Self-Assembly of Human Defensin 6 and Its Role in Innate Immunity and Siderophore-Based Strategies to Target Gram-Negative Bacteria

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ABSTRACT

The first part of this thesis focuses on studies of structural and functional properties of human defensin 6 (HD6). This peptide is produced and secreted by intestinal Paneth cells as a part of innate immune response to invading microbes. Instead of killing microbes, HD6 self-assembles into higher-order oligomers to entrap and prevent microbes from invading into the host cells. This activity of HD6 is unusual among defensins. Herein, we employed biophysical and biological techniques to decipher the molecular details of the unusual behavior of HD6. We demonstrate that the self-assembly of HD6 is driven by hydrophobicity and this work highlights how variable amino acid sequences among defensins afford different biological function. We further elucidated how HD6 is stored in Paneth cells such that its self-assembly is suppressed. Similar to zymogens, HD6 utilizes a pro region to control its self-assembly and upon secretion, trypsin cleaves the propeptide to unleash mature HD6 and trigger host-defense function. We also discovered that HD6 suppresses virulence traits of Candida albicans, an opportunistic fungal pathogen. This study expands the scope of the broad-spectrum function of HD6.

In the second part of this thesis, we present two siderophore-based strategies that target Gram-negative bacteria. Siderophores are small-molecule iron chelators that bacteria employ to sequester iron from the environment. Our approaches focus on enterobactin (Ent) and its glycosylated derivatives (GlcEnt), which are virulence factors of certain enteric pathogens, such as Salmonella spp. The first approach relies on the use of GlcEnt-β-lactam conjugates to target these pathogens. In addition to enhanced uptake efficiency of the drug into bacteria, GlcEnt specifically delivers the antibiotic to pathogens and leave commensal bacteria unaffected. In the second approach, we aim to use Ent/GlcEnt-specific antibodies to inhibit bacterial iron acquisition, and thereby prevent the bacteria from colonizing in the host. We demonstrate that antibodies against Ent/GlcEnt are produced in the mice immunized with a protein-siderophore conjugate. These mice exhibit reduced intestinal colonization, reduced systemic dissemination of S. Typhimurium, and increased resistance against the challenge of this pathogen.

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Chapter Abstracts

Chapter 1. Introduction of Human Defensins

The innate immune system is the first line of host-defense response to microbial invaders at the mucosa. This Chapter reviews the components of innate immune system in the small intestine and focuses on Paneth cells, which produce and release a cocktail of antimicrobial substances, including human defensins (HDs), into the lumen upon microbial stimuli. HDs are a family of small host-defense cysteine-rich peptides and they contribute to innate system by fighting against microbes with variable mechanism of action. The classification, expression, structural features, and host-defense function of HDs are discussed in this Chapter.

Chapter 2. Molecular Basis for Self-Assembly of a Host-Defense Peptide that Entraps Bacterial Pathogens

This Chapter describes molecular-level understanding for why HD6 functions differently from other human defensins. Native HD6 readily self-assembles into elongated fibrils, agglutinates bacteria, and prevents Listeria monocytogenes from invading cultured mammalian cells. Mutation of hydrophobic residues perturbs HD6 self-assembly and results in attenuated biological activity. In particular, the F2A and F29A variants do not form fibrils, agglutinate bacteria, or prevent L. monocytogenes invasion. In total, our results demonstrate that the hydrophobic effect is essential for promoting HD6 self-assembly and innate immune function. This work provides a description of how variations in amino acid sequence confer diverse physiological functions to members of the defensin family.
Chapter 3. Proteolysis Triggers Self-Assembly and Unmasks Innate Immune Function of a Human α-Defensin Peptide

In this Chapter, we elucidate critical steps in the self-assembly pathway of HD6. Our ex vivo analysis of human samples reveal that HD6 is stored in the granules of Paneth cells as an 81-residue propeptide, and is recovered from ileal lumen as a 32-residue mature peptide. The propeptide neither forms higher-order oligomers, nor agglutinates bacteria, nor prevents the human gastrointestinal pathogen *L. monocytogenes* invasion into epithelial cells. Trypsin, which is also expressed by Paneth cells, is the processing enzyme that cleaves the propeptide to afford mature HD6 and unmask its latent host-defense function.

Chapter 4. Human Defensin 6 Suppresses Virulence Traits of *Candida albicans*

This Chapter describes exploratory work that addresses the effect of HD6 on *Candida albicans* virulence traits. *C. albicans* is an opportunistic fungal pathogen that resides as a part of the gut microbiota in the intestine. Our work indicates that HD6 suppresses two *C. albicans* virulence traits, namely invasion into human epithelial cells and biofilm formation, by preventing the fungi from adhering to the surfaces. In addition, our preliminary morphological studies indicate that HD6 may suppress *Candida* transition from non-virulent yeast to virulent hyphae. To date, most studies about HD6 focus on its effect on bacteria. Our results expand the scope of HD6 host-defense function to fungi.
Chapter 5. Introduction to Siderophore-Based Strategies to Target Gram-Negative Bacteria

This Chapter presents an overview of iron homeostasis in humans and bacteria with emphasis on siderophore-mediated iron acquisition machinery in bacteria. Siderophores are small-molecule iron chelators that bacteria biosynthesize and employ to sequester iron from the environment. The chemistry and biology of siderophores as well as the biosynthesis and transport machinery of siderophores are discussed. New antimicrobial strategies are needed for combating bacterial infection due to the emergence of antibacterial resistance in hospital community settings and the paucity of new antibiotic in the drug pipeline. Targeting microbial virulence factors, including siderophores, has become one of the promising approaches for developing narrow-spectrum antimicrobial strategies. The overview and examples of antivirulence strategies with emphasis on the progress on siderophore-based approaches are reviewed in this Chapter.

