2013

Characterization of AmiBA2446, a Novel Bacteriolytic Enzyme Active against *Bacillus* Species

Krunal K. Mehta
*Rensselaer Polytechnic Institute*

Elena E. Paskaleva
*Rensselaer Polytechnic Institute*

Saba Azizi-Ghannad
*Rensselaer Polytechnic Institute*

Daniel J. Ley
*Construction Engineering Research Laboratory*

Martin A. Page
*Construction Engineering Research Laboratory*

*See next page for additional authors*

Follow this and additional works at: http://digitalcommons.unl.edu/usarmyresearch


This Article is brought to you for free and open access by the U.S. Department of Defense at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in US Army Research by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.
Characterization of AmiBA2446, a Novel Bacteriolytic Enzyme Active against *Bacillus* Species

Krunal K. Mehta,a,b Elena E. Paskaleva,a,b Saba Azizi-Ghannad,d Daniel J. Ley,e Martin A. Page,e Jonathan S. Dordick,a,b,c,d Ravi S. Kanea,b

Howard P. Isermann Department of Chemical and Biological Engineeringa and Center for Biotechnology and Interdisciplinary Studies,b Rensselaer Polytechnic Institute, Troy, New York, USA; Department of Materials Science and Engineering, Rensselaer Polytechnic Institute, Troy, New York, USA; Department of Biology, Rensselaer Polytechnic Institute, Troy, New York, USA; U.S. Army Corps of Engineers, Engineer Research and Development Center, Construction Engineering Research Laboratory, Champaign, Illinois, USA; Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, New York, USA

Bactericidal enzymes, including bacteriophage endolysins, and lytic enzymes encoded by bacterial genomes (autolysins), have been the subject of increased interest in the past decade, particularly in light of emerging antibiotic resistance (1–5). Bacteriophage endolysins are expressed late during the bacteriophage life cycle, hydrolyzing the bacterial cell wall peptidoglycan to allow viral progeny to escape the host cell. Practical interest in the use of these proteins against Gram-positive bacteria has emerged due to their ability to degrade the bacterial cell wall when applied exogenously (6). Their therapeutic use against human and animal pathogens (7) and their use for decontamination in the food industry (8, 9) have been proposed.

Recent analyses of gene sequences and protein structures of bacterium-derived lytic enzymes have shown that these enzymes share many of the attractive characteristics of bacteriophage endolysins (5, 10, 11). Lytic enzymes from a diverse spectrum of bacterial species have been characterized to date (2, 8, 12). Lytic enzymes are an enzymatically diverse group of proteins. Most of these enzymes have a modular structure, typically with an N-terminal catalytic domain and a C-terminal binding domain (2). The catalytic domain endows the enzyme with muramidase (13), glucosaminidase (14), transglycosylase (15), alanine-amidase (16), alanoyl-glutamate endopeptidase (17), glutaminyl-lysine endopeptidase (18), or cross-bridge endopeptidase (19) activity. The binding domain usually recognizes an epitope within the peptidoglycan layers of the bacterial cell wall or spore cortex and results in exquisite species specificity (16). The specificity against individual bacterial strains is so highly evolved that even a single amino acid substitution in the binding domain can alter the specificity of a lysin (19). This species selectivity (16), therefore, allows targeting of a single pathogenic strain while preserving commensal microbiota. Moreover, gained resistance against lytic enzymes (and bacteriophage endolysins) appears to be extremely low compared to that against antibiotics (2, 16, 20), which represents a significant motivation for further investigation.

