LAWRENCE
LIVERMORE
NATIONAL
LABORATORY

LLNL-JRNL-426611

CHARACTERIZATION OF A NOVEL ENDOLYTIC PROTEIN, AMPD BCZK2532 AS A BACILLUS ANTHRACIS ANTIMICROBIAL PROTEIN

Feliza A. Bourguet, Brian E. Souza, Matthew A. Coleman, Paul J. Jackson

March 30, 2010

Applied and Environmental Microbiology

Disclaimer

This document was prepared as an account of work sponsored by an agency of the United States government. Neither the United States government nor Lawrence Livermore National Security, LLC, nor any of their employees makes any warranty, expressed or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States government or Lawrence Livermore National Security, LLC. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States government or Lawrence Livermore National Security, LLC, and shall not be used for advertising or product endorsement purposes.

Title: Characterization of a novel endolytic protein, *AmpD BCZK2532* as a *Bacillus anthracis* antimicrobial protein.

Running Title: Lytic proteins encoded by bacterial genomes.

Feliza A. Bourguet, Brian E. Souza, Angela K. Hinz[1], Matthew A. Coleman and Paul J. Jackson*

Biosciences and Biotechnology Division, Physical and Life Sciences Directorate, Lawrence Livermore National Laboratory, Livermore, CA 94550

[1]Biochemistry and Biophysics, School of Molecular Biosciences, Washington State University, Pullman, WA 99164-7520

*Correspondence should be addressed to Paul J. Jackson, 7000 East Ave., L-172, Lawrence Livermore National Laboratory, Livermore, CA 94550; phone number: (925) 424-2725; FAX: (925) 422-8020; e-mail: jackson80@llnl.gov

ABSTRACT

Endolysins, proteins that cause bacterial cell lysis, are small proteins, often encoded by bacteriophage genomes, that induce cell lysis by attacking different components of the bacterial cell wall. However, a homologous but less well-studied class of lytic proteins that attack similar bacterial cell wall components are encoded by genes found in all bacterial genomes and have been implicated in aspects of cell wall biosynthesis and recycling. The ampD gene of *Bacillus cereus* E33L, BCZK2532, encodes a putative N-acetylmuramoyl-L-alanine amidase. This gene has better than 99% sequence homology to a *Bacillus anthracis* gene encoding an almost identical protein. We have expressed BCZK2532 *in vitro* and purified a very stable, highly active protein. Very low concentrations of this purified protein rapidly lyse vegetative *B. anthracis* cells with high efficiency. Lytic activity is specific to some members of the sub-group 1 Bacillus species including *B. anthracis* and selective *B. cereus* and *B. thuringiensis* isolates that are phylogenetically close to this pathogen. The implications of using such proteins encoded by genes found in the genomes of bacterial pathogens as antimicrobial agents specific for these pathogens are discussed.

INTRODUCTION

Lytic bacteriophage have been extensively studied because of their potential utility as antimicrobial agents. Following cell infection and production and assembly of new virus particles, endolyins encoded by phage genes are produced as part of the mechanism to release the newly synthesized phage particles from the infected cells. Phage and endolysins have both been used as antimicrobial agents with potential to treat bacterial infections (2, 4, 23) and reduce or eliminate harmful bacteria from foods (5, 19). Some endolysins are quite specific for the bacterial species they impact (2, 4) although none of the bacteriophage endolysins tested in our laboratory has been absolutely species-specific. Other endolysins, such as lysozyme, have much more general lytic activity. A survey of bacteriophage genomes shows significant hetogeneity among phage genes encoding these proteins even when the different phage infect a relatively specific set of bacteria (our own unpublished results).

Bacterial genes that were first implicated in cell wall metabolism have more recently been shown to exhibit bacterial endolytic activity (10, 11, 17). It has been reported that these proteins play a role in cell wall recycling and biosynthesis. There are five general classes of these proteins, N-acetylmuramidases, endo-β-N-acetylglucosaminidases, endopeptidases, lytic transglcosylases and N-acetylmuramoyl-L-alanine amidases, differentiated by the specific cell wall component that they attack (14, 15). Often, genes encoding multiple endolytic proteins are found within a single bacterial genome.

Because of their lytic function when applied externally, endolysins, primarily from bacteriophage, have been studied as potential bactericidal agents. Such applications include food preservation and treatment of infections. Several investigators have tested bacteriophage endolysins as antimicrobial agents in animal models, showing better than 60% survival of *B.*

anthracis-infected mice after 72 hours and 100% of *Streptococcus pneumoniae*-infected mice, 10 days after exposure (2, 4). Obesoa, *et al.* (19) showed that treatment of milk with a *S. aureus* bacteriophage-encoded endolysin reduced *S. aureus* growing in pasteurized milk to undetectable levels in 4 hours. Loessner, *et al*. reported that bacteriophage could be used to destroy *Listeria monocytogenes* in contaminated foods and phage cocktails were approved by the United States Food and Drug Administration in 2006 for treatment of ready-to-eat foods (5). By extension, these phage cocktails produce endolytic proteins that are ultimately responsible for cell lysis after they infect their bacterial hosts.

Identification of bacteriophage endolytic proteins requires availability of bacteriophage that target a specific bacterial species of interest as a source of a gene encoding such a protein. Bacteriophage that infect many bacteria, including a significant number of pathogens, have been identified and characterized. However, phage that infect and destroy *F. tularensis* and several other such pathogens have not been adequately described in the western scientific literature. If a new pathogenic bacterium is identified, significant effort would be required to identify, isolate and characterize bacteriophage that infect this new species. In contrast, every bacterial genome that has been sequenced and annotated contains sequences encoding endolytic proteins (our own unpublished observation). Unlike lysozyme, the first endolysin characterized and isolated from eukaryotic sources, those encoded by bacterial genomes show significant specificity for a relatively small group of microbes that are closely related to the source genome from which the endolysin gene was amplified. There is significant DNA sequence diversity across this set of genes, suggesting the presence of a large range of different endolytic proteins that may have utility as antimicrobial agents, each applicable to a relatively small number of bacterial species.

It is simply a matter of screening bacterial genomes to identify these genes. Once they are identified, it remains to clone and express these genes.

We describe here identification, cloning and *in vitro* expression of BCZK2532, a gene from *B. cereus* E33L that encodes N-acetylmuramoyl-L-alanine amidase, an endolysin with very high lytic activity against *B. anthracis* and several other closely related *B. cereus* and *B. thuringiensis* isolates. We discuss its enzymatic and physical properties and it potential utility as an antimicrobial agent, as a lytic agent in automated DNA-based assays, and its potential value, in combination with other processes, for remediation of *B. anthracis*-contaminated sites.

MATERIALS AND METHODS

Growth of bacterial cultures. Cultures of the different *Bacillus* isolates were started from single colonies and grown at 30°C or 35°C overnight in Nutrient Broth (EMD Chemicals, Gibbstown, NJ). The following day a small aliquot of the overnight culture was added to fresh media. The suspension was incubated at 30°C of 35°C with shaking at 150 rpm to an OD_{600} of 0.45 to 0.5 in Nutrient Broth. Prior to treatment with endolysins, cells were collected by centrifugation at *3,200g* for 10 min. The supernatant was decanted and the bacterial pellet was suspended in the same volume of ambient temperature phosphate buffered saline solution, pH 7.4 (1.06 mM KH_2PO_4, 155 mM NaCl, 2.97 mM $Na_2HPO_4.7H_20$), (Invitrogen, Inc., Carlsbad, CA) prior to endolysin treatment. The different bacterial species and strains used in this study are listed in Table 1.

DNA isolation and purification. Five milliliters of nutrient broth was inoculated with bacteria from a single colony and cultures were incubated overnight at 30 or 35°C with shaking at 150

rpm. Bacterial cells were collected by centrifugation at *1,000 x g* for 15 min. DNA was extracted from the pelleted bacteria using a MasterPure™ Gram Positive DNA Purification Kit from Epicentre Biotechnologies (Madison, WI) following the protocol provided by the manufacturer. If only small amounts of DNA was needed, a single bacterial colony was lifted from an agar plate using a sterile toothpick and suspended in 50 µl of a buffer containing 1% (v/v) Triton X-100, 20 mM Tris-HCl, pH 8.5 and 2 mM EDTA. Bacterial cells were released into this solution by briefly vortexing the sample. The tube containing the suspension was capped and incubated at 95°C for 10 minutes. Cells and debris were removed by centrifugation at *12,000 x g* for 5 minutes and the supernatant was collected and used as a source of DNA. Five µl of DNA prepared in this manner was ample for PCR amplification of any target.

