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Biochemical Characterization of β-Lactamases Bla1 and Bla2 from *Bacillus anthracis*

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The Sterne and Ames strains of *Bacillus anthracis* carry chromosomal genes *bla1* and *bla2*, which confer β-lactam resistance when expressed in *Escherichia coli*. MIC measurements and steady-state kinetic analyses indicate that Bla1 possesses penicillinase activity while Bla2 possesses penicillinase, cephalosporinase, and carbapenem-hydrolyzing activities.

Anthrax, caused by *Bacillus anthracis*, can be treated with antibiotics such as tetracyclines, macrolides, quinolones, and β-lactams. However, recent studies have shown that naturally occurring *B. anthracis* isolates can show variable sensitivity to multiple classes of antibiotics (5, 7, 20, 23; C. H. Choe, S. S. Bouhaouala, I. Brook, T. B. Elliot, and G. B. Knudson, Letter, Antimicrob. Agents Chemother. **44:**1766, 2000). The Sterne and Ames strains of *B. anthracis* carry chromosomal genes *bla1* and *bla2*, which are predicted to encode a group 2a penicillinase and a functional group 3 metalloenzyme (6, 8). Bla1 shares 94% amino acid identity with the *Bacillus cereus* group 2a β-lactamase (19) and contains three functional motifs, S_{70}XXK, S_{130}DN, and K_{234}SG (ABL numbering) (1, 14), present in group 2 β-lactamases. Bla2 shares 92% amino acid identity with the group 3 *B. cereus* 569H enzyme (17), shows conservation of the zinc-chelating motif common to group 3 enzymes (15), and is EDTA susceptible (data not shown).

The *bla1* and *bla2* genes were previously cloned from the *B. anthracis* Sterne strain and expressed in *Escherichia coli* (8). However, the β-lactamases were poorly expressed in the original constructs. In this work, a high-level expression system was modified to overexpress these enzymes as *ompA-bla1* and *ompA-bla2* gene fusion products (25). The gene for mature Bla1 or Bla2 enzyme was fused to the leader sequence of the *E. coli* OmpA protein, cloned into pET24a(+), and transformed into *E. coli* BL21Star(DE3). pET24a(+) contains an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible T7*lacUV5* promoter for large-scale protein expression and a Kan^r marker. Overlap extension PCR was employed as previously described (12) to construct pCMOB1 and pCMOB2. Briefly, four primers, *ompA-bla1-bot*, *ompA-bla1-top*, *bla1-Bam*HI, and *pET-Sph*I (Table 1), were used to PCR amplify two DNA fragments with overlapping ends from vectors pET24a(+)-TEM1 (containing the *ompA* signal sequence) and pUTE523 (containing *bla1*) (25). The two overlapping PCR products were combined in a single PCR and amplified by using the external primers *bla1-Bam*HI and *pET-Sph*I to generate the fusion product, *ompA-bla1*. The fusion product was digested with *Bam*HI and *Xba*I and ligated into pET-24a(+). Ligation products were transformed into *E. coli* BL21Star (DE3), and plasmid DNA was isolated from a set of transformants. The sequence of the *ompA-bla1* fusion gene was determined and confirmed by ABI3100 automated sequencing. The same procedure was used to construct pCMOB2. The four primers, *ompA-bla2-bot*, *ompA-bla2-top*, *bla2-Bam*HI, and *pET-Sph*I (Table 1), were used to PCR amplify two DNA fragments with overlapping ends from vectors pET24a(+)-TEM1 and pUTE490 (containing *bla2*) (25).

The MICs of β-lactam antibiotics were determined by using twofold dilutions in Luria-Bertani–kanamycin broth (LB-Kan; 25 μg/ml) (Table 2). Kanamycin was added to maintain the plasmid. In the absence of IPTG, expression of the cloned genes still occurs; therefore, IPTG was not added to the LB-Kan broth. An inoculum of 10^5 *E. coli* BL21Star(DE3) cells/ml containing pCMOB1, pCMOB2, or the pET24a(+) religated vector was used. The MICs were read after overnight incubation at 37°C. Bla1-producing *E. coli* possessed significant resistance to penicillins but remained susceptible to cephalosporins, imipenem, and aztreonam. Bla2-producing *E. coli* showed resistance to penicillins and some cephalosporins, reduced susceptibility to imipenem, and susceptibility to aztreonam.