A particularly relevant target of high economic, medical, and biodefense importance is the spore-forming pathogen *Bacillus anthracis*, the causative agent of anthrax. This pathogen naturally causes relatively rare infectious outbreaks in humans (21); however, it gained notoriety after its use as biological weapon in the Tokyo, Japan, release of 1995 (22) and the intentional spore release in the U.S. mail in 2001 (23). Given the high cost of decontamination (24) and the recalcitrance of dormant spores (25), there is acute interest in developing an efficient and targeted approach to decontamination and treatment. Schuch et al. have exploited the inherent binding specificity and activity of phage lytic enzymes for the rapid detection and killing of *B. anthracis* (16). They showed that the PlyG endolysin, isolated from the γ phage of *B. anthracis*, specifically kills *B. anthracis* isolates and other members of the *Bacillus cereus* group in vitro and in vivo. Both vegetative cells and germinating spores were found to be susceptible.

There continues to be a need for developing efficient and environmentally friendly treatments for *Bacillus anthracis*, the causative agent of anthrax. One emerging approach for inactivation of vegetative *B. anthracis* is the use of bacteriophage endolysins or lytic enzymes encoded by bacterial genomes (autolysins) with highly evolved specificity toward bacterium-specific peptidoglycan cell walls. In this work, we performed in silico analysis of the genome of *Bacillus anthracis* strain Ames, using a consensus binding domain amino acid sequence as a probe, and identified a novel lytic enzyme that we termed AmiBA2446. This enzyme exists as a homodimer, as determined by size exclusion studies. It possesses N-acetylmuramoyl-L-alanine amidase activity, as determined from liquid chromatography-mass spectrometry (LC-MS) analysis of muropeptides released due to the enzymatic digestion of peptidoglycan. Phylogenetic analysis suggested that AmiBA2446 was an autolysin of bacterial origin. We characterized the effects of enzyme concentration and phase of bacterial growth on bactericidal activity and observed close to a 5-log reduction in the viability of cells of *Bacillus cereus* 4342, a surrogate for *B. anthracis*. We further tested the bactericidal activity of AmiBA2446 against various *Bacillus* species and demonstrated significant activity against *B. anthracis* and *B. cereus* strains. We also demonstrated activity against *B. anthracis* spores after pretreatment with germinants. AmiBA2446 enzyme was also stable in solution, retaining its activity for 4 months of storage at room temperature.
Several other bacteriophage endolysins targeting bacillus strains have been reported. For example, genes encoding endolysins from *B. cereus* phages Bastille (PlyBa), TP21 (Ply21), and 12826 (Ply12) were shown to induce rapid and specific lysis of viable cells of several bacilli, with highest activity against *B. cereus* and *B. thuringiensis* (26). PlyL, a prophage Bat02 endolysin found in the genome of *Bacillus anthracis*, has N-acetylmuramoyl-L-alanine amidase activity and lyses the cell wall of *B. anthracis* cells (27). Other cell-lytic enzymes with reported activity against *Bacillus* strains include PlyPH and related enzymes (5, 28) and PlyB (13); these enzymes have been shown to have specific activity comparable to that of PlyG.

In the current work, using strictly *in silico* techniques to analyze the genome of *B. anthracis* strain Ames with a consensus cell wall binding domain sequence as a probe, we identified a novel lytic enzyme (autolysin). We termed the lysin AmiBA2446 based on its amidase catalytic activity (Ami) and the position of its gene in the genome of *B. anthracis* (locus BA2446). We expressed and purified this protein, confirmed its ability to disrupt the cell walls of various *Bacillus* species, and characterized its specificity and its catalytic properties. AmiBA2446 may be useful for the targeted inactivation of *Bacillus* cells in various applications.

**MATERIALS AND METHODS**

**Bacterial cell culture and plasmids.** *B. cereus* ATCC 4342, *B. cereus* Frankland and Frankland ATCC 10987, *Bacillus subtilis* ATCC 168, *Bacillus thuringiensis* subsp. *kurstaki* ATCC 33679, *Bacillus globigii* (*Bacillus atrophaeus* ATCC 9372), and *Staphylococcus aureus* ATCC 33807 were purchased from ATCC. *B. cereus* Frankland and Frankland 1887 AL (29) was a kind gift from P. Setlow, University of Connecticut. *B. anthracis* Sterne 34F2 was purchased from Colorado Serum Company, Denver, CO. *B. anthracis* Sterne was obtained from T. Buhr, NAVSEA Warfare Center, Dahlgren, VA.