Identification of genes encoding bacterial endolytic proteins. There is insufficient nucleotide sequence homology among endolysin genes from different bacteria and bacteriophage to allow identification of the majority of these genes by direct comparison of nucleotide sequences. The bacteriophage TP21-T (ply21 endolysin amino acid sequence was therefore used as a query sequence to identify related bacterial genome- and prophage-encoded proteins within published pathogenic bacterial pathogen genome sequences. A direct comparison of endolysin gene sequences from specific bacterial genomes was completed to demonstrate similarities and differences among these genes in closely related bacterial species. Bacterial genomes were screened with an emphasis on *B. anthracis* genome sequences and genome sequences from several *Bacillus* isolates that have previously been demonstrated to be very closely related to this pathogen (6, 9). BLASTP software provided by the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) was used to query available protein sequence

databases. BLASTN software from the same source was used to query available DNA sequence databases.

Cloning a putative endolytic protein gene. A putative endolytic protein gene, BCZK2532, was identified in the chromsome of *B. anthracis* E33L (Accession no. NC006274) (6). PCR primers suitable to amplify this gene sequence from total genomic DNA and containing NcoI-NdeI restriction endonuclease recognition sequences at the 5' end and SmaI-BamHI restriction endonuclease recognition sites at the 3' end were designed. Inclusion of these restriction sites allowed direct ligation of the amplified fragment containing the endolysin gene into the pIVEX2.4d *in vitro* translation (IVT) expression vector (Roche Applied Science, Indianapolis, IN). The forward (5'-GGCCATGGGGCATATGGGTTATATTGTAGATATTTCG-3') and reverse (5'-CCCCCGGGATCCTTTAACTTCATACCACCAAC-3') were purchased from Operon Biotechnologies, Inc. (Huntsville, AL). The *AmpD* BCZK2532 gene was amplified from *B. cereus* E33L DNA in a reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM Kcl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatine, 0.2 mM of each dNTP, 20 pmol of each primer, 2.5 U of Platinum *Taq* High Fidelity Polymerase (Invitrogen, Inc., Carlsbad, CA) and 20 ng template DNA. Template DNA was initially denatured by heating at 94°C for 2 min. This was followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and primer extension at 68°C for 2 minutes. Incubation at 68°C for 8 min followed to complete extension. PCR amplification was conducted in an Applied Biosystems GeneAmp™ PCR System 9700 (Applied Biosystems, Inc., Foster City, CA). The resulting amplicons were directly ligated into plasmid pCR2.1/TOPO and transformed into competent *E. coli* DH5α™-T1^R cells using the TOPO TA Cloning® kit (Invitrogen, Inc.). Following verification of the cloned

sequence by Sanger sequencing on an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA), the sequence-verified gene was sub-cloned into the pIVEX2.4d plasmid (Invitrogen, Inc.), adding a sequence encoding a 6 X histidine tag to the DNA sequence encoding the N-terminus of the protein. The pIVEX2.4d/BCZK2532 plasmid was transformed into competent *E. coli* DH5α™-T1^R cells. Cells containing this construct were identified and cultures were grown as a source of plasmid for subsequent IVT reactions.

Protein expression and purification. Two µg of purified pIVEX2.4d/BCZK2532 plasmid DNA was used to drive *in vitro* synthesis of endolytic enzyme using an RTS 500 ProteoMaster *E. coli* HY Kit (Roche Applied Science)(1). Reactions were incubated at 30°C for 18 hours with shaking (900 rpm). Newly synthesized histidine-tagged endolytic protein was purified from cell lysate under native conditions as follows. Ni-NTA Superflow resin (0.5 mL) (Qiagen, Inc, Valencia, CA) was equilibrated with Native Lysis Buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). The IVT lysate containing amplified BCZK2532 endolytic protein was mixed and incubated with the Ni-NTA Superflow resin for 2 hours at 4°C. The resin containing the bound BCZK2532 protein was then washed twice with 10 mL Native Lysis Buffer and four times with 10 mL Native Wash Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). The His-tagged BCZK2532 protein was eluted from the column with 2 mL Native Elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 400 mM imidazole, pH 8.0), collected in 0.5 mL fractions. One to ten µl of each fraction was combined with an equal volume of LDS loading buffer (Invitrogen, Inc.) and heated for 10 min at 70°C. Proteins were electrophoretically separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Purified proteins were transferred to 1 X protein storage buffer (50 mM sodium phosphate buffer, pH 8.0, 200 mM NaCl, 5% (v/v) glycerol, and

1 mM dithiothreitol) by dialysis using 6,000 – 8,000 molecular weight cut off dialysis tubes (EMD Chemicals). All buffers contained Complete, EDTA-free Protease Inhibitor cocktail (Roche Applied Science).

Protein characterization. IVT-expressed BCZK2532 protein, purified or as part of a total IVT *E. coli* lysate was initially assayed for lytic activity in the following manner. *B. anthracis* strain Sterne was grown to an OD_{600} of 0.45, collected by centrifugation at 3200 *x g* for 10 minutes and the bacterial pellet was suspended in one-fourth the original volume of fresh nutrient broth. One hundred microliters of this cell suspension was added to 3 mL warm (47°C) nutrient broth top agar (nutrient broth containing 0.75% w/v Difco agar) and immediately poured onto a nutrient broth agar plate at room temperature. The top agar was allowed to solidify for 5 minutes. Ten microliters of either the IVT *E. coli* lysate containing the BCZK2532 protein, purified BCZK2532 protein or some other endolysin was spotted onto the top agar. An IVT lysate expressing green fluorescent protein (GFP) or purified GFP from an IVT lysate generated as a control IVT reaction and purified using the same resins and buffers used to purify the lytic proteins, was used as a negative control. Nisaplin (Danisco, Inc, New Century, KS), a natural bacteriocin produced by fermentation of *Lactococcus lactis* that rapidly lyses bacteria, was used as a positive control. Plates were incubated overnight at 35°C and scored based the presence or absence of a bacterial lawn where the different products were placed on the plate. A lack of growth at the application site indicated lysis of the bacterial cells.

Flow cytometry. Flow cytometry was performed using a Becton-Dickenson FACSan flow cytometer. GFP fluorescence was recorded in the FL-1 channel. Flow data was analyzed using

CellQuest software (Becton-Dickenson, Franklin Lakes, NJ). A *B. anthracis* Sterne 7702 containing a plasmid (pUTE610) encoding expression of GFP (22) was kindly provided by Dr. Theresa Koehler. A single colony of this isolate was inoculated into 5 mL nutrient broth containing 5 µg/mL erythromycin (EMD Chemicals) and grown with shaking (150 rpm) at 30°C overnight. The following day, the overnight culture was used to start a new culture and the new culture was grown in nutrient broth containing erythromycin at 30°C with shaking to an OD_{600} of 0.45-0.5. Cells were collected by centrifugation as described above, washed by suspension of the cell pellet in 1 x PBS, pH 7.4, centrifuged as before then suspended again in the original culture volume of 1 x PBS, pH 7.4. Cells were divided into ten aliquots for the lysis experiments. Zero, 0.5, 1, 5, 10, or 50 nM BZCK2532 protein were added to the different cell aliquots. Cell suspensions containing the different amounts of this lytic protein were incubated at 30°C and cell lysis and viability were monitored by flow cytometry and by plating portions of each aliquot on nutrient agar plates, then incubating the plates overnight at 30°C to determine the number of surviving cells as measured by changes in the CFU of each suspension. The total number of fluorescent events in 10 seconds was counted for each flow cytometry data point.

Flow cytometry data were used to determine the enzyme kinetic properties of BCZK2532. The geometric mean of triplicate GFP-fluorescent events was divided by the pretreated geometric mean of the GFP-fluorescent events for each sample so that the percentage GFP fluorescence of each untreated sample equaled 100% and the treated samples had values between 0 and 100%. The K_m and V_{max} were determined by plotting the inverse of the slope at 5 minutes vs. the percentage of GFP fluorescence remaining for the different BCZK2532 endolytic protein concentrations. The dissociation constant, k_{-1}. Was determined using the decay equation

$$\%GFP_t = f_{max}(e^{-k-1t})$$

and fit by ByeGraph v.1.5 (Yasuyuki Hirai, http://members2.jcom.home.ne.jp/yasu.hirai/). Specific activity was calculated as the change in percentage of GFP in the FL-1 flow channel per mg protein per minute.

Measuring lytic activity on other *Bacillus* isolates. The impact of exposing different *Bacillus* species to BCZK2532 endolytic protein was determined by growing different *B. anthracis, B. cereus* and *B. thuringiensis* isolates to an OD_{600} of 0.45 to 0.5 in nutrient broth at 35°C or 30°C, collecting and washing the cells as described previously for the *B. anthracis* Sterne culture, the suspending the pellet in 1 x PBS. Cell suspensions (2.5 mL) were treated with 100 nM BCZK2532 or Ply21 or incubated without enzyme for 1 hour at 35°C. Cells were then diluted and different dilutions were plated on nutrient agar plates. Plates were incubated overnight at 30°C or 35°C, depending on the *Bacillus* isolate, to determine the number of cells surviving the treatment.

