Characterization of a Novel Lytic Protein Encoded by the *Bacillus cereus* E33L Gene *ampD* as a *Bacillus anthracis* Antimicrobial Protein

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Lytic proteins encoded by bacterial genomes have been implicated in cell wall biosynthesis and recycling. The *Bacillus cereus* E33L *ampD* gene encodes a putative N-acetylmuramoyl-1-alanine amidase. This gene, expressed *in vitro*, produced a very stable, highly active lytic protein. Very low concentrations rapidly and efficiently lyse vegetative *Bacillus anthracis* cells.

Lytic bacteriophages have long been studied as potential antimicrobial agents. Phages and their endolysins have potential to treat bacterial infections (2, 3, 19) and reduce or eliminate harmful bacteria in foods (4, 15). Bacterial genes first implicated in cell wall metabolism have recently been shown to exhibit bacterial lytic activity (8, 9, 13). Proteins encoded by these genes play such a role apparently by introducing nicks into the cell wall. Five protein classes are differentiated by the wall component they attack (11, 12). Every sequenced and annotated bacterial genome contains sequences encoding one or more of these putative lytic proteins. We describe the identification, cloning, *in vitro* expression, and preliminary characterization of the *Bacillus cereus* E33L *ampD* gene, encoding a putative N-acetylmuramoyl-1-alanine amidase with very high lytic activity against *Bacillus anthracis* and other closely related *Bacillus* isolates.

Table S1 in the supplemental material lists the different bacterial isolates used in this study, and cultures were grown as described previously (7). BLASTP and BLASTN software (NCBI; http://www.ncbi.nlm.nih.gov/) and the bacteriophage TP-21-T Ply21 protein amino acid sequence (see Fig. S1 in the supplemental material) were used to query protein and DNA sequence databases for genes encoding potential lytic proteins (10, 17). Sequence alignments (see Fig. S1 in the supplemental material) identified multiple putative glycosyl hydrolases (5). One such lytic gene, *ampD* (sequence tag BCZK2532), in the *B. cereus* E33L chromosome (GenBank accession no. NC_006274) (5) was chosen for further study.

The gene was PCR amplified to facilitate its direct cloning. The strategy used to clone and express this gene in an *in vitro* transcription/translation (IVT) expression system is outlined in reference 1. Newly synthesized histidine-tagged lytic protein was purified from lysates under native conditions using nickel-nitrilotriacetic acid (Ni-NTA) Superflow resin (Qiagen). Purified proteins were stored in a mixture of 50 mM sodium phosphate buffer, pH 8.0, 200 mM NaCl, 5% (vol/vol) glycerol, and 1 mM dithiothreitol. IVT-expressed protein had the predicted molecular weight (31.13 kDa) based on polyacrylamide gel electrophoresis (see Fig. S2 in the supplemental material). Screening for lytic activity was carried out as depicted in Fig. 1.

AmpD lytic activity was measured by exposing different *B. anthracis*, *B. cereus*, and *B. thuringiensis* isolates, chosen based on their phylogenetic proximity to *B. anthracis* (6, 7), to 100 nM AmpD, Ply21 endolysin, or a negative control for 1 h at 35°C. Cultures were then plated onto nutrient agar plates (Fig. 2). AmpD is much more active against isolates genetically similar to *B. cereus* E33L than against more distantly related isolates. There is
some correlation between the phylogenetic distance from *B. cereus* E33L and the effectiveness of AmpD against a microbe, but there is no direct correlation useful to measure phylogenetic distance based on lytic efficacy.

*B. anthracis* Sterne cells containing a plasmid encoding green fluorescent protein (GFP) (18) were analyzed by flow cytometry after exposure to AmpD protein. Following addition of the protein, cells were incubated at 30°C and cell lysis and viability were monitored by flow cytometry (Fig. 3a) and by plating (Fig. 3b). The CFU-based viability curves closely matched the flow cytometry results. Flow cytometric data were used to determine the enzyme kinetic properties. The geometric mean for triplicate GFP fluorescence events (Fig. 3) was divided by the geometric mean for GFP fluorescence events in untreated samples so that the GFP fluorescence of each untreated sample equaled 100% and the treated samples had values between 0 and 100%. The $K_m$, $V_{max}$, and dissociation constant were calculated based on fluorescence reduction (see the supplemental material). Specific activity was calculated as the change in the percentage of GFP in the FL-1 flow channel per milligram of protein per minute. More than 90% of the *B. anthracis* cells were lysed by 1 nM protein and 99% were lysed by 50 nM protein in 60 min. Exposure to 10 nM for 10 min resulted in significantly less than 1% survival (Fig. 3b).

Figure 3 shows micrographs of *B. anthracis* Sterne following exposure for 0 min (Fig. 4A) and 3 min (Fig. 4B) to 2.5 μg/ml AmpD lytic protein. The micrographs show the same field before and after exposure to the protein (see Movie S1 in the supplemental material). The change in culture morphology from chains to spheres is consistent with destruction of the bacterial cell wall. The *B. cereus* E33L ampD gene is chromosomally carried and is not part of a prophage sequence based on the absence of any other proximal phage-specific genes (see Fig. S1D in the supplemental material). Nanomolar concentrations of *in vitro*-expressed, purified AmpD lytic protein cause rapid, complete lysis of *B. anthracis* and *B. cereus* E33L cells. The *B. cereus* E33L BCZK2532 locus sequence is more than 99% similar to a homologous *B. anthracis*.
gene, resulting in a single arginine-to-isoleucine difference in the gene products. This amino acid may impact catalytic activity based on maps of the protein’s putative catalytic domain (14). The AmpD catalytic activity is significantly greater than those of comparable *Bacillus* phage-derived endolysins (16), causing lysis at low nanomolar concentrations. The protein is very stable. Purified preparations store at 4°C for at least 8 months without loss of activity, and active lyophilized protein has been stored for 2 years. The protein does not appear to be degraded by proteases and other components found in bacterial cell cultures.

As more bacterial pathogens become resistant to available antibiotics, new agents must be developed. A survey of all the available sequenced bacterial pathogen genomes reveals that all contain one or more genes encoding lytic proteins similar to the *B. cereus* AmpD protein. These proteins have been implicated in cell wall metabolism. If they fulfill a required metabolic function, it is unlikely that the microbes from which they were derived will develop mechanisms of resistance to their lytic properties.

In addition to their potential value as antimicrobial agents, these proteins have utility in DNA extraction protocols and automated DNA-based assays and in disinfection of contaminated materials when harsh disinfectants are not an option. The use of antimicrobial proteins as internal therapeutic agents may be limited. However, such agents may have utility in reducing surface infections and controlling the distribution and persistence of pathogenic microbes, especially drug-resistant bacteria, in a hospital or clinical environment. They may also have utility in other decontamination strategies.

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**REFERENCES**

5. Han CS, et al. 2006. Pathogenic sequence analysis of *Bacillus cereus* and *Bacillus thuringiensis* isolates closely related to *Bacillus anthracis*. J. Bacteriol. 188:3382–3390.