All bacterial strains used were stored frozen at −80°C in 20% (vol/vol) glycerol. The frozen glycerol stocks were used to grow cells in Difco nutrient broth (Becton Dickinson and Company) at 37°C with aeration at 220 rpm. Microbial cultures (bacilli and *S. aureus*) were grown at 37°C with aeration in a 14-ml Falcon tube for 6 h in 4 ml of nutrient broth (3 g/liter beef extract, 5 g/liter peptone) (Difco, Detroit, MI). From this growing culture, 1 ml was centrifuged at 12,000 rpm for 5 min; the pellet was then washed twice with phosphate-buffered saline (PBS) to remove culture medium. Bacteria were then suspended in PBS. The number of bacterial cells was counted under a microscope (magnification, ×400) using a bacterial cell counting chamber.

**Expression and purification of AmiBA2446.** The AmiBA2446 gene, after codon optimization, was cloned in *NcoI/Xhol* restriction sites in *Escherichia coli* plasmid pGS-21a with an N-terminal His tag, followed by an enterokinase cleavage site (GenScript). BL21 Star(DE3) chemically competent cells (Invitrogen), optimized for reduced mRNA degradation and full-length peptide synthesis, were used as an expression host.

AmiBA2446 was expressed and purified using the Native Invitrogen Pro-Bond protein purification system (Invitrogen). Nickel-chelating resin was used for purification of recombinant proteins expressed in *E. coli* strain BL21 Star(DE3). The overnight culture of *E. coli*, transformed with pGS-21a/AmiBA2446 and grown in LB-ampicillin selection medium, was subcultured at a 1:20 ratio in LB and propagated for 2 h (to reach an optical density of 600 nm [OD600] of ~0.4 to 0.6). Protein expression was induced by the addition of 0.25 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and the bacterial suspension was incubated for 2 h at 37°C and 220 rpm. Cells were then pelleted by centrifuging for 20 min at 4000 rpm at 4°C to remove the medium, resuspended in native purification buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and then frozen at −80°C until needed. The cell suspension was then thawed at 37°C and incubated with lysozyme (100 μg/ml) and DNase (10 μg/ml) at 4°C for 30 min. The cell suspension was subjected to two more freeze-thaw cycles and then clarified for 30 min at 4000 rpm at 4°C. The protein was purified from the clarified soluble fraction under native conditions according to the manufacturer’s instructions.

**Characterization of AmiBA2446 by SEC.** Five protein standards were loaded onto a size exclusion chromatography (SEC) column (Superdex 200 preparative grade; GE Healthcare) using PBS as the running buffer to obtain a standard equation relating the molecular weight of the protein to its elution volume. AmiBA2446 was then run on the standardized column to obtain its elution volume, which was then used to estimate the molecular weight of the protein and provide information about the oligomeric state of protein under native conditions.

**Isolation of peptidoglycan from cell walls of vegetative cells.** Preparation of purified cell wall substrate was carried out as described by Shah et al. (30). Briefly, cells were grown in 100 ml of nutrient broth medium to an OD600 of ~1.2 and were collected by centrifugation, washed with 0.8% (wt/vol) NaCl, resuspended in hot 4% (wt/vol) SDS, boiled for 30 min, and then left at room temperature (RT) overnight. The suspension was then boiled for 10 min, and the SDS-insoluble cell wall material was collected by centrifugation at 15,000 × g for 15 min at RT. The pellet containing cell wall peptidoglycan was washed four times with water and finally resuspended in 1 ml sterile water.