Chapter 6. Targeting Virulence: Salmochelin Modification Tunes the Antibacterial Activity Spectrum of β-Lactams for Pathogen-Selective Killing of Escherichia coli

In this Chapter, we present a strategy that specifically delivers antibiotics to bacterial pathogens, which utilizes the salmochelin (GlcEnt)-mediated iron uptake machinery. GlcEnt-β-lactam conjugates that harbor the antibiotics ampicillin (Amp) and amoxicillin (Amx), hereafter GlcEnt-Amp/Amx, were synthesized and these molecules exhibit up to 1000-fold enhanced antimicrobial activity against uropathogenic E. coli relative to the parent β-lactams. Moreover, GlcEnt-Amp/Amx based on a diglycosylated Ent platform selectively kill uropathogenic E. coli that express the salmochelin receptor IroN in the
presence of non-pathogenic *E. coli* and other bacterial strains that include the commensal microbe *Lactobacillus rhamnosus* GG. This work establishes that siderophore-antibiotic conjugates provide a strategy for targeting virulence, narrowing the activity spectrum of antibiotics in clinical use, and achieving selective delivery of antibacterial cargos to pathogenic bacteria on the basis of siderophore receptor expression.

Chapter 7. A Siderophore-Based Immunization Strategy to Inhibit Growth of Enteric Pathogens

An immunization approach that targets siderophores is discussed in this Chapter. Conjugates of Ent and the immunogenic carrier protein cholera toxin subunit B (CTB) were synthesized and used to immunize mice. The CTB-Ent-immunized mice produce anti-Ent and anti-DGE antibodies in the gut mucosa and when infected with the enteric pathogen *Salmonella*, the immunized mice exhibit reduced intestinal colonization, reduced systemic dissemination of the pathogen, and increased survival. Moreover, analysis of the gut microbiota reveals that the reduction of *Salmonella* colonization is accompanied by the expansion of *Lactobacillus* spp., beneficial commensal organisms that thrive in a similar environment as Enterobacteriaceae. Taken together, our results demonstrate that antibodies against siderophores confer protection against infection with *Salmonella* and possibly other Enterobacteriaceae that deploy similar molecules to acquire iron in the host.
Appendix A. The Effect of Methionine Oxidation on Self-Assembly of Human Defensin 6

This Appendix describes the synthesis, purification, and preliminary functional characterization of the Met(O)23-HD6. Met23 is surface-exposed and the oxidation of Met23 in HD6 is catalyzed by acid. Our preliminary biophysical studies reveal that the methionine oxidation impairs the HD6 self-assembly possibly due to an increase in polarity of Met23. These results are in agreement with our work with the HD6 variants described in Chapter 2. We speculate that the methionine oxidation may be employed to control the self-assembly of HD6 during infection and may confer additional roles of HD6 in innate immune system.

Appendix B. An N-Terminal Extension of Human Defensin 6 Attenuates Its Self-Assembly and Biological Function

This Appendix presents the preparation, purification, and functional studies of RALGSTR-HD6 (proHD662-100). In Chapter 3, we show that the pro region of proHD6 temporally and spatially controls the HD6 self-assembly. Subsequently, we question why proHD6 contains a long flexible pro region. Our results indicate that a 7-residue N-terminal extension is sufficient to suppress the HD6 self-assembly and biological function. Therefore, we speculate that there are additional roles of the pro region besides controlling the HD6 self-assembly. Further investigation is required to address this notion.
To Mom, Dad, and Grandma
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My graduate study at MIT has provided me with an invaluable experience for my future. I feel grateful for a chance of learning and conducting scientific research in the environment filled with great scientists and inexhaustible resources. In addition, I had a great opportunity to meet many people from different parts of the world and having conversation with these people expanded my horizon not only about science, but also life in general. I would not have completed my graduate journey without the following people and I would like to take this opportunity to thank all of them.

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The Ent-based immunization work was achieved because of our strong collaboration with Professor Manuela Raffatellu at UC Irvine and her graduate student, Martina Sassone-Corsi. They conducted in vivo experiments with the Ent conjugates
that I made. We shared data and had great discussions about science and future career. Working on the immunization project also exposed me to the microbiology and immunology world and made me realize how fascinating these topics are. I would like to thank them for these. I want to thank Professor Charles L. Bevins at UC Davis and his former graduate students, Patricia Castillo and Hiutung Chu, for our collaboration on the proHD6 work and for letting me visit your laboratory. I really had a great week there.