RESULTS

Cloning, expression and purification of putative endolysins from the *B. cereus* E33L genome. The amino acid sequence encoded by the Ply21 gene from bacteriophage TP-21-T (Figure 1) was used as the base sequence to identify genes encoding novel endolytic proteins (21). Genes identified in the *B. anthracis* Ames ancestor (Accession no. NC007530) and *B. cereus* E33L (Accession no. NC006274) were chosen for further evaluation.

Global sequence alignments (Figure 1 for an example) identified multiple putative glycosyl hydrolases (endolysins) in *B. anthracis* and its close relative, *B. cereus* E33L (6). BCZK2195 and BCZK2532, from the *B. cereus* E33L genome, encode bacterial endolytic

proteins. BA2446, with close nucleotide sequence homology to BCZK2195; and BA2805, with close homology to BCZK2532 are both encoded by the *B. anthracis* chromosome. In contrast, BA4073, with sequence homology to the bacteriophage TP-21-L Ply21 gene, appears to be associated with a prophage sequence within the *B. anthracis* genome and probably is a phage-encoded endolysin. BA4073 was previously reported as PlyL (18).

BA2446 was reported by Rollins, *et al.* (20) to have *B. anthracis*-specific lytic activity although the enzyme was tested only against *B. anthracis,* one *B. cereus* isolate, one *B. subtilis* isolate and a single *E. coli* strain and the concentrations of purified protein needed to generate lysis were significantly higher than those reported here. Six genes were chosen for further study. BA2446, BA2805 and BA4073, encoding putative endolysin genes were chosen from *B. anthracis.* BCZK2195 and BCZK2532, encoding very similar proteins, were chosen from the *B. cereus* E33L genome, and BALH_3313, encoding an endolysin in the *B. thuringiensis* Al Hakam genome (Accession nos. CP000485 and CP000486) (3). All of these genes were amplified from their respective genomes, cloned as described in Materials and Methods and expressed in a cell-free IVT system. Figure 2 shows the results of protein synthesis in IVT reactions labeled with the tRNA-Lysine-BODIPY conjugate FluoroTect GreenLys (Promega, Inc., Madison, WI). Results of amplifying four different endolytic proteins are shown. With the exception of one clone encoding the BA2446, all reactions generated proteins of the expected molecular weights. Nickel-affinity purified proteins were assayed for activity on a lawn of *B. anthracis* Sterne cells (Figure 3). Endolysins encoded by BA4073 and BA2446 (spots 1 and 3) showed very limited lytic activity. The endolysin encoded by BALH_3313 from *B. thuringiensis* Al Hakam showed no measurable activity. The endolysins encoded by two almost identical genes, BA2805 and BCZK2532, showed significant activity compared to a highly purified preparation of the

BCZK2532 enzyme. As expected, IVT-produced GFP had no affect on the cultures. *Bacillus* bacteriophage LAB80, a soil isolate shown to infect *B. anthracis* (our own unpublished results) was included as a positive control and cleared the bacterial lawn as expected. The endolysin encoded by BCZK2532 showed the highest level of activity and was selected for further analysis.

The BCZK2532 endolysin (BCZK2532) was synthesized in an IVT reaction (Figure 4a), purified through Nickel affinity columns (Figure 4b) and shown to be 93% pure based on LC-MS peptide sequencing (12). A 1 mL cell-free transcription-translation reaction generated 4 mg of affinity-purified protein. The calculated molecular weight of this His-tagged protein is 31.13 kDa. The apparent measured molecular weight, based on comparison to molecular weight standards on a polyacrylamide gel is 33 KDa. This purified protein was used for the characterization studies. PlyL (BA4073), the prophage endolysin, was also expressed in the IVT system and purified to approximately 68% purity as determined by SDS-PAGE (Figure 4c). This enzyme was previously purified to 90% purity after expression of the BA4073 gene in an *E. coli in vivo* expression system (20).

BCZK2532-mediated lysis of *B. anthracis* Sterne. Purified BCZK2532 was diluted three orders of magnitude and tested for specific activity using a *B. anthracis* Sterne isolate (7702) containing a GFP-encoding plasmid (pUTE610) (22). Analysis of the results used flow cytometry to monitor the change in cell fluorescence with exposure to the endolysin and culture plating to measure the impact of endolysin activity on survival of the culture. Greater that 90% of the *B. anthracis* cells were impacted by exposure to 1 nM (33 ng/ml) BCZK2532 for 60 minutes at 30°C (Figure 5a). Exposure to 50 nM protein impacted greater than 99% of the cells. Exposure to 1 nM BCZK2532 for 10 minutes at 30°C resulted in significantly less than 10%

survival of the cells in the culture as determined by the number of CFU and exposure to as little as 10 nM for as little as 10 minutes resulted in significantly less than 1% survival (Figure 5b). The viability curves shown in Figure 5b closely matched the flow cytometry results. Figure 6 shows micrographs of *B. anthracis* Sterne following exposure for 0 (6A) and 3 min (6B) exposure to 2.5 µg/mL BCZK2532. Both micrographs show the same field before and after endolysin treatment. The change in cell morphology from chains of *B. anthracis* cells to a spherical shape is consistent with destruction of the bacterial cell wall. Such cells are not viable as measured by culturing and plating methods.

BCZK2532 protein stability and enzyme properties. A Nickel-column purified BCZK2532 was stored at 4°C in 1 X protein storage buffer and tested by polyacrylamide gel electrophoresis and by measuring *B. anthracis* lytic activity at different times over eight months. There was no detectible protein degradation or loss of endolytic activity following eight months of protein storage at 4°C (data not shown).

Specificity of BCZK2532 for different *Bacillus* species. BCZK2532 was applied to very close relatives of *B. anthracis* to understand the range of different closely related species and strains that this lytic protein could impact. In addition to testing against *B. anthracis* and *B. cereus* E33L, the ability to lyse *B. thuringiensis* 97-27, *B. thuringiensis* HD1, *B. thuringiensis* HD560 and *B. thuringiensis* HD658 was also tested. *B. thuringiensis* serovar *konkukian* 97-27 is a very unusual *B. thuringiensis* isolate. It is a close relative of *B. anthracis* isolated from a severely infected wound of a French soldier and shown to be infectious in animal models (7). In contrast, *B. thuringiensis* serovar *kurstaki* HD1, serovar *israelensis* HD560 and serovar *kenyae* are all

used to control insects and are not very closely related to *B. anthracis* phylogenetically (8).

BCZK2532 showed lytic activity at a concentration of 100 nM for 1 hour against all of the *Bacillus* isolates tested based on a reduction in CFU of cultures exposed to (Figure 7). However, it is much more effective against *B. anthracis* and *B. cereus* E33L than it is against the *B. thuringiensis* isolates. Only 0.1% of the *B. anthracis* Sterne and 3.7% of the *B. cereus* E33L survived this treatment. It is not surprising that this lytic protein has similar activities against these two pathogens because, while the gene sequence differs by less than 1% (six point mutations), only one of the six point mutations results in a change in an amino acid. Only 3.1% of the *B. thuringiensis* serovar *konkukian* 97-27 survived this treatment. In contrast, 15% of the treated *B. thuringiensis* serovar *israelensis* HD658 cells, 40% of the *B. thuringiensis* serovar *kenyae* HD560 cells and 48% of the *B. thuringiensis* serovar *kurstaki* HD1 survived exposure to the endolysin. There appears to be some correlation between the phylogenetic distance of the *B. thuringiensis* isolate (*B. thuringiensis konkukian* 97-27 is a close relative of *B. anthracis* while the other *B. thuringiensis* isolates are only distantly related) but there is no direct correlation between the efficacy of this endolysin and the phylogenetic distance of the treated cells from *B. cereus* E33L. The Ply21 endolysin used in these experiments was expressed from the genome of the bacteriophage TP-21-L, a known *B. anthracis* phage. It is therefore surprising that this endolysin shows less lytic activity against *B. anthracis* and *B. cereus* (E33L) than it does against all but one of the *B. thuringiensis* isolates. However, none of the other *B. thuringiensis* strains have been tested as hosts for this phage.