**Cell wall hydrolysis assay.** The lytic activity of purified recombinant endolysins was determined spectrophotometrically, as previously described (12, 31). The assays were carried out in polystyrene cuvettes (Greiner) with a total volume of 1 ml in phosphate-buffered saline (pH 7.4) at room temperature, unless otherwise mentioned. Substrate was added to an initial OD600 of 0.3 of 0.4 with purified peptidoglycan from cell walls. At time zero, enzymes were mixed into substrate suspensions to achieve enzyme concentrations ranging from 3 nM to 3,200 nM, and the OD600 in each reactor was recorded for up to 30 min (UV-2401PC UV-visible recording spectrophotometer; Shimadzu). As a negative control, buffer alone was added and monitored for the same duration.

**Kinetics of killing of vegetative bacterial cells.** Kinetics were determined by testing three different concentrations of AmiBA2446 (6 nM, 30 nM, and 150 nM) dosed at time zero into 1 ml of microbial suspensions, each containing 10^8 CFU/ml collected at mid-log growth phase (OD600 = 0.5). The number of live cells was determined by removing aliquots from the microbial suspension after different incubation times (up to 180 min), diluting them (ca. 50-fold), and spreading them on nutrient agar.

**Dose-dependent bacterial killing.** The dose dependence of the anti-microbial activity of AmiBA2446 was determined by treating 1 ml of a microbial suspension containing 10^8 CFU/ml with 2 to 480 nM AmiBA2446 for 3 h. Aliquots from the microbial suspension were collected after 3 h of incubation, diluted (ca. 50-fold), and plated on nutrient agar. The number of surviving colony-forming bacteria was counted after overnight incubation. The lytic activity of the enzyme was calculated in comparison with the colony numbers on untreated controls that were collected and plated the same way.

**Influence of cell density on bacterial killing.** The antimicrobial activity of fixed concentrations of AmiBA2446 enzyme was determined against 10^3-CFU/ml, 10^4-CFU/ml, and 10^5-CFU/ml suspensions of *B. cereus* cells by treating 1 ml of the microbial suspension with 60 nM, 600 nM, and 6 μM concentrations of AmiBA2446 for 3 h. Colony counts were determined by plating aliquots from the microbial suspension after 3 h of incubation on nutrient agar.

**Enzyme thermostability analysis.** Native enzyme AmiBA2446 was incubated at different temperatures, and residual activity of the enzyme was measured using a cell wall hydrolysis assay at regular intervals. A working enzyme concentration of 60 nM was used to measure the residual activity, which was expressed as a percentage of the initial activity. To quantify activity for this comparison, the first-order rate constant, k, of inactivation was obtained from a semilogarithmic plot of percent residual activity versus time. Per the Arrhenius relationship, the activation energy
of inactivation ($E_a$) was obtained by plotting ln $k_i$ against the reciprocal of temperature, as described by Rajalakshmi and Sundaram (32). The activation enthalpy ($\Delta H_f$), activation entropy ($\Delta S_f$), and activation free energy ($\Delta G_f$) of inactivation were calculated using methods described by Gouda et al. (33).

Enzymatic digestion of peptidoglycan and separation and detection of released muropeptides using liquid chromatography-mass spectrometry (LC-MS). Cell wall peptidoglycan (2 mg/ml) was incubated with 500 µg of AmiBA2446 in 1 ml of total solution for 16 h. The supernatant was collected by spinning the mixture at high speed ($\sim 18,000 \times g$) for 15 min. The supernatant was then ultrafiltered using a 10-kDa-cutoff membrane to remove the enzyme in the retentate, while the muropeptide released due to digestion was collected as filtrate. Prior to analysis, the collected muropeptide was reduced with sodium borohydride to prevent anomerization at the C1 position (34). The digested peptidoglycan was mixed with 0.5 M borate buffer (pH 8.0) in which NaBH$_4$ (650 mM) had been freshly dissolved to a final NaBH$_4$ concentration of 130 mM. After 20 min, the reaction was terminated by the addition of phosphoric acid, and the reaction mixture was brought to a final pH of ca. 4 and filtered (0.2-