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### Abbreviations

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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>A. baumannii</td>
<td>Acinetobacter baumannii</td>
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<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>AHL</td>
<td>acyl homoserine lactone</td>
</tr>
<tr>
<td>AMA</td>
<td>Antimicrobial activity</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
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<tr>
<td>AMPs</td>
<td>Antimicrobial peptides</td>
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<td>AmpB</td>
<td>Amphotericin B</td>
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<td>Amx</td>
<td>Amoxicillin</td>
</tr>
<tr>
<td>APMSF</td>
<td>4-Amidinophenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>Aq</td>
<td>Aqueous</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Analytical ultracentrifugation</td>
</tr>
<tr>
<td>B. anthracis</td>
<td>Bacillus anthracis</td>
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<tr>
<td>B. subtilis</td>
<td>Bacillus subtilis</td>
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<tr>
<td>BHI</td>
<td>Brain heart infusion medium</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>C. albicans</td>
<td>Candida albicans</td>
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<td>C. tetani</td>
<td>Clostridium tetani</td>
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<td>C. trachomatis</td>
<td>Chlamydia trachomatis</td>
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<td>CD</td>
<td>Circular dichroism</td>
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<td>CFU</td>
<td>Colony forming unit</td>
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<td>CI</td>
<td>Competitive index</td>
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<td>Cholera toxin</td>
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<td>CTB</td>
<td>Cholera toxin subunit B</td>
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<td>DGE</td>
<td>Diglycosylated enterobactin</td>
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<td>DHB</td>
<td>2,3-Dihydroxybenzoic acid</td>
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<td>DIPEA</td>
<td>N,N-diisopropylethylamine</td>
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<td>DMEM</td>
<td>Dulbecco's modified Eagle medium</td>
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<td>Dimethylformamide</td>
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<td>Dimethyl sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DP</td>
<td>2,2’-Dipyridyl</td>
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<td>2,2’-Dithiodipyridine</td>
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<td>E. coli</td>
<td><em>Escherichia coli</em></td>
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<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>Ent</td>
<td>Enterobactin</td>
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<td>ESI</td>
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<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
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<td>GI</td>
<td>Gastrointestinal</td>
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<td>GlcEnt</td>
<td>Glycosylated enterobactin</td>
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<td>High resolution mass spectrometry</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>HSV</td>
<td>Herpes simplex virus</td>
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<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half maximal inhibitory concentration</td>
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<td>ICP-OES</td>
<td>Inductively coupled plasma atomic emission spectroscopy</td>
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<td>Abbreviation</td>
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<td>LB</td>
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<td>N-acetylmuramic acid</td>
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<td>Sodium phosphate</td>
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<td>Ni-NTA</td>
<td>Nickel-nitritriacetic acid</td>
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<td>NIS</td>
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<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NRAMP</td>
<td>Natural resistant macrophage protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NRPS</td>
<td>Non-ribosomal peptide synthetase</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td>P. syringae</td>
<td>Pseudomonas syringae</td>
</tr>
<tr>
<td>PBP</td>
<td>Penicillin-binding protein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PITC</td>
<td>Phenylisothiocyanate</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear neutrophils</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
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<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
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<tr>
<td>QS</td>
<td>Quorum-sensing</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RP</td>
<td>Reverse phase</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>S. aureus</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>S. dysenteriae</td>
<td>Shigella dysenteriae</td>
</tr>
<tr>
<td>S. flexneri</td>
<td>Shigella flexneri</td>
</tr>
<tr>
<td>S. Typhimurium</td>
<td>Salmonella enterica serovar Typhimurium</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SE</td>
<td>Sedimentation equilibrium</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>sIgA</td>
<td>Secretory immunoglobulin A</td>
</tr>
<tr>
<td>sPLA$_2$</td>
<td>Secretory group IIA phospholipase A$_2$</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>SV</td>
<td>Sedimentation velocity</td>
</tr>
<tr>
<td>tBu</td>
<td>tert-Butyl</td>
</tr>
<tr>
<td>TBTA</td>
<td>Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>TCP</td>
<td>Toxin co-regulated pilus</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>Tf</td>
<td>Transferrin</td>
</tr>
</tbody>
</table>
TFA  Trifluoroacetic acid  
TfR  Transferrin receptor  
TGE  Triglycosylated enterobactin  
TLC  Thin-layer chromatography  
TPCK  Tosyl phenylalanyl chloromethyl ketone  
Tris  Tris(hydroxymethyl)aminomethane  
TRR  Tandem repeat region  
TSB  Tryptic soy broth  
TTSS  Type III secretion system  
UA  Uranyl acetate  
UDP-Glc  Uridine diphosphate glucose  
UTI  Urinary tract infection  
*V. cholerae*  *Vibrio cholerae*  
*Y. enterocolytica*  *Yersinia enterocolytica*  
*Y. pseudotuberculosis*  *Yersinia pseudotuberculosis*  
YPD  Yeast extract-peptone-dextrose
Chapter 1