DISCUSSION

The BCZK2532 gene is chromosomally encoded and is clearly not a part of a prophage sequence based on the absence of any other phage-specific genes in the vicinity of this gene

within the *B. cereus* E33L genome. The BCZK2532 protein is thought to be associated with cell wall metabolism and turnover based on its homology to other gene sequences that have independently been implicated in these processes (10, 11, 17). This gene encodes a protein that has a similar structure and probably a similar function to several other proteins encoded by the *B. cereus* E33L genome.

Nanomolar concentrations of *in vitro* expressed, purified BCZK2532 endolysin cause rapid, complete lysis of *B. anthracis* and *B. cereus* E33L cells. Very low concentrations of this protein (2.5 μg/mL) can completely lyse a *B. anthracis* Sterne culture containing approximately 5×10^8 cells/mL in several minutes (Figures 7a and b). The BCZK2532 gene sequence is greater than 99% similar to a homologous gene in *B. anthracis*. There are six nucleotide differences between the *B. anthracis* and *B. cereus* E33L (BCZK2532) gene sequences. However, five of these differences are synonymous, leading to no differences in the amino acid sequences of the two proteins. The sixth difference at position 100, results in an arginine to isoleucine substitution. BCZK2532 has slightly lower lytic activity against *B. anthracis* relative to *B. cereus* E33L (data not shown). This amino acid residue may have some importance in catalytic activity because it maps to the catalytic domain of this protein (18), however there is no known reason why this change in protein sequences would cause a difference in lytic activity between the two bacterial isolates. It may be that there are differences in the *B. anthracis* cell wall relative to *B. cereus* E33L that are responsible for this.

BCZK2532 is not absolutely specific for its host and *B. anthracis* but has a relatively narrow range of activity against other Bacillus isolates, at least at the very low concentrations that are effective against *B. anthracis* and *B. cereus* E33L. The BCZK2532 catalytic activity is significantly greater than that of comparable *Bacillus* phage-derived endolysins. The

bacteriophage endolysin PlyL will lyse a *B. anthracis* culture (5 x 10^8 cells/mL in exponential phase) within 10 minutes at 2 μM concentrations (20). The PlyB bacteriophage endolysin will lyse a similar culture of *B. cereus* ATCC 4342 cells in 10 minutes at 625 nM concentrations. The BCZK2532 endolytic protein will lyse a *B. anthracis* Sterne culture in 10 minutes at 10 nM concentrations.

This enolysin is a very stable protein. Purified preparations were stored at 4°C with no special protection for at least eight months without loss of activity. Moreover, the protein does not appear to be degraded when added to cultured cells, suggesting that it is relatively resistant to the proteases and other components found in a bacterial cell culture.

The cell-free *in vitro* translation used in this study produced a recombinant, easily purified protein with very high lytic activity. A single IVT reaction produced 6 mg of purified protein. The approach provided significantly better results than those obtained by using a traditional *E. coli* protein expression system. The latter method produced some active lytic protein but generated large amounts of insoluble protein with very low specific activity. The IVT approach coupled with single-step affinity chromatography allowed synthesis and purification of large, high quality protein preparations in a relatively short time period. It may eventually be useful to optimize expression of such proteins if they have therapeutic value, in a cell-based system. However, for initial testing and characterization of such proteins, the IVT-based methods have significant advantages.

Efforts to identify new therapeutic compounds that are highly specific for a particular bacterial pathogen are increasing due to the emergence or more targeted therapy. As less costly, more rapid methods of specifically identifying bacterial pathogens are developed, it is more rational to use therapeutic agents that are more specific. Moreover, as more bacterial pathogens

become resistant to available antibiotics, new agents must be developed that cannot be circumvented or destroyed by the targeted pathogens. Bacteriophage have long been considered potential antibacterial agents while bacteriophage endolysins, the small proteins responsible for lysis of the infected host cell, have been studied more recently (2, 4, 16 for examples). Bacteriophage are known that infect many of the bacterial pathogens. However, phage have not been identified for all of these microbes and, in the event of the emergence of a new pathogen, identifying phage that infect these as a source of therapeutically useful endolysins could be problematic.

A survey of all the available sequenced bacterial pathogens genomes reveals that all the genomes available carry one or more genes encoding endolytic proteins similar to those involved in bacteriophage-mediated bacterial cell lysis. *B. cereus* E33L contains several other genes encoding potential lytic proteins besides those described here. These proteins have been implicated in cell wall metabolism and, as such, may be required for cell wall synthesis and maintenance. If they fulfill a required need in cell metabolism, then it is unlikely that the microbes from which they were derived will develop mechanisms of resistance to these lytic agents. It is therefore not necessary to mine bacteriophage genomes for genes encoding these potentially antimicrobial proteins. Instead, one can identify these sequences in the bacterial genomes. Then it is simply a matter of designing the appropriate primers to amplify the gene from a very small amount of pathogen DNA and cloning and expressing the gene to produce a potential antimicrobial protein. Rapid genomic sequencing of newly identified pathogens will facilitate identification of genes encoding such proteins in these newly identified microbes. Many bacterial genomes contain prophage sequences, another potential source of genes encoding lytic proteins.

In addition to their potential value as antimicrobial agents, bacterial endolytic proteins may have utility in development of automated DNA-based assays. It is often difficult to quantitatively extract DNA from bacterial species, especially the Bacilli. Automated methods have previously relied on a combination of mechanical and chemical methods to extract DNA necessitating complex machinery to faciliate DNA extraction. None of these methods provide high yields of nucleic acids. Endolytic proteins have utility in such assays because the addition of these proteins results is almost immediate, complete lysis of the cell population without mechanical disruption, making subsequent DNA purification significantly less challenging. If applied to a complex environmental sample, these will lyse only those cells susceptible to the endolysin, providing a potential means of enriching for DNA from targeted organisms. These lytic proteins may also have utility in the decontamination of materials accidentally or intentionally contaminated with bacterial pathogens, especially when harsh disinfectants cannot be applied.

We have demonstrated that bioinformatics tools can be used to mine the genome sequences of Gram-positive pathogenic bacteria to locate, isolate and express genes whose products are highly lethal to the microbes from which the genes were derived. Polyamines (i.e., protamine, polyethylenimine, and polymyxin B nonapeptide) increase the permeability of the outer membrane of Gram-negative bacteria. Adding such compounds to endolytic protein preparations may significantly increase sensitivity of Gram-negative bacteria to these lytic proteins (13) facilitating their use as antimicrobial agents against these pathogens. The use of antimicrobial proteins as therapeutic agents may be limited by methods of application and delivery. However, such lytic 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 also have demonstrated utility in treating food stocks to eliminate food-borne pathogens.

Information about crucial microbial metabolic pathways has long been used as a means to identify potential antimicrobial agents that severely impact the growth and reproduction of bacterial pathogens. The results presented here suggest that DNA sequences mined from available pathogenic microbial genome sequences can themselves be used directly in strategies to control or destroy these pathogens.

ACKNOWLEDGEMENTS

We thank Dr. Theresa Koehler for providing the *B. anthracis* Sterne isolates containing genes encoding green fluorescent protein and for the directions for their growth and use. This work performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344. Funding for this project was provided by the Lawrence Livermore National Laboratory Directed Research and Development Program (LDRD).

REFERENCES

1. **Beernink, P. T., B. W. Segelke, and M. A. Coleman.** 2003. High-Throughput, Cell-Free Protein Expression Screening Using the RTS 100 *E. coli* HY Kit. Biochemica 1:4-5.
2. **Borysowski, J., B. Weber-Dabrowska, and A. Górski.** 2006. Bacteriophage Endolysins as a Novel Class of Antibacterial Agents. Exp. Biol. Med. 231:366-377.
3. **Challacomb, J. F., M. R. Altherr, G. Xie, S. S. Bhotika, N. Brown, D. Bruce, C. S. Campbell, M. L. Campbell, J. Chen, O. Chertkov, C. Cleland, M. Dimitrijevic, N. A. Doggett, J. J. Fawcett, T. Glavina, L. A. Goodwin, L. D. Green, C. S. Han, K. K. Hill, P.**

Hitchcock, P. J. Jackson, P. Keim, A. R. Kewalramani, J. Longmire, S. Lucas, S. Malfatti, D. Martinez, K. McMurry, L. J. Meincke, M. Misra, B. L. Moseman, M. Mundt, A. C. Munk, R. T. Okinaka, B. Parson-Quintana, L. P. Reilly, P. Richardson, D. L. Robinson, E. Saunders, R. Tapia, J. G. Tesmer, N. Thayer, L. S. Thompson, H. Tice, L. O. Ticknor, P. L. Wills, P. Gilna, and T. S. Brettin. 2007. The Complete Genome Sequence of *Bacillus thuringiensis* Al Hakam. J. Bacteriol. 189:3680-3681.