To determine the exact molecular weight of the muropeptides released during digestion, the digested peptidoglycan was analyzed by LC-tandem MS (LC-MS/MS) using an Agilent 1200 high-pressure liquid chromatograph (HPLC) (Agilent Technologies, CA) coupled to an LTQ-Orbitrap hybrid mass spectrometer (Thermo Scientific, Bremen, Germany). Muropeptide separation was accomplished using a C$_{18}$ reverse-phase column (Thermo Scientific Bio Basic C$_{18}$ 100 by 2.1 mm, 5 μm) at a flow rate of 200 μl/min. The solvents were 0.2% formic acid in water (buffer A) and 20% acetonitrile–0.2% formic acid (buffer B). A linear gradient from 0% to 67% (vol/vol) of buffer B (for 0 to 60 min) was used. The mass spectrometer was operated in electrospray ionization mode with detection of positively charged ions in the m/z range 300 to 1800 at a m/z ratio of 1.79 × 10$^{-6}$ min$^{-1}$. The enzyme activity was measured by monitoring the absorbance at 4342 cells (OD 600 $\sim 0.25$).

RESULTS

Identification of AmiBA2446. We based our strategy to identify novel phage lytic enzymes targeting B. anthracis on the observation that the majority of lytic enzymes have a modular structure that consists of a catalytic domain and a binding domain, connected by a short linker. The binding domain confers specificity by recognizing peptidoglycan motifs in the bacterial cell wall (35). We built a consensus binding domain sequence to recognize the cell wall of B. anthracis by aligning the sequences of the known enzymes PlyG (16) and PlyPH (28) (see Fig. S1 in the supplemental material). Next, we performed a homology search against the genome of B. anthracis strain Ames in the NCBI database (ASM784v1, bioproject PRJNA57909) (36). We identified four highly homologous hits with E values of $\leq 10^{-20}$. The low E value indicates a low probability that these homologies occurred by chance (see Fig. S1c in the supplemental material).

Three of the four sequences were either reported or were highly homologous to already reported lytic enzymes. Sequence NP844822, however, was dissimilar to both PlyG and PlyPH. In silico analysis of the protein sequence using the Conserved Domain Database at NCBI (37–39) led to the supposition that the protein was an N-acetylmuramoyl-1- alanine amidase (also known as MurNAC-LAA, peptidoglycan amidase; EC 3.5.1.28). We termed the lysin AmiBA2446 based on its amidase catalytic activity and the position of its gene in the genome of B. anthracis (locus BA2446). The sequence of AmiBA2446 can be retrieved from the GenBank database (ncbi.nlm.nih.gov/GenBank) as NP844822 or as ZP00392698 within the genome of B. anthracis Ames strain A2012. In summary, AmiBA2446 is a 245-amino-acid protein that shares homology of the C-terminal binding domain with PlyPH and PlyG but has a highly dissimilar catalytic domain (see Fig. S2 in the supplemental material).

Recombinant AmiBA2446 expressed in E. coli and purified from other lysate proteins showed a single band on SDS-PAGE, at a molecular weight corresponding to that of a monomeric protein (see Fig. S3 in the supplemental material). Size exclusion studies suggested that AmiBA2446 existed in its native form as a dimer (see Fig. S4 in the supplemental material).

Phylogenic analysis. A phylogenetic tree was constructed to establish the evolutionary localization of AmiBA2446 relative to the detected open reading frames (ORFs) in the NCBI genome database (Fig. 1). Over 90% of the AmiBA2446 homologs were auto-lysins of predicted bacterial origin. Only seven of the evolutionarily closest enzymes were of bacteriophage origin.