Introduction to Human Defensins
1.1 Innate Immunity in the Intestine

The immune system is a network of cells, tissues, and organs that work together to protect the host from infections caused by pathogens.\(^1\) This system can be divided into innate and adaptive immunity.\(^1\) Both immune systems can distinguish between self and nonself, but the recognition mechanisms are different. The innate immune system relies on certain receptors and secreted proteins that recognize common features among several pathogens.\(^2\) On the other hand, the adaptive immune system employs a process of somatic cell gene rearrangement to generate a vast repertoire of antigen receptors that are able to discriminate closely related molecules.\(^3\) Although adaptive immune responses are more specific to the pathogenic invaders than innate immune responses, it takes much longer time for the adaptive immune system to generate responses to the pathogens.\(^2,3\) As a result, the innate immune system functions as the first line of a host defense to counteract pathogenic invasion at the mucosa.\(^2,4,5\)

The small intestine is the largest organ in the human body and is essential for absorbing nutrients. It is constantly exposed to dietary and foreign materials, including pathogenic microorganisms.\(^4\) In addition, trillions of commensal microbes, also known as the microbiota, reside in the intestinal tract. The commensal provide colonization resistance because they compete with pathogenic invaders for nutrients and for attachment sites on epithelial cells, and thereby prevent the pathogenic invaders from colonizing in the host.\(^6,7\) Under some circumstances, commensals can cause infections if they grow excessively or if the immune system is compromised.\(^8\) To control the commensal microbes as well as to prevent the pathogens from colonizing and causing infections, the host has to maintain the intestinal homeostasis tightly. One of the key
contributors to intestinal innate immunity is the intestinal epithelium (Figure 1.1), which creates a host defense against microbial invasion by forming physical and chemical barriers. The intestinal epithelial cells are held together by tight junctions, which effectively form a seal against the external milieu. Moreover, the intestinal epithelial cells sense and respond to microbial stimuli by reinforcing their barrier function, secreting a cocktail of antimicrobial substances, and inducing appropriate immune responses, varying from tolerance to anti-pathogen immunity. 4,5

![Diagram of intestinal epithelial cells](image)

Figure 1.1. The physical and chemical barriers in a crypt of small intestine. Intestinal epithelial cells form physical and chemical barriers that maintain segregation between luminal microbial communities and the mucosal immune system. AMP = antimicrobial peptide and sIgA = secretory immunoglobulin A.

The intestinal epithelium (Figure 1.1) is composed of four cell lineages, namely enterocytes, enteroendocrine cells, goblet cells, and Paneth cells, all of which are
derived from a common stem cell progenitor in the crypts. The majority of cells bordering the intestinal lumen are absorptive enterocytes, which are important for metabolic and digestive function. Secretory intestinal epithelial cells, which include enteroendocrine cells, goblet cells, and Paneth cells, are specialized for maintaining the barrier function of the epithelium. Enteroendocrine cells provide communication between the central and enteric neuroendocrine systems via the secretion of several hormones that regulate digestive function. Goblet cells and Paneth cells secrete mucus and antimicrobial peptides (AMPs), respectively, to promote the removal of microbes from the mucosal surface. The transcytosis and luminal release of secretory immunoglobulin A (sIgA) further reinforce this barrier function. These molecules will be further discussed later in Section 1.6. Beneath the epithelium is the lumina propria, where macrophages and dendritic cells (DCs) reside. Macrophages function as innate effector cells by engulfing and removing invading microbes, secreting cytokines, and maintaining the intestinal homeostasis. In contrast, DCs migrate from the intestine to the mesenteric lymph nodes (MLNs) and initiate adaptive immune responses by priming naïve T cells.

In summary, the innate immune system is one of the primary driving forces that maintain intestinal homeostasis. It consists of a complex network of different types of cells working together to promote physical and chemical barriers against microbial invasion. The importance of these barriers is exemplified by defects in the components of the intestinal innate immune system leading to increased susceptibility to diseases such as Crohn's disease and microbial infections, even in the presence of a functional adaptive immune system.
1.2 Paneth Cells and Antimicrobials

1.2.1 Paneth Cells and Innate Immunity

Paneth cells were described by Josef Paneth in 1888 as granule-rich epithelial cells that are located at the base of crypts of Lieberkühn in the small intestine (Figure 1.1). Paneth cells are found in humans, marsupials, and mice and rats, whereas some mammals (e.g. cows and sheep) lack them. The extensive endoplasmic reticulum and Golgi networks in Paneth cells are ultrastructural hallmarks of intensive secretory activity. Indeed, Paneth cells express and store a wide variety of proteins in secretory granules. The most abundant proteins in these granules are host-defense compounds, including α-defensins (cryptdins in mice), lysozyme C, phospholipases, and the C-type lectin REG3α (REG3γ in mice). Paneth cell degranulation takes place in response to several stimuli, including cholinergic agonists, bacteria, and bacterial products such as lipopolysaccharide (LPS) and lipoteichoic acid. Because the intestine is constantly exposed to bacteria and bacterial products, it is possible that Paneth cells continuously secrete antimicrobials at baseline levels and that higher amounts are released upon stronger stimulation. These host-defense molecules are released into the intestinal lumen to prevent microbial invasion of the crypt. They also disseminate into the mucus layer and contribute to the mucosal barrier at the epithelium.