4. **Fischetti, V. A.** 2004. The use of phage lytic enzymes to control bacterial infections. p. 321-334. *In:* E. Kutter, A. Sulakvelidze, (ed.), Bacteriophages: Biology and Applications. CRC Press, Boca Raton.
5. **Guenther, S., D. Huwyler, S. Richard, and M. J. Leossner.** 2009. Virulent bacteriophage for efficient biocontrol of *Listeria monocytogenes* in Ready-To-Eat Foods. Appl. Environ. Microbiol. 75:93-100.
6. **Han, C. S., G. Xie, J. F. Challacombe, M. R. Altherr, S. S. Bhotika, D. Bruce, C. S. Campbell, M. L. Campbell, J. Chen, O. Chertkov, C. Cleland, M. Dimitrijevic, N. A. Doggett, J. J. Fawcett, T. Glavina, L. A. Goodwin, K. K. Hill, P. Hitchcock, P. J. Jackson, P. Keim, A. R. Kewalramani, J. Longmire, S. Lucas, S. Malfatti, K. McMurry, L. J. Meincke, M. Misra, B. L. Moseman, M. Mundt, A.C. Munk. R. T. Okinaka, B. Parson-Quintana, L. P. Reilly, P. Richardson, D. L. Robinson, E. Rubin, E. Saunders, R. Tapia, J. G. Tesmer, N. Thayer, L. S. Thompson, H. Tice, L. O. Ticknor, P. L. Wills, T. S. Brettin and P. Gilna.** 2006. Pathogenic sequence analysis of *Bacillus cereus* and *Bacillus thuringiensis* isolates closely related to *Bacillus anthracis*. J. Bacteriol. 188:3382-3390.

7. **Hernandez, E., F. Ramisse, J. P. Ducoureau, T. Cruel, and J. D. Cavallo.** 1998. *Bacillus thuringiensis* subsp. *konkukian* (serotype H34) superinfection: case report and experimental evidence of pathogenicity in immunosuppressed mice. J. Clin. Microbiol. **36:**2138–2139.
8. **Hill, K. K., L. O. Ticknor, R. T. Okinaka, M. Asay, H. Blair, K. A. Bliss, M. Laker, P. E. Pardington, A. P. Richardson, M. Tonks, D. J. Beecher, J. D. Kemp, A.-B. Kolstø, A. C. L. Wong, P. Keim and P. J. Jackson.** 2004. Fluorescent Amplified Fragment Length Polymorphism Analysis of *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* Isolates. Appl. Environ. Microbiol. 70:1068-1080.
9. **Hoffmaster, A. R., K. K. Hill, J. E. Gee, C. K. Marston, B. K. De, T. Popovic, D. Sue, P. P. Wilkins, S. B. Avashia, R. Drumgoole, C. H. Helma, L. O. Ticknor, R.T. Okinaka, and P. J. Jackson.** 2006. Characterization of *Bacillus cereus* isolates associated with fatal pneumonias: Strains are closely related to *Bacillus anthracis* and harbor *B. anthracis* virulence genes. J. Clin. MIcrobiol. 44:3342-3360.
10. **Jacobs, C., L. J. Huang, E. Bartowsky, S. Normark, and J. T. Park.** 1994. Bacterial cell wall recycling provides cytosolic muropeptides as effectors for beta-lactamase induction. EMBO J. 13:4684-4694.
11. J**acobs, C., B. Joris, M. Jami, K. Klarsov, J. Van Beeumen, D. Mengin-Lecreulx, J. van Heijenoort, J. T. Park, S. Normark, and J. M. Frère.** 1995. AmpD, essential for both beta-lactamase regulation and cell wall recycling, is a novel cytosolic N-acetylmuramyl-L-alanine amidase. Mol Microbiol. 15:553-559.
12. **Jensen, O. N., W. Matthias, A. Shevckenko, and M. Mann.** 1998. Peptide sequencing of 2-DE Gel-Isolated Proteins by Nanoelectrospray Tandem Mass Spectrometry. Methods Mol. Biol. 112:571-588.

13. **Kwon, D. H. and L. Chung-Dar. 2006.** Polyamines increase antibiotic susceptibility in *pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 50:1623-1627.

14. **Loessner, M. J.** 2005. Bacteriophage endolysins-current state of research and applications. Curr. Opin. Microbiol. 8:480–487, 2005

15. **Loessner, M. J., K. Kramer, F. Ebel, and S. Scherer. 2002.** C-terminal domains of *Listeria monocytogenes* bacteriophage murein hydrolases determine specific recognition and high-affinity binding to bacterial cell wall carbohydrates. Mol. Microbiol. 44:335-339.

16. **Loessner, M. J., A. Schneider, and S. Scherer.** 1996. Modified *Listeria* bacteriophage lysin genes (*ply*) allow efficient overexpression and one-step purification of biochemically active fusion proteins. Appl. Environ. Microbiol. 62:3057-3060.

17. **López, R., E. García, and J. L. García.** 1997. The Pneumococcal Cell Wall Degrading Enzymes: A Modular Design to Create New Lysins? Microb. Drug Resist. 3:199-211.

18. **Low, L. Y., C. Yang, M. Perego, A. Osterman, and R. C. Liddington.** 2005. Structure and Lytic Activity of a *Bacillus anthracis* Prophage Endolysin. J. Biol. Chem. 280:35433-35439.

19. **Obesoa, J. M., B. Martíneza, A. Rodrigueza, and P. García.** 2008. Lytic activity of the recombinant staphylococcal bacteriophage ΦH5 endolysin active against *Staphylococcus aureus* in milk. Int. J. Food Microbiol. 128:212-218.

20. **Rollins, S. M., A. Peppercorn, J. S. Young, M. Drysdale, A. Baresch, M. V. Bikowski, D. A. Ashford, C. P. Quinn, M. Handfield, J. D. Hillman, C. R. Lyons, T. M. Koehler, S. B. Calderwood, and E. T. Ryan.** 2008. Application of in vivo induced antigen technology (IVIAT) to *Bacillus anthracis.* PLoS ONE 3:e1824.

21. **Ruhfel, R. E., and C. B. Thorne. 1988.** Physical and genetic characterization of the *Bacillus thuringiensis* subsp. *kurstaki* HD-1 extrachromosomal temperate phage TP-21. Abst. Ann. Meet. American Society for Microbiology, p. 145.

22. **Saile, E., and T. M. Koehler. 2006.** *Bacillus anthracis* multiplication, persistence, and genetic exchange in the rhizosphere of grass plants. Appl. Environ. Microbiol. 72:3168-3174.

23. **Summers, W. C.** 2001. Bacteriophage therapy. Annu. Rev. Microbiol. 55:437-451.

Table 1. *Bacillus* species and strains used in this study.

Species	Strain	GFP Fluorescence	Source
Bacillus anthracis	Sterne	Negative	Dugway Proving Grounds, Utah
Bacillus anthracis	Sterne UT238*	Positive	Theresa Koehler, UT, Houston Medical Center, TX
Bacillus anthracis	Sterne 7702/pUTE610**	Positive	Theresa Koehler, UT, Houston Medical Center, TX
Bacillus cereus	E33L	Negative	Tetracore, Inc., Rockville, MD
Bacillus thuringiensis	serovar *konkukian* 97-27	Negative	Laboratoire de Biologie, Hopital des Armees Begin, Paris, France
Bacillus thuringiensis	serovar *kurstaki* HD1	Negative	ARS Culture Collection (NRRL), Peoria, IL
Bacillus thuringiensis	serovar *israelensis* HD658	Negative	ARS Culture Collection (NRRL), Peoria, IL
Bacillus thuringiensis	serovar *kenyae*	Negative	ARS Culture

	HD560		Collection (NRRL), Peoria, IL

* This isolate contains a single copy of the gene for green fluorescent protein (GFP) gfpmut3a fused to a 645-bp PCR product containing the constitutively active promoter of the *B. anthracis* metalloprotease gene, inhA (BA1295) ().

** Plasmid pUTE610 carries the gene for green fluorescent protein (GFP) gfpmut3a fused to a 645-bp PCR product containing the constitutively active promoter of the *B. anthracis* metalloprotease gene, inhA (BA1295) (22).

Figure Legends.

Figure 1. Amino acid sequence alignments of endloysins encoded by genes from the *B. anthracis* and *B. cereus* E33L genomes. The amino acid sequence encoded by the Ply21 gene from the *B. anthracis* bacteriophage TP-21-T (21) was used to identify genes with similar sequence in the *B. anthracis* and *B. cereus* E33L genomes. Three homologous pairs were identified; BKCK2532 and BA2805; BCZK2195 and BA2446; and BA4073 and Ply21.