The Bla1 and Bla2 enzymes were purified to >95% homogeneity by using previously described methods (18) with the following changes: (i) cultures were inoculated into LB-Kan broth (25 μg/ml), induced at a final concentration of 0.1 mM IPTG, and allowed to grow overnight at 25°C; (ii) 50 μM $ZnSO_4$ was not used for Bla1 protein purification; (iii) enzymes were eluted using a 0 to 1 M NaCl linear gradient; and (iv) Bla2, after collection from cation exchange chromatography, was loaded onto an S-200 Sepharose high-performance liquid chromatography gel permeation column at a flow rate of 1 ml/min, while Bla1 was loaded onto the G-75 Sephadex gel filtration column. Using amino acid analysis, we determined the concentration of Bla2 to be 0.4 mg/ml. The concentration of Bla1 protein was determined by use of the Bradford assay to be 0.5 mg/ml (4). Bla1 migrated at about 31,000 Da and Bla2

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TABLE 1. Primers used in this study

Primer	Nucleotide sequence[a]
ompA-bla1-bot	5′-CACTTGTCCAGTCTTTTCTTTGGCCTGCGCTACGGTAGCGAAACCAGCCAG-3′
ompA-bla1-top	5′-CGCTACCGTAGCGCAGGCCAAAGAAAAGACTGGACAAGTGAAACATAAAAATC-3′
*bla1-Bam*HI	5′-GGGCGCGGGATCCGAGATTATCACCTAAAAGCATTAACTATAACTTCAGCCG-3′
*pET-Sph*I	5′-GAGCACCGCCGCCGCAAGGAATGGTGCATG-3′
ompA-bla2-bot	5′-TACTTTATGCTCTACCTTTCGTTCGGCCTGCGCTACGGTAGCGAAACCAGCCAG-3′
ompA-bla2-top	5′-CGCTACCGTAGCGCAGGCCGAACGAAAGGTAGAGCATAAAGTAATAAAAAATGAGACAG-3′
*bla2-Bam*HI	5′-GGGCGCGGGATCCTCCTCGCTTTTTTATTTTAACAAATCCAATGTATGTAAAAG-3′

[a] The underlined sequence indicates the *Bam*HI restriction site present in the primer sequence.

migrated at approximately 28,000 Da by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). The SignalP version 1.1 program (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the cleavage sites for Bla1 (VEA_{15}-$K_{16}EK$) and Bla2 (VQA_{26}-$E_{27}RK$) signal sequences, which were positively confirmed by N-terminal protein sequencing. β-Lactam hydrolysis by Bla1, performed at Johnson & Johnson, Raritan, N.J., was assayed in 1-ml reaction mixtures at 25°C by using a Shimadzu UV-1601 spectrophotometer (26). The concentrated Bla1 enzyme was diluted into 50 mM phosphate buffer (pH 7.0) with 10 μg of bovine serum albumin/ml when enzyme concentrations of ≤0.05 mg/ml were used. Substrates were prepared fresh in 50 mM phosphate buffer (pH 7.0). Substrates were assayed at least twice, with benzylpenicillin included as a daily reference. K_m and V_{max} values were obtained by averaging the results of a least-squares fit to the Michaelis-Menten equation and Hanes, Eadie-Hofstee, and Cornish-Bowden direct linear plots. Inhibition data for clavulanic acid, tazobactam, and EDTA were obtained by preincubating 0.81 pM Bla1 with various concentrations of inhibitor in 946 μl of phosphate buffer at 25°C for 5 min, adding nitrocefin to a final concentration of 100 μM in 1 ml, and measuring initial hydrolysis rates at 495 nm. The 50% inhibitory concentrations (IC_{50}s) were determined from graphs plotting the percentage of control activity versus inhibitor concentration. Hydrolysis of phenoxymethylpenicillin (16), nitrocefin, imipenem, and cefotaxime (15) by Bla2, performed at the Baylor College of Medicine, Houston, Tex., was measured by using a DU 640 Beckman Coulter spectrophotometer. The reactions were performed in a total volume of 500 μl. The substrate and buffer (50 mM HEPES, 50 μM $ZnSO_4$ [pH 7.5], 20 μg of bovine serum albumin/ml) were preincubated at 30°C for 5 min. Substrates were prepared fresh in assay buffer. K_m and k_{cat} values were derived from at least four independent initial-velocity measurements by nonlinear regression using EnzymeKinetics Pro version 2.34. Inhibition data for EDTA were obtained by preincubating 15 nM Bla2 with or without 50 μM EDTA in 110 μl of assay buffer at 25°C for 5 min, and 20 μl of either of these solutions was then added to 81 μM nitrocefin in 120 μl and incubated at 25°C for 5 min or overnight (≥16 h).

Bla1 is a penicillinase with most efficient hydrolysis for benzylpenicillin, ampicillin, amoxicillin, and piperacillin (Table 3). The Bla1 k_{cat} value of 1,900 s^{-1} for benzylpenicillin is similar to published values of 1,920 and 2,000 s^{-1} for the *B. cereus* β-lactamase I (9, 11). Cephaloridine and nitrocefin demonstrated measurable hydrolysis rates. Bla1 had K_m values of <70 μM for most penicillins, cephaloridine, and nitrocefin. Hydrolysis values of <0.2 s^{-1} were observed for cefepime, cefotaxime, cefoxitin, cefpodoxime, ceftazidime and ceftriaxone, and imipenem (data not shown). The tazobactam IC_{50} value for Bla1, 0.086 ± 0.03 μM, was 80-fold lower than the value obtained for clavulanic acid, 6.9 ± 0.8 μM. The IC_{50} value obtained for EDTA for Bla1 was >250 mM.

