M. tuberculosis* BlaC, especially to penicillin type substrates. The critical role of Asn[132] in the activity of class A β-lactamase has been proven by the study of an N132A mutant of *S. albus* G β-lactamase (28), which showed a 100- to 200-fold decrease of penicillinase and cephalosporinase activities. Unlike the N132A mutant of *S. albus* G β-lactamase, *M. tuberculosis* BlaC has relatively good activity against cephalothin. The notable R164A substitution in the neck of the Ω loop is likely the reason why BlaC maintains good cephalothin activity. In other class A β-lactamases with known structures, this position is always occupied by Arg[164]. Normally, Arg[164] hydrogen bonds to another highly conserved residue, Asp[179], to form a salt bridge in the neck of the Ω loop, which holds the Ω loop closed and rigid (Fig. 7B). Loss of this hydrogen bond could potentially destabilize the Ω loop (Fig. 7A), which has been shown to play an important role in the expansion of activity against cephalosporins, based on a mutagenesis study (54). Indeed, compared to many other class A β-lactamases, part of the Ω loop region, Asn[170] to Pro[173], of BlaC is curved away from B4 by ∼2 Å.

Several other residue substitutions could contribute to the boost of cephalosporinase activity. Superimposition to structures of ESBLs in complex with cephalosporins indicated that the hydroxyl group of Thr[237] (equivalent to Ala[237] or Ser[237] in other class A β-lactamases) of *M. tuberculosis* BlaC occupies the same position as the side chain of Ser[237] in the ESBLs and that the hydroxyl group is within the hydrogen bonding distance (2.5 Å) for the carboxyl group of β substituents of cephalosporins. This could help to stabilize the acyl intermediate of cephalosporins and increase the activity. This is consistent with the finding that the A237T mutation increased the relative cephalosporinase activity in TEM β-lactamase (non-ESBL) (20). Since *S. albus* G β-lactamase is a non-ESBL at the equivalent position, it has alanine, not serine or threonine. The loss of the hydrogen bonding interactions at both sites could be the reason why the N132A mutant totally lost cephalosporinase activity.

Class A β-lactamase inhibitors. Clavulanate is a suicide inhibitor specific for class A β-lactamases. This potent ($K_i <$ 0.1 μM) inhibitor forms a covalent acyl enzyme complex with class A β-lactamase. After acylation, the intermediate can either tautomerize to form a transient inhibitor to the enzyme or acylate another active-site serine, Ser[130], to form a cross-linked, irreversibly inactivated enzyme (58). Our inhibition studies show that clavulanate is a relatively poor inhibitor of *M. tuberculosis* BlaC ($K_i = 2.4$ μM) with a 24-fold-higher K_i compared to TEM-1. Class A β-lactamases with amino acid substitutions at Arg[244], Asn[276], Arg[275], Met[69], Met[182], and Trp[165] have been reported to be resistant to clavulanate and other mechanism-based inhibitors (52). Among these residues, the substitutions R244A, N278E, M69C, and M182T are all found in BlaC. It is interesting that clavulanate is produced by *S. clavuligerus*, whose β-lactamase is the closest homolog of *M. tuberculosis* BlaC. Not surprisingly, *S. clavuligerus* β-lactamase is resistant to clavulanate ($K_i = 12$ μM) (44). The superimposition of an E166A variant of SHV-1 with tazobactam bound (43) and *M. tuberculosis* BlaC structures reveals that the N132G substitution would also cause the loss of a hydrogen
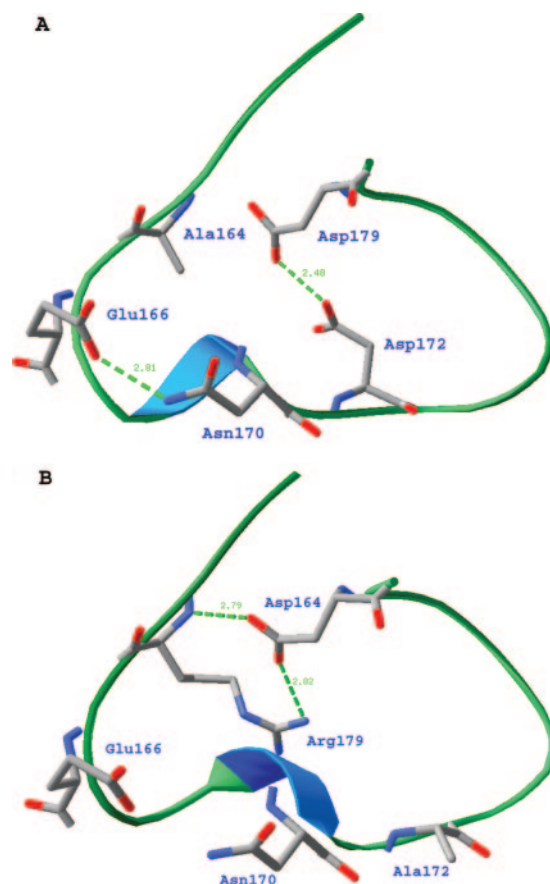
FIG. 7. Comparison of the Ω loops comprising residues 160 to 179 of *M. tuberculosis* BlaC (A) and SHV-1 (B).