Figure 2. Analysis of IVT results expressing four different *Bacillus* endolytic proteins. Two clones each of four different genes encoding endolytic proteins were used to amplify the corresponding proteins in a cell-free IVT system labeled with BODIPY (1). Newly synthesized BODIPY-labeled proteins were separated by electrophoresis through a 4-12% polyacrylamide gel and the gel was scanned for BODIPY fluorescence. Lane 1 contains a molecular weight marker. Lanes 2 and 3 contain products from reactions containing two different plasmid clones of BCZK2195; lanes 4 and 5 contain products from reactions containing two different plasmid clones of BA2446; lanes 6 and 7 contain products from reactions containing two different plasmid clones of BCZK2532; lanes 8 and 9 contain products from reactions containing two different plasmid clones of BA2805. Lane 10 contains products from a reaction containing a cloned GFP sequence and lane 11 contains products from a no-DNA control reaction. Note that one BA2446 reaction (lane 5) generated a product that was not the expected molecular weight.

Figure 3. Endolysin spot tests. Nickel affinity column-purified endolysin preparations were tested by placing each purified protein on a freshly plated suspension of *B. anthracis* Sterne cells. Five minutes after plating the bacterial cells, 10 μl of each protein was spotted onto the

plate and the plate was incubated overnight at 35°C overnight. The following day, spots were assayed for evidence of bacterial lysis. Each spot is labeled with the protein deposited on that spot. Ten µl of IVT synthesized green fluorescent protein (GFP) was spotted on the plate as a negative control and 10 µl of bacteriophage LAB80, a bacteriophage that infects *B. anthracis*, was spotted as a positive control.

Figure 4. *In vitro* synthesis and purification of BCZK2532 endolysin. Two µg of purified pIVEX2.4d/BCZK2532 plasmid DNA, encoding the BCZK2532 containing a 6 residue Histidine tag was used to drive *in vitro* synthesis of the BCZK2532 endolytic enzyme using an RTS 500 ProteoMaster *E. coli* HY Kit (Roche Applied Science)(1). Figure 4a shows a Coomassie Blue stained SDS-PAGE gel analysis of an IVT synthesis. Lane 1, molecular weight marker; lanes 2, 3 and 4, total proteins from IVT reactions containing the pIVEX2.4d/BCZK2532 plasmid DNA; lane 5; results of flow-through upon loading a reaction on a Nickel-affinity column to purify the endolysin from other IVT components; lane 6, 0 mM imidazole wash 1; lane 7, 0 imidazole wash 2. Figure 4b shows purification of the BCZK2532 from the Nickel-affinity column. Lane 1, molecular weight marker, lanes 2 through 5, 4 successive 10 mM imidazole washes; lanes 6 through 8, elution of purified endolysin from the column. Figure 4c, purified BCZK2532 and Ply21 endolysins. Lane 1, molecular weight marker; lane 2, purified BCZK2532 endolysin; lane 3, purified Ply21 endolysin.

Figure 5. Lysis of *B. anthracis* Sterne cells expressing the Green Fluorescent Protein (GFP) with BCZK2532. *B. anthracis* Sterne cells expressing GFP were grown and prepared as described in Materials and Methods. One culture was divided into five aliquots and differing concentrations

of BCZK2532 endolysin were added to each aliquot. Cells were analyzed by flow cytometry. Figure 5a shows flow cytometic analysis of *B. anthracis* cells exposed for different times to the endolysin. - ○- , no endolysin; - ■-, 1 nM endolysin; - ●-, 10 nM endolysin; - ▲-, 50 nM endolysin; - ◆-, 100 nM endolysin. Figure 5b shows the percent survival of *B. anthracis* cells based on the number of colony forming units (CFU) that remain after treatment BCZK2532 endolysin. - ○-, no endolysin; - ■ -, 1 nM endolysin; - ●-, 10 nM endolysin; - ▲ -, 50 nM endolysin; - ◆-, 100 nM endolysin.

Figure 6. Microscopic visualization of BCZK2532 endolysin treatment. Micrographs of *B. anthracis* Sterne cells after exposure to 2.5 µg/mL for 0 minutes (Panel 6A) and after 3 minutes of exposure (Panel 6B). Both images, frames taken from a movie of cell lysis, are of the same microscopic field before and after treatment with the endolysin.

Figure 7. Six different *Bacillus* isolates were treated with 100 nM BCZK2532 or Ply21 endolysin for 1 hour, then cells were plated at different dilutions on nutrient broth plates and incubated overnight to determine the number of surviving cells. Cells treated with BCZK2532 are labeled in black and cells treated with Ply21 are labeled in gray. Results shown are from two different plating experiments. Results are plotted as percent survival compared to untreated cells. Black bars show results of cells treated with BCZK2532 endolysin. Gray bars show results of cells treated with Ply21 endolysin.

Figure 1.

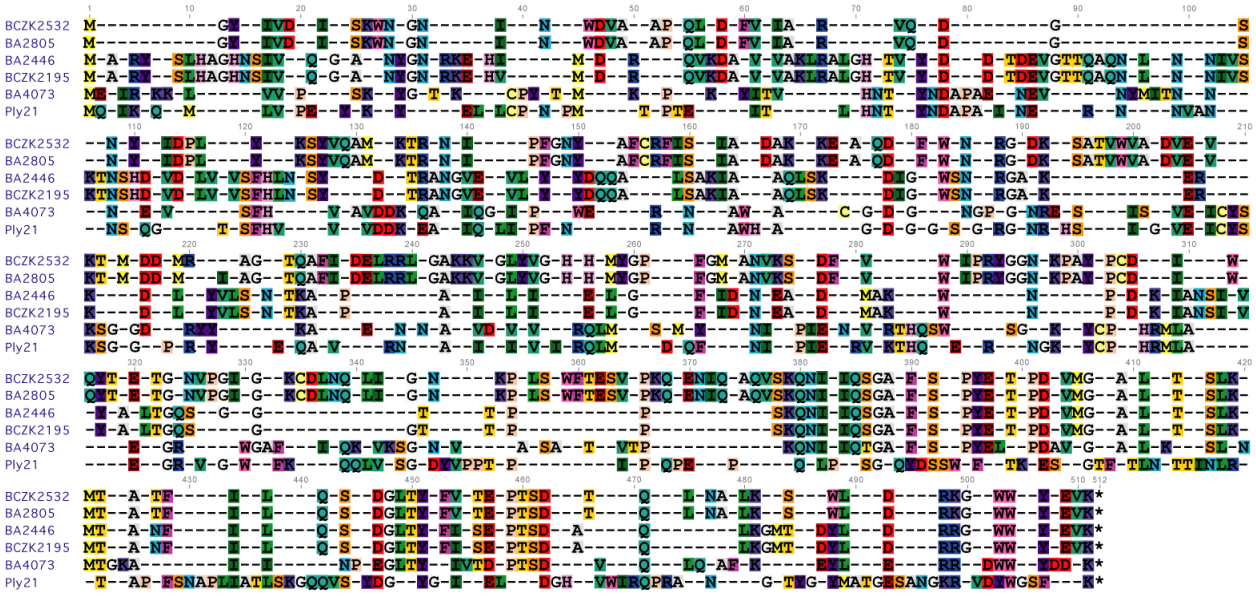


Figure 2.

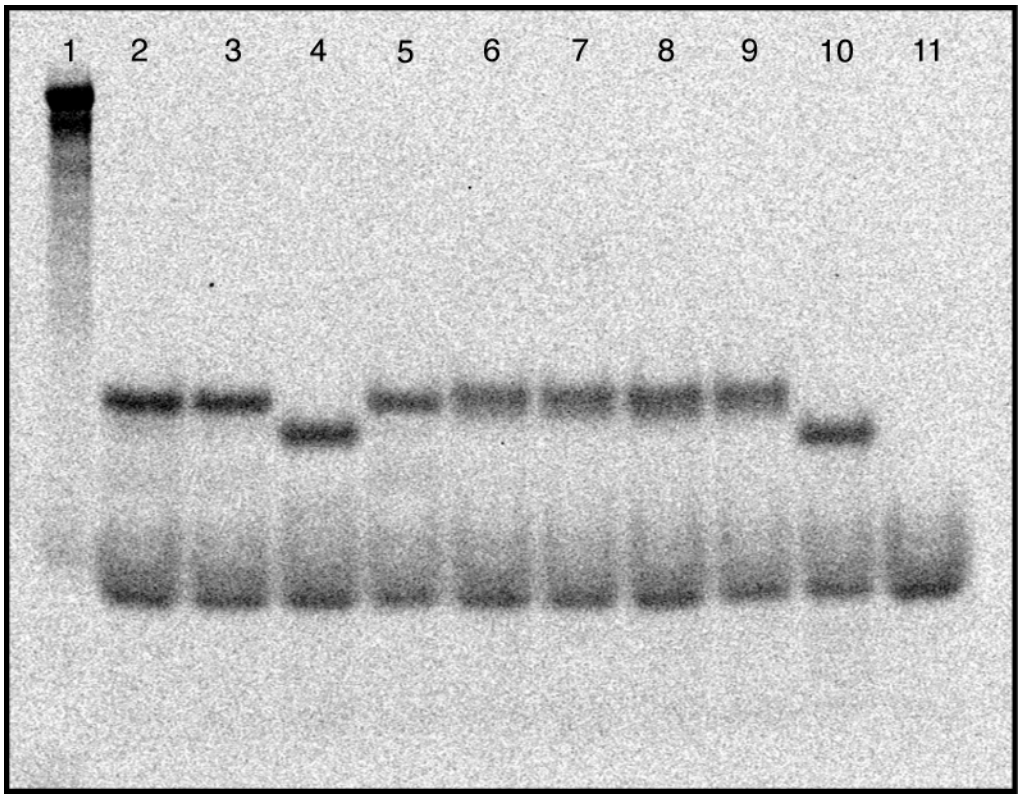


Figure 3.