Biochemical properties of AmiBA2446. The lytic activity of AmiBA2446 was first quantified using the B. cereus cell wall hydrolysis assay by monitoring changes in the optical density of a suspension of cell wall peptidoglycan fragments. Absorbance at 600 nm was observed to decrease over time due to the action of AmiBA2446. The enzymatic degradation of B. cereus cell wall material was highly nonlinear as a function of AmiBA2446 concentration (see Fig. S5a in the supplemental material). AmiBA2446 did not follow the typical saturation kinetics (see Fig. S5b in the supplemental material). Based on the initial rate data, the observed $V_{max}/K_m$ ratio was 1.79 × 10$^{-6}$ min$^{-1}$. The enzyme activity (based on the cell wall hydrolysis assay) was optimal at pH 7.0 (see Fig. S6a in the supplemental material). Interestingly, AmiBA2446 activity was highly dependent on the concentration of NaCl, with activity decreasing by 80% as the NaCl concentration increased from 50 to 500 mM (see Fig. S6b in the supplemental material).

Antimicrobial activity of AmiBA2446. The bactericidal activity of AmiBA2446 was demonstrated by treating 10$^6$ CFU/ml of B. cereus 4342 cells (OD$_{600} = 0.5$) with enzyme at concentrations ranging from 2 nM to 480 nM. This strain is commonly used as a surrogate for pathogenic B. anthracis strains (40, 41). We observed up to ca. 99.9% (3-log) killing (Fig. 2a). To quantify AmiBA2446 kinetics, we treated 10$^6$ CFU/ml B. cereus cells with three different enzyme concentrations (6, 30, and 150 nM) and then determined the number of live cells at each concentration. Enzyme quantities expected to yield stronger concentration dependence were selected based on the results in Fig. 2a. Indeed, as the concentration of enzyme increased, so did the rate of cell killing, with up to an ~3-log reduction in viable bacterial cells obtained with 150 nM AmiBA2446 after 3 h (Fig. 2b).

We observed a correlation between the phase of bacterial growth and the bactericidal effect exerted by the enzyme. A rapidly dividing bacterial culture in mid-log phase (OD$_{600}$ = 0.5) during the initial hours of expansion was markedly more sensitive than an overnight culture in stationary phase (Fig. 3). We further explored the maximal bactericidal activity achievable with this treatment on a rapidly dividing bacterial culture. We varied both the AmiBA2446 concentration and the initial concentration of B. cereus cells and found that the reduction in viable cells was ~3 to 4 logs (Fig. 4). Moreover, the extent of cell killing achieved was even higher, close to 5 logs, during the very early growth phase (OD ~ 0.25).
We tested AmiBA2446 against a panel of closely related and unrelated species (Table 1). AmiBA2446 was highly effective against both tested strains of *B. anthracis*, *B. cereus* 4342, and *B. thuringiensis*; however, it was not effective against the more distant *B. subtilis*, *B. globigii*, two other strains of *B. cereus*, and *S. aureus*.

We also tested the activity of AmiBA2446 against spores of the *B. anthracis* Sterne 34F2 after treating them with germinants. As expected, the enzyme had no activity against the spores in the absence of germinants (Fig. 5). However, exposure of the spores to tryptic soy broth containing 10 mM L-alanine and 5 mM inosine for 2 h, followed by treatment with enzyme, resulted in a ~84% decrease in the number of viable spores. We note that enzyme-based sporicidal activity relies on spore germination and that the surviving spores may not have germinated in the 2 h of pretreatment used in this experiment.

**Storage and thermal stability.** AmiBA2446 retained ~95% of its initial activity after storage at room temperature for more than 4 months (Fig. 6). The residual enzymatic activity of AmiBA2446 at different temperatures over a period of time (see Fig. S7 in the supplemental material) showed its activation energy of inactivation (*E_a*) to be ~ 174 kJ mol⁻¹ (see Fig. S8 in the supplemental material). The *E_a* of AmiBA2446 is comparable to those reported for trypsin, chymotrypsin, and papain (32, 42, 43), suggesting that its thermostability is comparable to those of these moderately stable proteases. The activation enthalpy (*ΔH‡*), activation entropy (*ΔS‡*), and activation free energy (*ΔG‡*) at different temperatures were calculated and did not show any significant dependence on temperature (see Table S1 in the supplemental material). Interestingly, comparing the thermostability of AmiBA2446 with those of other bacteriolytic enzymes highlights its superior thermostability. For instance, AmiBA2446 retained its activity for at least 24 h at 50°C, while Cpl-1, a phage lytic enzyme against *Streptococcus pneumoniae*, has been shown to be stable for only 30 min at 45°C (44).

