Anthrax, the infection caused by the Gram-positive pathogen *Bacillus anthracis* (*B.anthracis*), is fatal if untreated, and some strains of *B. anthracis* have been found to be resistant to currently available antibiotics. The development of broad spectrum antibiotics is needed to treat the resistive strains. In antibiotic development, we have targeted *B. anthracis* Class I PurE enzyme (*BaPurE*) as a unique and necessary enzyme in the de novo purine biosynthesis pathway, since the inactivation of this gene prevents *B. anthracis* growth in human serum, resulting in decreased bacterial proliferation. To identify inhibitors to *BaPurE*, structural information on the substrate binding to its active site is needed. However, it is difficult to obtain crystals of *BaPurE* with the substrate molecule in its binding site since upon binding to PurE, the substrate molecule is converted to the product molecule. An alternative approach is to create mutants of PurE that exhibit no enzymatic activity and do not convert the substrate to product, but still allow the substrate to bind to the active site. Then, the structure of mutant PurE with bound substrate can be obtained. We have identified a histidine residue at position 70 as the target of mutation to give an inactive enzyme. After successfully preparing the recombinant protein H70N, we have found that it exhibited no enzyme activity. This mutant will be useful in future experimentation to identify inhibitors of *BaPurE*.

## Materials and Methods

### Primer design

The sequence of the *BaPurE* gene BA0288\(^9\) was used (Figure 1), and the segment consisting of nucleotides 196-242 with CAT in the middle (GGT GGA GCA GCG CAT TTA CCA GGA ATG) was selected for primer design to give the desired mutation, replacing a histidine residue at position 70, or replacing the codon 70 (nucleotides 208-210) from CAT to AAT.
A silent mutation was also introduced to provide a unique restriction enzyme digestion site near the mutation site to allow quick analysis of the mutated plasmid. The complementary reverse primer sequence was also designed. The primer oligonucleotides were ordered from the Research Resources Center at the University of Illinois at Chicago (RRC) and used without further processing.

**Cell culture**

*E. coli* cells (either DH5α or BL21(de)C+ strains) were cultured in lysogeny broth (LB) medium, often also referred to as Lauria Broth or Lauria-Bertani medium, containing ampicillin, in a shaking-incubator (C25 Incubator - Shaker, New Brunswick Scientific; Enfield, CT) at 250 rpm and 37 °C for a specified time period. Values of optical density at 600 nm (OD 600) were obtained as a function of time. Usually, cells in 2 L medium were grown in a 4-L flask, and cells in 4 mL medium were grown in a 13-mL test tube. Cells from transformation (see below) were grown on agar plate with LB medium and ampicillin to screen for isolated colonies.

**cDNA plasmid isolation**

DH5α cells harboring a plasmid that expresses glutathione S-transferase (GST) and BaPurE fusion protein (pDEST-15 - BaPurE) were grown for 16 hrs. Cells were harvested for plasmid DNA extraction, using the PureYield Plasmid Miniprep System (Promega, Madison, Wisconsin). pDEST-15 - BaPurE plasmid was used as the template in polymerase chain reaction.

**Polymerase chain reaction**

Polymerase chain reactions (PCR) were carried out with a thermal cycler (Eppendorf, Brinkman Instrument, Westbury, New York) to give a modified pDEST-15 - BaPurE plasmid, pDEST-15 - H70N, to express PurE with the H70N mutation. Each PCR run started with an initiation step, at 95 °C for 5 min, followed by 20 cycles of 95 °C for 1 min, 55 °C for 30 sec and 72 °C for 14 min, and ended with the final step at 72 °C for 10 min. The reaction mixture consisted of the template (pDEST-15 - BaPurE) at 0.6 ng/µL (final concentration), forward and reverse primers at 2 ng/µL, a mixed dNTP solution (Promega, Madison, WI), with each nucleotide at 200 µM, and the DNA polymerase, Pfu Turbo (Strategene, Santa Clara, California) in Pfu buffer (2.5 enzyme units). The total volume of the reaction mixture was 50 µL.

After each PCR run, the sample was mixed with 1 µL of DpnI restriction endonuclease (New England Biolabs, Beverly, MA) and incubated at 37 °C for 1 hr to digest the methylated parent template and give pDEST-15 - H70N.