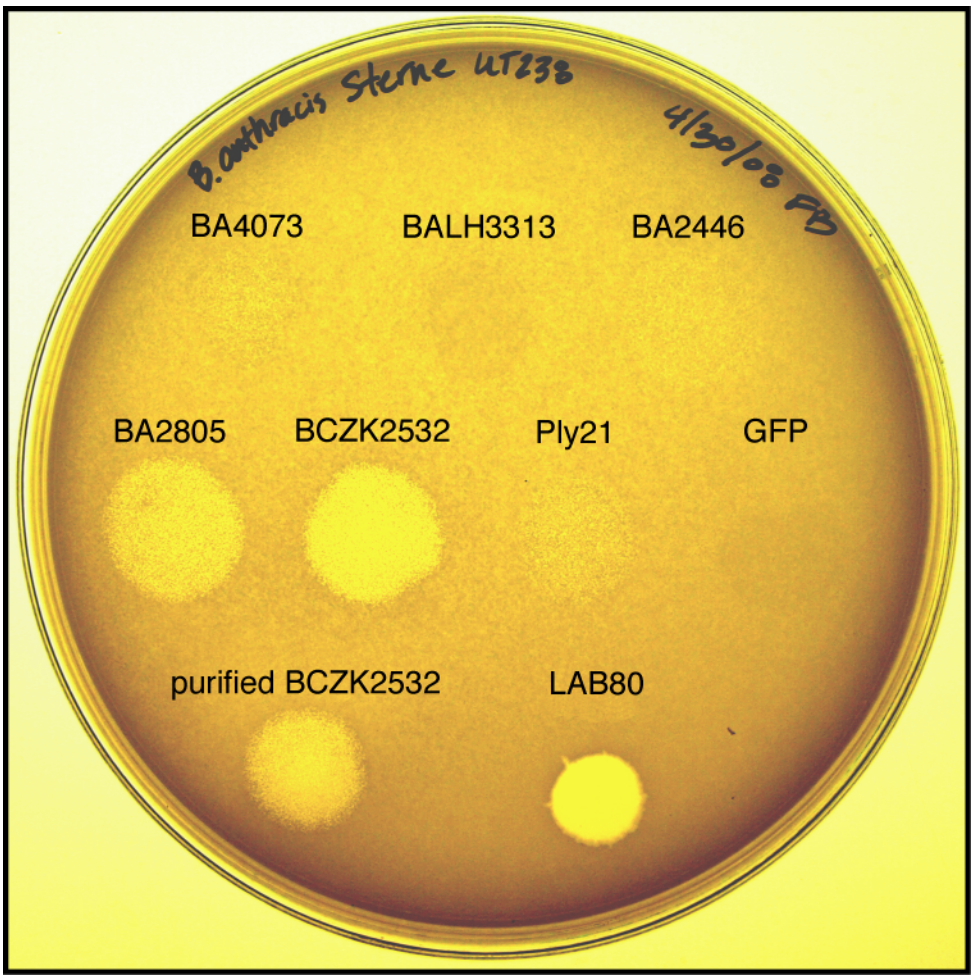


Figure 4.

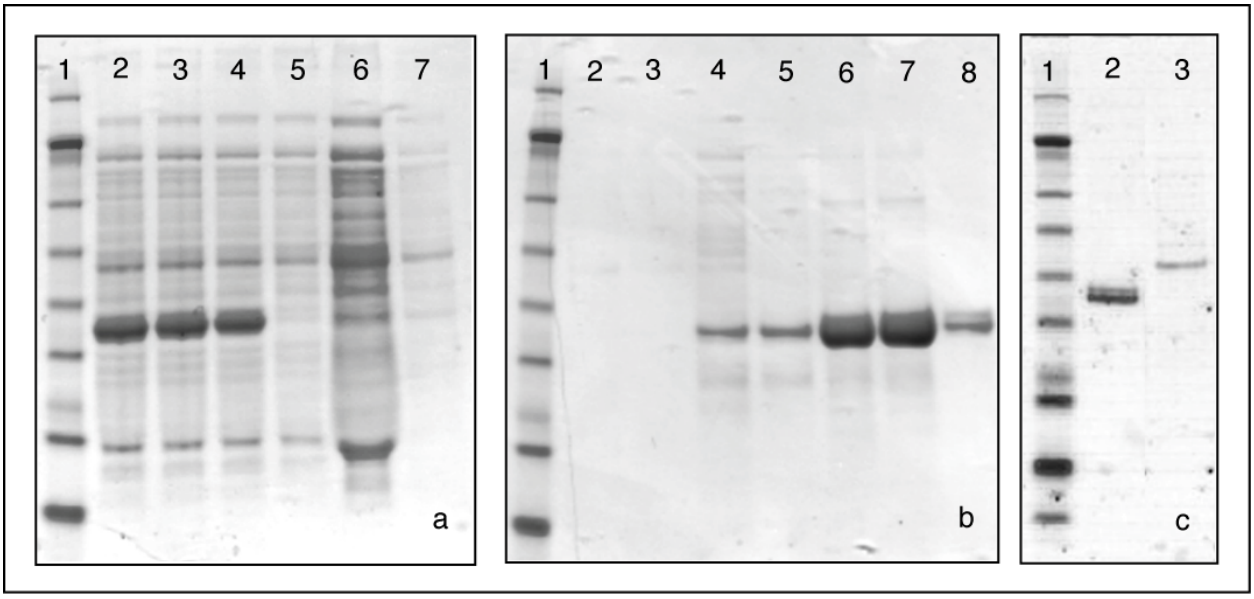


Figure 5.

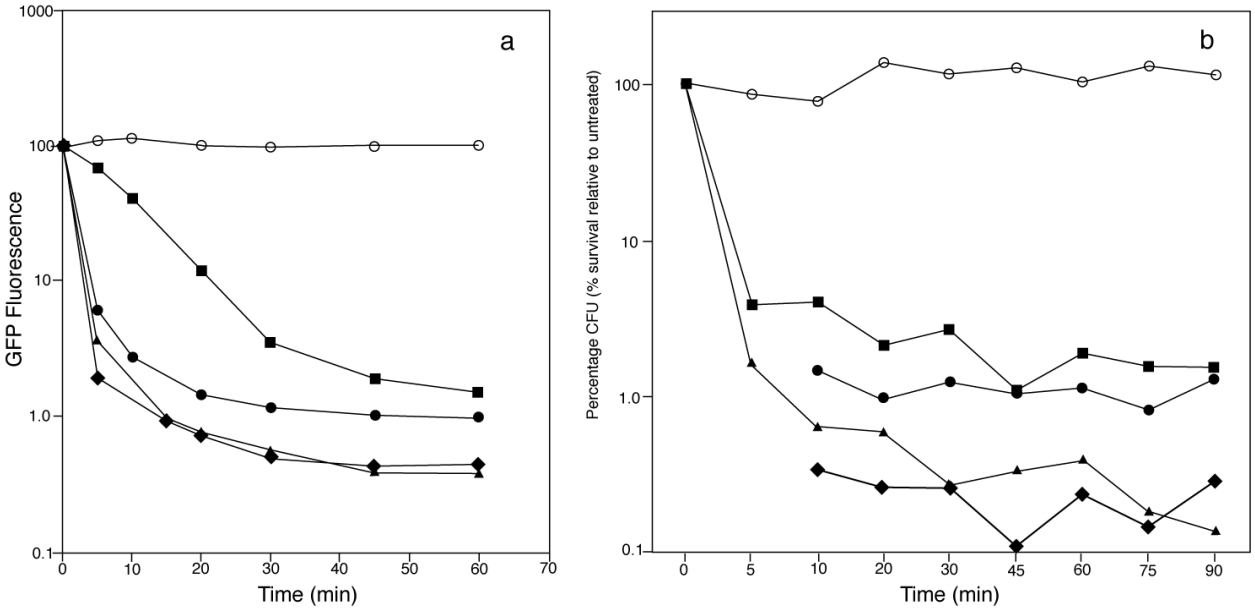

a
GFP Fluorescence
1000
100
10
1.0
0.1
0
10
20
30
40
50
60
70
Time (min)
b
Percentage CFU (% survival relative to untreated)
100
10
1.0
0.1
0
5
10
20
30
45
60
75
90
Time (min)

Figure 6.

