Abstract
Serine Protease Autoantibodies of Enterobacteriaceae (SPAE) are multidomain proteins found in pathogenic enteric bacteria. They are characterized by a conserved serine protease domain. These proteins are comparatively large (> 100 kDa) and comprised of a signal sequence, a passenger domain, and a translocase domain. The passenger domain is responsible for the protein's virulence activity. Temperature sensitive hemagglutinin (Tbh) is used as a model SPAE in our studies. In the present study we aim to define the substrate binding site of Tbh. Sequence alignment was performed using Tbh and another autoantibody called Hap, for which the potential substrate binding site is already public. From this we identified five potential residues: G254, L279, T280, A281, and N290. Based on our hypothesis, mutations introduced at these five residues are expected to deform the substrate groove and thereby inhibit the correct positioning of the substrate at the active site. This would result in decreased proteolytic function. Therefore, to test this hypothesis we introduced single point mutations at these five residues using PCR based site directed mutagenesis. SDS-PAGE and western blotting were then used to analyze the secretion pattern of the mutants. A functional assay was performed using a Tbh-specific oligopeptide assay and a muca assay. The secretion analysis showed that the mutations did not impact the proper secretion of the mutants.

The results of the cleavage assays showed a significant reduction in the proteolytic activity of the mutants. These results show that the five residues that we have identified are crucial for the proteolytic activity of Tbh, which suggest a role of these residues in forming the Tbh substrate binding site.

Introduction
Gram-negative bacteria have an outer membrane encompassing their cell walls which confers the secretion and subversion of substances such as proteins. As a result, gram-negative bacteria have developed systems to aid in the secretion of proteins to the extracellular space. One of the protein secretion pathways is called the Autotransporter (AT) pathway. This is a two-step pathway that transports virulence factors to the extracellular environment. These virulence factors contribute greatly to bacterial pathogenicity. The autoantibodies proteases secreted via this pathway are fairly large (>100 kDa). These proteins are comprised of three domains: a N-terminal signal sequence, a passenger domain, and a translocase domain. The N-terminal signal sequence is recognized by the Sec translocase allowing the protein to traverse the outer membrane. The passenger domain folds into a functional product upon secretion and is responsible for virulence activity, and lastly the C-terminal translocase domain translocates across the outer membrane.

Research Overview
The goal of our research is to determine the amino acid residues making up the substrate binding site of Tbh. In order to determine the potential binding site residues, the crystal structures of the serine protease alpha-muramylocayotenovar bound to a tosyl-ester inhibitor and the serine protease domain of Hbp were superimposed in Swiss PDB Viewer. The coordinates of the tosyl-ester in the superimposed conformation were then merged with the Hbp serine protease domain coordinates, enabling us to search for residues around the inhibitor. Sequence alignment was also performed between Tbh and Hap, another autoantibody for which the potential substrate binding site has been identified. Using these tools, we were able to identify the following residues as potential residues of the Tbh substrate groove: G254, L279, T280, A281, and N290. Based on our hypothesis, mutations introduced at these five residues are expected to deform the substrate groove and thereby inhibit the correct positioning of the substrate at the active site. This would result in decreased proteolytic function.

To test these hypotheses, PCR based site-directed mutagenesis will be used to mutate the binding site residues of Tbh. After mutation, the residues will be subjected to a secretion profile analysis using SDS-PAGE and western blotting to confirm secretion of Tbh. Cleavage assays such as the Tbh-specific oligopeptide assay and muca assay will then be used to study the proteolytic function of the mutants.

Mutagenesis
1. Primers for the mutant were constructed.
2. PCR was used to amplify the mutant DNA.
3. Mutated plasmid was used to transform bacteria (E. coli K-12 strains)
4. Extraction of plasmid to verify sequence using Quagen miniprep kit.
5. DNA sequencing was done at City of Hope

Results

Table 1: Examples of serine proteases of the enterobacteriaceae

<table>
<thead>
<tr>
<th>Protein</th>
<th>Organism</th>
<th>Disease Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>EspP</td>
<td>EHEC</td>
<td>Bacteria diarrhea, hemolytic colitis</td>
</tr>
<tr>
<td>EspC</td>
<td>EPEC</td>
<td>Diarrhea, enterotoxin</td>
</tr>
<tr>
<td>EspF</td>
<td>STEC</td>
<td>Diarrhea, hemolytic colitis, failure</td>
</tr>
<tr>
<td>EspG</td>
<td>EHEC</td>
<td>Bloody diarrhea, hemolytic colitis</td>
</tr>
<tr>
<td>EtaA</td>
<td>EPEC</td>
<td>Diarrhea</td>
</tr>
<tr>
<td>Hbp</td>
<td>E. coli</td>
<td>Wound infections, colitis</td>
</tr>
<tr>
<td>Tbh</td>
<td>APEC</td>
<td>Colibacillosis, enteropathia, mesenteric ischemia</td>
</tr>
<tr>
<td>Sept</td>
<td>Shigella</td>
<td>Fecleni</td>
</tr>
<tr>
<td>Pic</td>
<td>EADEL</td>
<td>Shigella flexneri</td>
</tr>
</tbody>
</table>

Oligopeptide Cleavage Assay
Table 2: Cleavage activity of a conserved and non-conserved mutant on a porcine gastric mucin gel. Culture supernatant of each mutant were collected and concentrated using a 10-K exclusion gel: Iband PMF is used as a control protease inhibitor. The samples are resolved by SDS-PAGE (10% acrylamide gels) and stained with Schiffs reagent. Wild type (wt) serves as a negative control.

Conclusions
This results show that at the mutations at the five residues, G254, L279, T280, A281, and N290 lead to extremely low proteolysis of the Tbh specific substrate, indicating that these residues are crucial for the protein's proteolytic function. This supports our hypothesis and strongly suggests a role of these residues in forming the substrate binding site of Tbh.

Methods

Molecular modeling: Graphics were generated using the coordinate of the published crystal structure of EspB (Ortiz, Stivala et al. 2003, PDB ID: 1YAK) and the VMD program (Humphrey et al. 1996) version 3.7.

Site-directed Mutagenesis: Site-directed mutagenesis of plasmid (pJAG433) carrying ctb E. coli was performed according to a Quagen based protocol (Humphrey et al. each construct was performed at the DNA sequencing lab of City of Hope.

Culture Preparation and Infection: E. coli K-12 cells were grown at 37°C in Luria broth medium containing 0.1% glycerol at an optical density of 0.6-1.0 reached at 600nm after further expression of Tbh was induced by the addition of IPTG for 3 hours. Supernatants were then filtered through 0.22-μm 10-350 mm filters and concentrated 500-fold using a 10-K exclusion gel. The concentration of the supernatant was checked by measuring the Bradford assay. SDS-PAGE gel Electrophoresis and Silver Staining (5% polyacrylamide gels) were prepared. The samples were run on gels and visualized using silver staining.

Oligopeptide Cleavage Assay: Peptides were incubated with wild-type and mutant autotransporter in SDS-PAGE gel at pH 7.4 in the wells. After the bands were resolved for 40 min and the absorbance readings taken at 520 nm.

Acknowledgements
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