Dysfunction of Paneth cell secretory pathways and reduced expression of Paneth cell host-defense substances are often associated with diseases. For example, Crohn's disease (CD) is a major category of inflammatory bowel disease (IBD) and often involves the distal portion (ileum) of the small intestine. CD is associated with
excessive bacterial adherence and growth on the intestinal mucosal surface, along with an abnormal composition of the intestinal microbiota.\textsuperscript{31,32} Studies of several genes that increase susceptibility to CD implicate Paneth cells and their antimicrobial substances in the pathophysiology of the disease.\textsuperscript{33-37} Hence, dysfunction of Paneth cells may limit the ability of these cells to prevent pathogenic invaders from colonization or may cause changes in the intestinal microbiota. Eventually, these two phenomena could lead to chronic inflammation as a result of disturbed intestinal homeostasis.

1.2.2 Mechanism of Action of the Antimicrobials from Paneth Cells

Lysozyme C is a 14-kDa glycosidase that catalyzes hydrolysis of 1,4-\(\beta\)-linkages between the \(N\)-acetylmuramic acid (NAM) and \(N\)-acetyl-\(D\)-glucosamine (NAG) moieties (Figure 1.2) in a peptidoglycan, which is one of the key components of the bacterial cell wall.\textsuperscript{38} The antibacterial activity of lysozyme was first observed in chicken egg albumin by Laschtschenko in 1909,\textsuperscript{39} but it was not until when Fleming identified and named the enzyme "lysozyme" in 1922.\textsuperscript{40} Fleming observed the antibacterial property of lysozyme when he treated an agar plate covered with \textit{Micrococcus lysodeikticus} with aqueous extract of nasal mucus obtained from a patient suffering from a head cold.\textsuperscript{40} Detection of lysozyme C in Paneth cells provided the first clue for an antimicrobial function of these cells.\textsuperscript{25} In addition to Paneth cells, lysozyme C is also found in the mucosal fluid of other organs, as well as in granules of macrophages and neutrophils.\textsuperscript{41}
Figure 1.2. Mechanism of hydrolysis of peptidoglycan catalyzed by lysozyme. Glu35 and Asp52 are found to be important for the activity of lysozyme. Glu35 catalyzes a nucleophilic substitution at the C1 position of NAM by Asp52 to break the 1,4-β-linkage. Glu35 then deprotonates a water molecule, which subsequently replaces Asp52 at the C1 position of NAM to form the products.

Similar to lysozyme C, Secretory group IIA phospholipase A2 (sPLA2) is constitutively expressed by Paneth cells. Phospholipase activity was first studied in pancreatic juice and cobra venom in the early 1900s. Subsequently, PLA2 isolated from various snake and bee venoms, as well as from the mammalian pancreas, have been characterized for their structure and mechanism of action. The phospholipase A2 enzymes catalyze the hydrolysis of the fatty acid ester bond at position sn-2 of the glycerol backbone of membrane phosphoglycerides (Figure 1.3). Some of these enzymes, including sPLA2, exhibit antibacterial activity, especially against Gram-positive bacteria, because bacterial membranes are composed of phosphatidylethanolamine and phosphatidylglycerol, which are the two preferred substrates of sPLA2. The other two major components of Paneth cell granules, which are α-defensins and lectins, will be discussed in the following sections.
**Figure 1.3.** Mechanism of hydrolysis of phosphoglyceride catalyzed by PLA$_2$. Ca$^{2+}$, His48, and Asp99 are key components for the catalytic activity of sPLA$_2$. Asp99 increases the basicity of His48 so that His48 deprotonates a water molecule, which subsequently adds to the carbonyl group to form a tetrahedral intermediate. The oxygen of the glycerol backbone is then protonated by His48 and leave to afford the products.

### 1.3 Defensins

Since the discovery of lysozyme by Fleming,$^{40}$ many host-defense peptides and proteins have been identified and characterized from most living organisms, including bacteria, fungi, plants, invertebrates, and vertebrates. These host-defense molecules play an important role in innate immune system as the first line of defense against pathogens. In 1956, aqueous extracts of rabbit polymorphonuclear neutrophils (PMNs) were found to exhibit bactericidal activity, especially on Gram-negative enteric bacilli.$^{46}$ This bactericidal substance, which was first named “phagocytin,” was mainly localized in the cytoplasmic granules of PMNs.$^{47}$ Later in 1963, Zeya and Spitznagel identified phagocytin as a group of cationic proteins that possess antimicrobial activity.$^{48}$ They discovered that the proteins were distinct from and more basic than lysozyme and ribonucleases, the bioactive molecules were described as arginine- and cysteine-rich antimicrobial proteins. Two decades later, Lehrer and coworkers purified and characterized these factors and found that some of them had low molecular weights ($\sim$4 kDa) and exhibited broad-spectrum antibacterial activity.$^{49}$ They subsequently determined the primary sequences of these molecules$^{50}$ and renamed these host-
defense substances as "defensins." Apart from leukocytes, defensins are also expressed by various types of epithelial cells.

Defensins are a class of small cationic cysteine-rich host-defense peptides produced by several eukaryotes, including fungi, plants, fish, arthropods, birds, and mammals. Defensins are ribosomally synthesized and consist of <50 residues in length with regiospecific disulfide linkages. Vertebrate defensins, insect defensins, and some fungal defensins contain three disulfide bonds, whereas plant defensins have four disulfide linkages.