Table 4 lists the kinetic parameters for β-lactam hydrolysis by Bla2. Bla2 hydrolyzed nitrocefin with values similar to those for the *B. cereus* metallo-β-lactamase (10, 21, 24), and it efficiently hydrolyzed phenoxymethylpenicillin. The catalytic efficiency (k_{cat}/K_m) for cefotaxime hydrolysis by Bla2 was about sixfold greater than that of β-lactamase II and *B. cereus* 569/

TABLE 2. Antibiotic susceptibilities of *E. coli* BL21Star(DE3) expressing *B. anthracis* Bla1, Bla2, or the pET24a(+) vector

Antibiotic[b]	MIC (μg/ml)[a]		
	pET24a(+)	Bla1	Bla2
Amoxicillin	0.25	≥256	16
Ampicillin	0.125	≥256	8
Benzylpenicillin	2	≥256	16
Carbenicillin	0.25	≥256	64
Cefotaxime	<0.125	<0.125	16
Ceftazidime	<0.125	<0.125	0.5
Cephalothin	0.25	0.5	4
Cefoxitin	1	2	1
Imipenem	<0.125	<0.125	2
Aztreonam	<0.125	<0.125	<0.125

[a] The MIC determinations were performed in duplicate by using the twofold dilution method in LB-Kan broth (25 μg/ml).
[b] Antibiotic concentrations ranged from 0.125 to 256 μg/ml.

TABLE 3. Hydrolysis parameters of *B. anthracis* Bla1 β-lactamase

Antibiotic	k_{cat} (s^{-1})	Relative k_{cat} value[a]	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})	Relative k_{cat}/K_m value[a]
Benzylpenicillin	1,900 ± 120	100	31 ± 2	61	100
Amoxicillin	2,700 ± 330	140	68 ± 10	40	65
Ampicillin	2,600 ± 140	140	50 ± 1	52	85
Carbenicillin	340 ± 15	18	38 ± 2	8.9	15
Cloxacillin	20 ± 5	1.1	NA[b]	NA	NA
Oxacillin	230 ± 2	12	120 ± 6	1.9	3.1
Piperacillin	660 ± 50	35	13 ± 0.6	51	83
Methicillin	58 ± 1	3.1	19 ± 4	3.1	5.0
Cephaloridine	36 ± 1	1.9	32 ± 4	1.1	1.8
Nitrocefin	42 ± 1	2.2	19 ± 2	2.2	3.6
Aztreonam	≤11	≤0.58	NA	NA	NA

[a] Relative k_{cat} and k_{cat}/K_m values were calculated with benzylpenicillin as the reference compound.
[b] NA, not available. Rates were too low to determine a K_m value.

TABLE 4. Hydrolysis parameters of the purified *B. anthracis* Bla2

Antibiotic	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)[a]	Relative k_{cat}/K_m value[b]
Nitrocefin	313 ± 21	75 ± 5	4.18 ± 0.32	100
Phenoxymethylpenicillin	NA[c]	>830	4.41 ± 0.12	106
Cefotaxime	NA	>300	6.59 ± 0.62	158
Imipenem	NA	>500	1.51 ± 0.09	36

[a] The k_{cat}/K_m ratio was calculated, except for nitrocefin, from the initial slope by using the equation $v = k_{cat}/K_m$ ($[E]_0[S]$), where $[E]_0$ is total enzyme concentration and $[S]$ is substrate concentration.

[b] Relative k_{cat} and k_{cat}/K_m values were calculated with nitrocefin as the reference compound.

[c] NA, not available. K_m was too high.

H/9 metallo-β-lactamases and was similar to that of IMP-1, -3, and -6 metalloenzymes (13, 18, 27). The catalytic efficiency of imipenem hydrolysis by Bla2 was approximately eightfold higher than that of the *B. cereus* metallo-β-lactamases (10, 21, 24). Bla2 possessed an imipenem catalytic efficiency similar to that of the group 3 enzymes BlaB, FEZ-1, and VIM-2 (2, 3, 22). Some differences in catalytic efficiencies may be due to experimental conditions such as pH and zinc ion concentration. In the absence of EDTA, Bla2 exhibited hydrolysis of chromogenic nitrocefin within 5 min, while in the presence of EDTA, Bla2 showed slight hydrolysis of nitrocefin only after overnight incubation (data not shown); therefore, Bla2 is inhibited by EDTA.

In this work, we characterized the enzymes encoded by the silent chromosomal *bla1* and *bla2* genes from the nonvirulent *B. anthracis* Sterne strain. Bla1 preferentially hydrolyzed penicillins and was inhibited by tazobactam and clavulanic acid. Bla2 exhibited carbapenem-, penicillin-, and cephalosporin-hydrolyzing activities and was inhibited by EDTA. Further investigations of *bla* gene expression in prototypical β-lactam-susceptible *B. anthracis* strains and in rare β-lactam-resistant isolates will reveal whether increased expression of these genes is associated with resistance to these antibiotics.

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