**Transformation**

pDEST-15 - H70N plasmid (2 µL) was transformed into DH5α *E. coli* cells (Z-Competent™ Cells from Zymo Research Corporation, Irvine, California) (50 µL), using the transformation kit provided by the company. Briefly, the DH5α Z-Competent cells (50 µL) were mixed with pDEST-15- H70N (1 µL), by tapping the container of the mixture lightly. The mixture (25 µL) was spread on a pre-warmed (37 °C) agar plate with LB medium and ampicillin, and incubated at 37 °C for 16 hr. Distinct colonies appeared on the plate. Individual colonies were inoculated into a liquid LB medium with ampicillin, and grown for 6 hr. The harvested cells were either used to extract the DNA plasmid for sequencing, or were mixed with glycerol (100 µL).

Similarly, pDEST-15 - H70N was also transformed into *E. coli* BL21(de3)C+ competent cells (New England Biolabs, Ipswich, MA). These cells were used for protein expression.

Cells for storage were mixed with glycerol (16% ; equal volume).

**DNA gel electrophoresis**

DNA gel electrophoresis was used to analyze PCR products and DNA fragments. Agarose (1%) gels with ethidium bromide were used with an electrophoresis unit (GEL XL Ultra V-2, Labnet International, Inc, Woodbridge, NJ). Each DNA sample (7 µL), with DNA loading dye (5 µL), was loaded to the gel. Two DNA ladders (100 bp and 1 kb plus) were also loaded to the gel. The electrophoresis was run for 30 min at 100 mV and constant current.

**Protein preparation**

Cells were grown in LB medium with ampicillin (2L in 4 L flasks) to active growth stage, and isopropyl β-D-thiogalactoside (IPTG) was added to the cells to induce GST- H70N fusion protein expression. Cells were allowed to continue to grow until the value of an optical density reading at 600 nm (OD₆₀₀) of about 1.3 and harvested for protein purification.

Standard procedures used in our laboratory for affinity column chromatography purification were used. Briefly, the cells were lyzed and the protein GST - H70N, as well as many other cellular proteins, was solubilized. The soluble cell extract was loaded to a GSH affinity column, and the fusion protein was eluted with buffer containing GSH. Thrombin was used to cleave the fusion proteins.
protein, and GST was removed by the affinity column to give pure H70N.

### Protein analysis

The purity of H70N was monitored by PAGE gel electrophoresis\(^\text{11}\). The mass of H70N was analyzed by high resolution mass spectrometry (RRC). The enzymatic activity of the mutant enzyme was measured using a published method\(^\text{12}\) with a temperature controlled UV spectrophotometer. Briefly, the conversion of substrate CAIR to product by the wild-type BaPurE (used as a control) or H70N was followed by monitoring the absorbance at 260 nm.

### Results

#### Primer design and PCR product

Based on the sequence of BaPurE cDNA (486 nucleotides, Figure 1), the following sequence was designed for the forward primer for His to Asn mutation at position 70: 5'- GGT GGA GCA GCG AAT TTA CCG GA ATG -3', with the bolded part as replacement of His with Asn. We also changed codon 72 from CCA to CCG, a silent mutation but introducing a restriction site for the enzyme NciI, which recognizes a CCGGG sequence and cleaves between C and G. The sequence for the complementary reverse primer was 5'- CAT TCC CGG TAA ATT CGC TGC TCC ACC -3'.

The product of a typical PCR run using the designed primers and plasmid pDEST-15 - BaPurE as the template appeared as a single band at about 7,000 base-pairs (bp) in DNA electrophoresis gel (Figure 2, Lane 2). The DNA ladders are shown in Lane 1 for the 100 bp marker and in Lane 6 for the 1 kbp plus marker. A PCR product of a previously prepared PurE mutant (R41E), used as a positive control (Lane 3), showing a band at about 7,000 bp, (4) a negative control sample, the same as the sample in Lane 2, but without PCR (Lane 4), showing no PCR DNA product, and (5) a 1 kbp plus DNA ladder marker (Lane 5).

#### Cell growth profile

Cell growth studies show normal and reproducible cell growth curves, with OD\(_{600}\) values of 0.5 to 1.0 at active growth stage (Figure 3). We also showed that IPTG, added during active growth stage to induce protein expression, produced no apparent effect on cell growth, as expected, for cells harboring either the wild-type or the mutant plasmids (Figure 3).