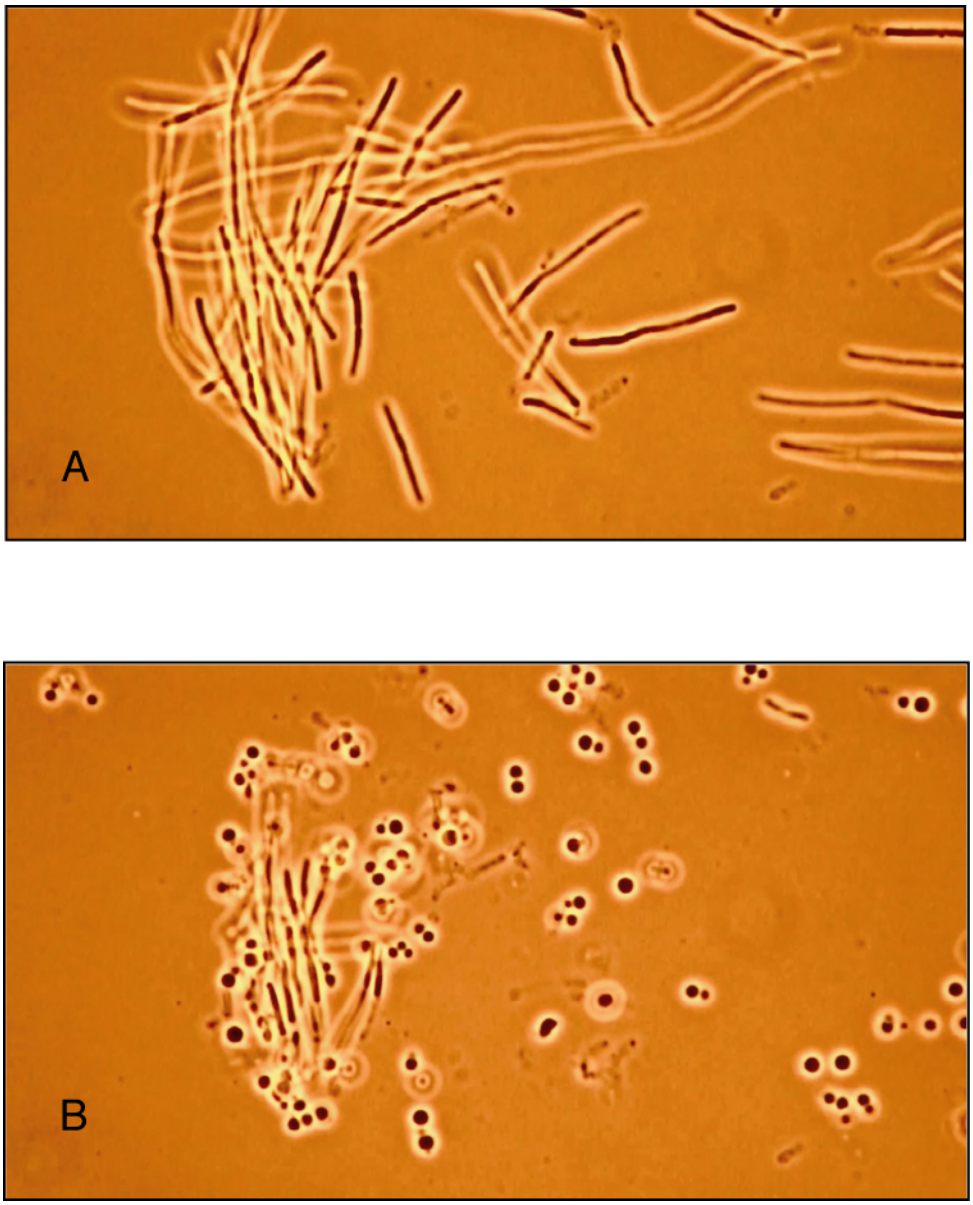
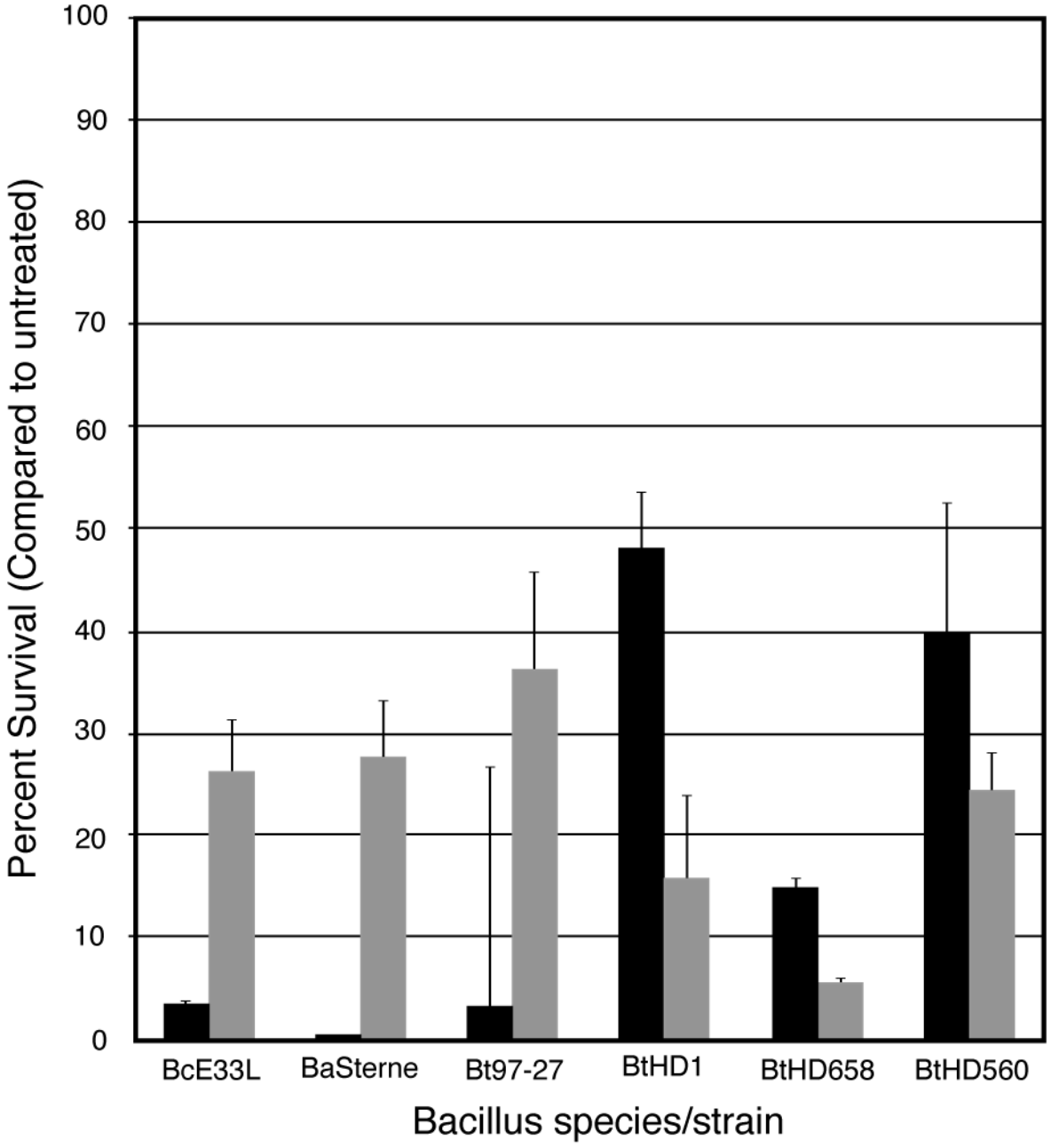


Figure 7.


100
90
80
70
60
50
40
30
20
10
0
Percent Survival (Compared to untreated)
BcE33L
BaSterne
Bt97-27
BtHD1
BtHD658
BtHD560
Bacillus species/strain