**N-AcetylMuramoyl-l-alanine amidase activity of AmiBA2446.** Analysis of the AmiBA2446 sequence using BLASTP revealed conserved domains that suggested an enzymatic activity identical to that of an N-acetylMuramoyl-l-alanine amidase (also known as

---

**FIG 1** Phylogenetic relationship of AmiBA2446 to lytic enzymes originating from *Bacillus* species and their phages. The scale bar indicates the evolutionary distance between the sequences, 20 amino acids, relative to the hypothetical common ancestor. The location of AmiBA2446 is indicated with filled arrow, and the enzymes of bacteriophage origin are indicated with empty arrows; the rest of the homologs are predicted autolysins.
MurNAc-LAA, peptidoglycan amidase; EC 3.5.1.28), a putative endolysin that hydrolyzes the amide bonds between \( N^-\)acetylmuramic acid and L-amino acids in the cell wall. We analyzed muro-peptides released from the cell wall peptidoglycan due to enzymatic treatment using LC-MS (Fig. 7a and b) and confirmed that AmiBA2446 has \( N^-\)acetylmuramoyl-L-alanine amidase activity.

**DISCUSSION**

Lytic enzymes from both phage and bacterial origin have been successfully shown to be potent bactericidal agents (5–8). Our strategy to use a consensus binding domain sequence that recognizes the cell wall of \( B.\) anthracis as a probe and the subsequent *in silico* analysis of the \( B.\) anthracis genome led to the identification of a novel lytic enzyme, AmiBA2446. We performed a database-wide analysis to determine whether any closely related species had similar protein sequences encoded in their genomes. We found that \( B.\) cereus ATCC 4342, which is genetically similar to \( B.\) anthracis, has an identical sequence, termed ZP04284115. In bacteriophages, the *Bacillus* phage BCP78 has a 272-amino-acid sequence (GenBank accession number AEW47021) with 74% homology to AmiBA2446 (45), and the phage M19-encoded lytic protein had ~70% homology to AmiBA2446. The protein encoded by environmental sample phage M19 (accession number ADF97544) was shown to be catalytically active in a clearing zone assay when tested against autoclaved *Pseudomonas aeruginosa* cells (20).

Our experiments demonstrated that AmiBA2446 exists as a homodimer. This result is consistent with previous reports of the dimerization of the consensus binding domain of another enzyme, PlyG (Protein Data Bank [PDB] number 2L48) (J. S. Dias, F. C. Peterson, and B. F. Volkman, unpublished results). PlyG and AmiBA2446 have the same modular structure and share highly homologous binding domain sequences, rendering the comparison valid. The dimerization of the binding domains of PlyG and AmiBA2446 does not prove that these enzymes necessarily function as dimers in nature. For instance, the full-length PlyG has been reported to exist as a stable monomer based on size exclusion

<table>
<thead>
<tr>
<th>Bactericidal specificity of AmiBA2446(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strain</strong></td>
</tr>
<tr>
<td><em>Bacillus cereus</em> ATCC 4342</td>
</tr>
<tr>
<td><em>Bacillus anthracis</em> ÅSterne</td>
</tr>
<tr>
<td><em>Bacillus anthracis</em> Sterne 34F2</td>
</tr>
<tr>
<td><em>Bacillus thuringiensis</em> subsp. <em>kurstaki</em> ATCC 33679</td>
</tr>
<tr>
<td><em>B. cereus</em> Frankland and Frankland 1887 AL</td>
</tr>
<tr>
<td><em>B. cereus</em> Frankland and Frankland ATCC 10987</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> ATCC 168</td>
</tr>
<tr>
<td><em>Bacillus globigii</em> (Bacillus <em>atrosphaea</em> ATCC 9372)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> ATCC 33807</td>
</tr>
</tbody>
</table>

\(^a\) All bacteria were grown in nutrient broth to an OD\(_{600}\) of ~0.5, and for each strain, \(10^6\) CFU/ml of its cell suspension was treated with 600 nM AmiBA2446 for 3 h.
studies (16), while our work suggests that AmiBA2446 exists as a stable homodimer.