The vertebrate defensins are further categorized into three subfamilies, α-, β-, θ-defensins, based on the regiospecific disulfide-bond connectivity (Figure 1.4). Both α- and β-defensins consist of a triple-stranded β-sheet fold with six cysteine residues linked as CysI—CysVI, CysII—CysIV, CysIII—CysIV in α-defensins or CysI—CysV, CysII—CysIV, CysIII—CysVI in β-defensins. θ-Defensins are structurally distinct from α- and β-defensins. The backbone of the θ-defensins is a cyclic peptide that is formed via splicing and cyclization from two of the nine-residue segments of α-defensin-like precursor peptides. The six cysteine residues in θ-defensins are linked as a cyclic cysteine ladder motif.
The expression and localization of each class of defensins vary from organism to organism. α-Defensins are only found in mammals and marsupials, whereas β-defensins are more commonly found among living organisms. Humans express α-defensins in neutrophils and intestinal Paneth cells, whereas mice do not have any defensins in neutrophils, but mice express several α-defensins that are called cryptdins in the gut. Cows and chickens do not produce α-defensins, but both express β-defensins in neutrophils and epithelial cells. θ-defensins were first identified in rhesus macaque monkey leukocytes and the peptides have only been isolated from non-human primates thus far. Humans possess the genes encoding θ-defensins, but they are not expressed due to a premature stop codon.

In humans, six α-defensins and four β-defensins have been well characterized, although there are more β-defensin genes identified in humans. All genes encoding human defensins are localized at chromosome 8, location 8p23. Of the six α-defensins, DEFA1 encodes HNP1 and HNP2 and DEFA3 encodes HNP3. Both DEFA1
and DEFA3 are multiple-copy genes. On the contrary, DEFA4, DEFA5, and DEFA6 encodes HNP4, HD5, HD6, respectively and they are single-copy genes.\textsuperscript{75} For the four characterized human \(\beta\)-defensins, DEFB1 encoding HBD1 is single-copy gene, whereas the others (DEFB4 encoding HBD2, DEFB103A encoding HBD3, and DEFB104A encoding HBD4) are multi-copy genes due to copy number polymorphisms.\textsuperscript{75} Variations of the copy number and the expression level of these peptides are associated with certain diseases; however, this correlation is still controversial.\textsuperscript{76-78}

1.4 Human \(\alpha\)-Defensins

1.4.1 Human Neutrophil Peptides (HNPs)

In 1985, Lehrer and coworkers isolated and characterized the first group of HNPs (HNP1-3) from human neutrophils.\textsuperscript{50,51} HNP1-3 represent about 30-50\% of the proteins in azurophilic granules and 5-7\% of total protein content in the neutrophil.\textsuperscript{79} HNP4 is less abundant. It accounts only for 1-2\% of the total defensins in the neutrophil.\textsuperscript{80} HNPs are expressed as prepropeptides (\textbf{Figure 1.5a}) in the promyelocytes and the pro region is cleaved during the maturation of bone marrow cells.\textsuperscript{81} Consequently, HNPs are stored in their mature form in the azurophilic granules.\textsuperscript{82} In addition, HNPs are also detected in monocytes\textsuperscript{83} and natural killer cells.\textsuperscript{84} Neutrophils produce \(\approx\)250 mg of HNPs per day. The concentrations of HNPs are elevated in plasma, blood, and body fluids from patients with infections as a result of increased neutrophil influx and enhanced expression level of HNPs in response to infections.\textsuperscript{85}
HNP1 and HNP3 are both 30 amino acids in length, whereas HNP2 is 29 amino acids in length. HNP2 is only different from HNP1 and HNP3 by the first amino acid at the N-terminus (Figure 1.5b).\textsuperscript{50} In contrast, HNP4 is a 34-residue peptide and is more cationic than HNP1-3 (+4 versus +3 overall charge at neutral pH).\textsuperscript{86} The conserved features among α-defensins include the glycine residue in a (GXC) motif that forms the β-bulge, the Arg-Glu salt bridge, and the three disulfide-bond connectivity.\textsuperscript{87} The first reported crystal structure of an α-defensin is that of HNP3 by Eisenberg and coworkers.\textsuperscript{88} This structural study revealed the characteristic triple-stranded β-sheet fold of α-defensins. In addition, this work demonstrated dimerization of a defensin for the first time and provided a foundation for further studies of defensin oligomerization.\textsuperscript{89,90} Subsequently, Lu and coworkers showed that dimerization is essential for the antimicrobial activity of HNP1.\textsuperscript{89} Solid-state NMR studies on HNP1 revealed its
membrane-bound structure and topology, indicating a dimer pore mechanism for membrane disruption.\textsuperscript{91}