bond between the side chain of Asn[132] and 3-β-carboxyl group of the inhibitor (Fig. 6C and D). In the complex structure of wild-type SHV-1 with tazobactam bound, Asn[132] was also observed hydrogen bonding with the sulfinic acid group of the *trans*-enamine intermediate (30). Based on the structure and activity relationship, it is quite clear that *M. tuberculosis* and *S. clavuligerus* share very similar strategies in the resistance of general class A β-lactamase inhibitors, that is, to weaken the binding of the enzyme to those inhibitors mainly through the single amino acid substitution N132G, even though this will also sacrifice enzyme activity to other β-lactam substrates.

BlaC has Ala[244] on β strand B4. This position is usually occupied by arginine in non-ESBL enzymes and threonine in ESBL enzymes. Interestingly, in *M. tuberculosis* BlaC, the side chain of Arg[220] is almost in the same position usually occupied by the side chain of Arg[244] in other class A β-lactamases. This Arg[220]-Ala[244] residue pair exists only in *M. tuberculosis* BlaC, carbapenemase NMC-A (*E. cloacae*), and SCLA (*S. clavuligerus*). Mutation of Arg[244] to Ser in the TEM-1 β-lactamase has been shown to produce resistance to inactivation by clavulanate in the mutant enzyme and resistance to ampicillin plus clavulanate in a strain of *E. coli* producing this enzyme (27). The modeling studies indicated that the guanidinyl group of Arg[244] and an ordered water molecule stabilize the carboxyl group of clavulanate and its acylated intermediate through hydrogen bonding (26). In the R244S mutant, these hydrogen bonds

would be lost, which could potentially cause resistance to clavulanate. It is tempting to speculate that the R244A substitution in BlaC would have a similar mechanism of resistance to clavulanate. However, the position of the Arg[244] side chain in TEM-1 is occupied by the guanidinyl group of Arg[220] in BlaC, which does not exist in TEM-1 (Fig. 5); this complicates the situation. Even though the water molecule in the TEM-1 structure that is believed to be important to clavulanate inhibition is not observed in the BlaC structure, and the guanidinyl group of Arg[220] in BlaC does not have the same orientation as that of Arg[244] in TEM-1, it is possible that the presence of the guanidinyl group of Arg[220] could to some degree restore the sensitivity of BlaC to clavulanate. Indeed, the resistance of BlaC to clavulanate (K_i = 2.4 μM) is about 10-fold less than the R244S mutant of TEM-1 (K_i = 33 μM) (26). However, the clavulanate inhibition is biphasic, and K_i reflects only the first phase. Arg[244] in TEM-1 has been shown to be important to both the binding of clavulanate and the chemistry of inactivation (26). Arg[220] in BlaC may be able to help the binding of clavulanate by a hydrogen bonding interaction in the first phase but is not likely to facilitate the chemistry of inactivation in the second phase.

Although clavulanate is not an ideal inhibitor to *M. tuberculosis* BlaC, it has been demonstrated that the combinations of β-lactam antibiotics and mechanism-based inhibitors such as clavulanate are effective in the treatment of *M. tuberculosis*, even MDR strains. This makes *M. tuberculosis* BlaC a promising target for the design of new inhibitors that are specific for and potent against *M. tuberculosis* BlaC. Based on the structure of *M. tuberculosis* BlaC, it is now possible to either modify current inhibitors to increase inhibitory potency or design novel inhibitors that are specific for the enzyme. In combination with the highly potent *M. tuberculosis* BlaC inhibitors, β-lactam antibiotics could become a new regimen to treat tuberculosis.

ACKNOWLEDGMENTS

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