Our biochemical studies showed that the enzymatic degradation of *B. cereus* cell wall material was highly nonlinear as a function of AmiBA2446 concentration (see Fig. S4a in the supplemental material). This result was not unexpected, as the cell wall represents a highly heterogeneous substrate. Thus, easily hydrolyzable linkages (e.g., those that may have greater accessibility) are easily cleaved, leading to a relatively high rate of hydrolysis. Less reactive/accessible bonds remain recalcitrant to hydrolysis even at higher enzyme concentrations (46). AmiBA2446 did not follow typical saturation kinetics (see Fig. S5b in the supplemental material). Due to substrate heterogeneity and the varying degree of polymerization seen in the cell wall, there are relatively few sites that are easily accessible, and there are likely far more sites that are poorly accessible. Such a situation is similar to that observed with heterogeneous cellulose hydrolysis catalyzed by cellulases, which would favor more linear reactivity as a function of substrate concentration (46–48).

The species specificity of the lytic enzymes is an advantage over broad-spectrum antibiotics, which can adversely affect commensal microflora. We found that AmiBA2446 was active against a few closely related *Bacillus* strains. The bactericidal activity against *B. cereus* 4342 and *B. thuringiensis* is likely attributable to their close genetic similarity to *B. anthracis* (40, 41, 49). Indeed, *B. cereus* 4342 is commonly used as an experimental surrogate strain for *B. anthracis* (40). The relatively modest activity (50% killing) against *B. cereus* Frankland and Frankland 1887 AL and the lack of activity against *B. cereus* Frankland and Frankland ATCC 10987 is likely due to the considerable difference between the structures of its cell wall peptidoglycan and that of *B. anthracis* (50). Moreover, AmiBA2446 was not effective against the more distant *B. subtilis*, *B. globigii*, and *S. aureus*, thereby confirming the specificity of the enzyme. Our results are consistent with those observed for other lytic enzymes, PlyG (16) and PlyPH (28), which is expected, given the homology between the binding domains of AmiBA2446, PlyG, and PlyPH (see Fig. S1 and S2 in the supplemental material).

In conclusion, as more bacterial pathogens become resistant to available antibiotics, new agents must be developed. The approach used to identify and characterize AmiBA2446 represents an example of how to expand, perhaps dramatically, the repertoire of highly selective bactericidal agents. The high stability of AmiBA2446 of at least 4 months in solution further expands the potential for its commercial use. Indeed, the approach used in this work could enable the effective decontamination of any resistant bacterial pathogen by sequencing its genome and using bioinformatics to identify putative lytic enzymes. Given that such an enzyme would be found encoded within the genome of a
target pathogen and likely would serve a metabolic function, it is unlikely that gained resistance will rapidly emerge. It has been proposed that lytic enzymes have evolved to target nonredundant defense features within the peptidoglycan cell wall, thus making it unlikely for the target microbes to develop efficient mechanisms of resistance to lytic enzyme activity (2, 12). This approach, therefore, may be important for addressing the growing concern posed by antimicrobial resistance and could be very useful in future applications (51–53).

ACKNOWLEDGMENTS

We acknowledge financial support from the Defense Threat Reduction Agency (W912ST-11-C-00) via a cooperative research agreement with the U.S. Army Corps of Engineers Engineer Research and Development Center.

We thank Dmitri Zagorevski for his help with LC-MS experiments and analyses, and we gratefully acknowledge discussions with K. Solanki.

REFERENCES