HNPs display broad-spectrum antimicrobial activity against bacteria and fungi along with antiviral activity. The cationic nature of these peptides was proposed to account for such broad-spectrum activity of HNPs.\textsuperscript{92} Some early mechanistic insight on the antibacterial activity of HNP1-3 was obtained from the study of \textit{E. coli} ML-35 and ML-35p strains treated with HNP1-3.\textsuperscript{92} \textit{E. coli} ML-35 is a lactose permease-deficient strain, which express cytoplasmic $\beta$-galactosidase, whereas \textit{E. coli} ML-35p express both cytoplasmic $\beta$-galactosidase and a periplasmic lactase. Using this strain, Lehrer and coworkers revealed that membrane permeabilization is important for the antibacterial activity of HNPs.\textsuperscript{92} HNP1 was also shown to exhibit lectin-like behavior.\textsuperscript{93} The peptides weakly bind to lipid II, a precursor for bacterial cell wall biosynthesis.\textsuperscript{94} Moreover, HNP1-3 also function as chemoattractants for naive T cells and immature dendritic cells, suggesting that HNPs may contribute to linkage between innate and adaptive immunity.\textsuperscript{95}

1.4.2 \textit{Human $\alpha$-Defensin 5 (HD5)}

HD5 was first detected in the human small intestine;\textsuperscript{54} however, HD5 is also produced by other tissues and organs, including the urogenital tract\textsuperscript{96} and female reproductive tract.\textsuperscript{97} In the small intestine, HD5 is constitutively expressed and it is the most abundant antimicrobial peptide in the intestinal Paneth cells (\textit{Figure 1.1}).\textsuperscript{23} The transcriptional level of HD5 is 4-6-fold higher than that of HD6 and is higher than other antimicrobial substances in the granules of intestinal Paneth cells.\textsuperscript{98} In the female
reproductive tract, HD5 is expressed in a distinct pattern during each menstrual cycle, which is possibly regulated by hormone levels. This study also demonstrated that HD5 is upregulated during inflammation in the female reproductive tract.

HD5 is expressed as a prepropeptide that contains an N-terminal 19-residue signal peptide, a 43-residue pro region, and a 32-residue mature peptide. Following the cleavage of the signal sequence, HD5 is stored in Paneth cell granules as a propeptide (proHD5), which is cationic (pI ≈9.5). Trypsin is also detected in Paneth cells and hence it is identified as the protease that cleaves proHD5 to afford mature HD5 with an overall charge of +4 at neutral pH. Lu and coworkers demonstrated that the presence of the pro region attenuates the antibacterial activity of HD5. In the same study, the conserved salt bridge between Arg6 and Glu14 was shown to be essential for the proper folding of HD5 in the absence of the pro domain. In addition, the salt bridge in HD5 confers proteolytic stability against trypsin digestion although the salt bridge is not required for the antimicrobial activity of HD5.

In addition, the disulfide-bond connectivity of HD5 is essential for the antimicrobial activity. Nolan and coworkers prepared a family of Cys-to-Ser mutants and demonstrated that the disulfide linkages are important for the stability of HD5 against various proteases. In addition, mutating one or more disulfide bonds resulted in complete loss of antibacterial activity against *Staphylococcus aureus*; however, many of these HD5 variants remained active against *Escherichia coli*. The significance of the disulfide linkages for the antimicrobial activity of HD5 is also supported by the work done by Lu and coworkers, where they employed Cys-to-Abu (α-aminobutyric acid) variants of HD5. Taken together, these results indicated the importance of proper
folding of HD5 on its antibacterial activity against Gram-positive bacteria such as *S. aureus*; whereas, the overall structure of HD5 has less impact on the antibacterial activity against Gram-negative bacteria like *E. coli*. These results indicate that HD5 may exhibit species-specific mechanism of action against microbes.

HD5 is amphipathic with all the cationic residues on one side of the monomer and most hydrophobic residues on the other side of the monomer. HD5 forms a non-covalent dimer by utilizing hydrophobic interactions at the dimer interface.\(^1\) HD5 also exists as a tetramer in solution at neutral pH.\(^9\) Furthermore, Nolan and coworkers reported the formation of HD5-CD, a non-canonical C\(_2\)-symmetric \(\beta\)-barrel-like covalent dimer of HD5, under certain oxidizing conditions.\(^1\)\(^0\)

HD5 and HD5-CD display broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, and fungi.\(^1\)\(^0\)-\(^7\) Bevins and coworkers demonstrated that transgenic mice expressing HD5 confer resistance to oral *Salmonella* challenge.\(^1\)\(^6\) Studies of the intestinal microbiota revealed a homeostatic role of HD5 in regulating the composition of the commensal microbiota.\(^1\)\(^9\) This seminal work provided evidence for the importance of HD5 in the maintenance of intestinal homeostasis and protection against bacterial pathogens. More recently, HD5 was shown to be active against hypervirulent *Clostridium difficile* strains that are resistant to several AMPs such as polymyxin and nisin.\(^1\)\(^0\) Many studies addressing the mechanism of antibacterial activity of defensins have focused on the interaction between the cationic defensins and the negatively charged bacterial membrane, suggesting that the mechanism involves pore formation and membrane disruption.\(^1\)\(^0\),\(^2\)\(^1\)\(^1\) Nonetheless, more recent studies suggest that defensins exhibit antimicrobial action by targeting multiple cellular targets
and pathways in bacteria. For example, Nolan and coworkers demonstrated that HD5 causes distinct morphological changes to E. coli and other Gram-negative bacteria and by employing fluorophore-HD5 conjugates, they found that HD5 localizes at the site of cell division and cell poles.