#### Transformation

Cells transformed with the plasmid pDEST-15 - H70N showed growth of distinct colonies on agar plate (Figure 4(a)), whereas the negative control, cells without the transformation step and thus harbored no pDEST-15-H70N plasmid, showed no growth (Figure 4(b)). Four colonies with plasmid were selected for DNA sequencing.
FIG. 3: Frozen *E. coli* cells harboring the pDEST-15-H70N plasmid were introduced into lysogeny broth and allowed to grow in a shaking incubator at 250 rpm and 37 °C. Values of optical density at 600 nm (OD$_{600}$) were obtained as a function of time. A typical growth curve shows active growth from about 300 min to about 450 min. The addition of IPTG during the active growth period to induce protein expression did not affect cell growth.

FIG. 4: DH5α Z-Competent™ Cells from Zymo Research Corporation and pDEST-15 - H70N mixture were spread on an agar plate consisting of LB medium and ampicillin, and pre-warmed to 37 °C. Distinct colonies appeared on agar plate after incubation at 37 °C for 16 hrs (A), whereas similar cells without transformation and thus harboring no pDEST-15 - H70N plasmid showed no colonies (B).

FIG. 5: Polyacrylamide (16%) gel with low molecular weight standards (Lane 1), wild-type *BaPurE* (Lane 2) and mutant H70N protein (Lane 3) showed that H70N with electrophoretic mass similar to that of wild-type at about 17 kDa, with a purity of about 90%. The protein samples (both Lane 2 and Lane 3) were overloaded to show minor impurity band(s).

DNA sequencing

Sequencing results of DNA extracted from the four distinct DH5α *E. coli* colonies obtained from transformation, each cultured in 24 mL medium to give about 13 µg (60 µL at 221 ng/µL) of the DNA plasmid, showed only Colony 3 consisting of the proper DNA sequence of H70N as designed, with nucleotide 208 as A rather than C to give a codon of AAT for asparagine, and with nucleotide 216 as G rather than A to give a restriction site CCGGG for enzyme NciI. The sequences of plasmids in Colonies 1, 2 and 4 were not as designed. Thus, these colonies were discarded.

Protein expression and analysis

Generally, from 6 g cells, about 2 mg of H70N protein was obtained at a purity about 90% (Figure 5, Lane 3). The purification result was similar to that of the wild-type (Lane 2).

The expected mass of H70N calculated from the amino acid sequence was 17,299.9 Da, and the mass spectrometry analysis indicated a mass of 17,299.0 Da. The mass difference in of a histidine residue (137.1 Da) in the wild-type PurE (17,322.9 Da) and of a asparagine residue (114.1 Da) in H70N is 23 Da. Thus, the masses of H70N and of the wild type is easily resolved by mass spectrometry analysis. Our mass spectrometry results clearly indicate that the mutant protein H70N, and not the wild-type PurE, was expressed and purified.

The enzyme activity assay (Figure 6) showed that the mutant exhibited no change in absorbance as a function of time, thus demonstrating no detectable enzymatic ac-
FIG. 6: The conversion of substrate CAIR to product by BaPurE (wild-type) or H70N was followed by monitoring the absorbance at 260 nm ($A_{260}$). The decrease in $A_{260}$ indicates the disappearance of CAIR, and thus an active enzyme for WT. However, the $A_{260}$ values for H70N remained unchanged, indicating an inactive enzyme for H70N.

activity. The wild-type enzyme exhibited a decrease in absorbance as a function of time, demonstrating its ability to convert the substrate molecule to product molecule.

Discussion

Our goal is to prepare a mutant BaPurE with the mutation at the active site to inactivate the enzyme and thus to allow the substrate to bind to the active site for structural studies. Our sequence alignment suggested the histidine residue at position 70 was a good candidate for the mutation. We designed and prepared a pair of primers to introduce the H70N mutation into the BaPurE cDNA. We successfully obtained an expression plasmid for H70N and used it to express the mutant protein H70N. To our delight, the newly prepared mutant exhibited no enzymatic activity.

Conclusion

In identifying inhibitors to BaPurE, structural information on the substrate binding to its active site is needed. However, it is difficult to obtain crystals of BaPurE with its substrate molecule in its binding site since upon binding to PurE, the substrate molecule is converted to the product molecule. An alternative approach is to obtain the structure of a mutant PurE that exhibits no enzymatic activity to allow the substrate molecule to remain bound in the active site of the mutant PurE, and not be converted to a product molecule. We have identified a histidine residue at position 70 as the target of mutation to give an inactive enzyme. We successfully prepared the recombinant protein H70N and found that it exhibited no enzyme activity. This mutant will be useful in future experimentation to identify inhibitors of BaPurE.

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