HD5 displays a lectin-like behavior and bind to glycosylated proteins, including bacterial toxins. HD5 also possesses antiviral property. The oligomerization of HD5 and the orientation of its arginine residues at the surface interacting with the viral capsid are found to be essential for its antiviral activity against non-enveloped viruses (e.g. human adenoviruses). HD5 along with other defensins can also function as chemoattractants for macrophages, T lymphocytes, and mast cells. Taken together, further investigation is required to probe the exact mechanism of action of HD5 against these microbes and viruses.

The relationship between enteric defensins (HD5 and HD6) and labile zinc is another area that is not well understood. Paneth cells also contain high concentration of labile zinc, the function of which is not well understood. Zinc deficiency leads to depletion of granules and morphological abnormality, degeneration, and apoptosis of Paneth cells. Moreover, the expression of HD5 and HD6 is also detected in the pancreas, which is another organ that contains labile zinc. These observations suggest that the enteric defensins may interact with labile zinc. For example, Nolan and coworkers reported that the reduced form of HD5 binds zinc, resulting in formation of a complex between one HD5 molecule and two zinc ions. In the same study, the thioredoxin system was shown to reduce HD5 and the redox mid-point potential of HD5 was determined to be -257 mV, which fits in the redox potential range under
physiological conditions, implying the relevance of the reduced form of HD5 in the biological system as well as possible interactions between HD5 and labile zinc.\textsuperscript{120}

1.4.3 Human \( \alpha \)-Defensin 6 (HD6)

HD6, the focus of the first part of this thesis, is primarily produced by Paneth cells of the small intestine.\textsuperscript{121} Both HD5 and HD6 are expressed in duodenum, jejunum, and ileum of the small intestine with the highest expression observed in the ileum; however, the enteric defensins are not produced in the colon.\textsuperscript{54} Moreover, the expression of enteric defensins is reduced in patients suffering from Crohn’s disease.\textsuperscript{33}

The structure and function of HD6 have been less extensively studied compared to other human \( \alpha \)-defensins. Analysis of human mRNA indicated that HD6 is expressed as a prepropeptide (pI \( \approx \) 4.5) with an N-terminal 19-residue signal peptide, a 49-residue pro region, and a 32-residue mature peptide (Figure 3.1).\textsuperscript{121} Nonetheless, in contrast to other \( \alpha \)-defensins, it was not known which HD6 isoform existed in the granules of Paneth cells and prior work precludes prediction between these storage forms.\textsuperscript{99,122,123} Recently, Nolan and coworkers reported that similar to HD5, HD6 is stored in the granules of Paneth cells as a propeptide and trypsin is the processing enzyme that cleave the pro domain to release the mature HD6 into the intestinal lumen. This work is further discussed in Chapter 3.

The tertiary structure of HD6 monomer looks similar to that of HD5 and other characterized \( \alpha \)-defensins (Figure 1.6), HD6 behaves differently from HD5 by exerting negligible \textit{in vitro} antimicrobial activity.\textsuperscript{65,106,124} One possible explanation is that HD6 only possesses an overall charge of +2 at neutral pH, which is considered to be lower
than other human α-defensins. This notion is supported by a recent work, where HD6 analogs containing an N-terminal segment were shown to be active against several bacterial strains.\textsuperscript{125} Wehkamp and coworkers recently reported that the reduced form of HD6 variant that lack two N-terminal residues exhibits antimicrobial activity against \textit{Bifidobacterium adolescentis}.\textsuperscript{126}

![Diagram of HD6 primary sequence and secondary structure](image)

\textbf{Figure 1.6.} (a) Primary sequence of HD6 and a cartoon showing its secondary structure. (b) Crystal structures of HD5 (PDB 1ZMP) and HD6 (PDB 1ZMQ) monomers\textsuperscript{65} shows that their monomers look similar.

The contribution of HD6 to the mucosal immunity had been enigmatic for several years until Bevins and coworkers reported that HD6 functions by an unprecedented host-defense mechanism among defensins.\textsuperscript{124} This work demonstrated that transgenic mice expressing HD6 confer resistance to oral \textit{Salmonella} challenge; however, their feces still contained high \textit{Salmonella} burden. By employing scanning electron microscopy (SEM), they showed that \textit{Salmonella} was entrapped in the intestine of HD6-transgenic mice. As a result, they proposed the working model, where HD6 self-assembles into higher-order oligomers that are called “nanonets” to entrap pathogens and prevent them from invading into host cells.\textsuperscript{124} This seminal work suggested that the
two enteric defensins, which are colocalized in the intestinal Paneth cells, contribute to mucosal innate immunity by different host-defense mechanisms. HD5 involves direct killing of bacteria, whereas HD6 protects host epithelial cells from bacterial invasion. More recently, de Leeuw and coworkers reported the first evidence for functional synergism of HD5 and HD6. This work showed that the HD5-induced interleukin 8 (IL-8) secretion from epithelial cells is further enhanced by the presence of HD6. This synergistic effect of HD6 was observed only with HD5 but not with other α-defensins.

The antiviral property of HD6 has also been investigated. HD6 along with other human α-defensins were shown to prevent Herpes simplex virus (HSV) infection. However, both HD5 and HD6 promote HIV-1 infectivity by enhancing HIV attachment to target cells. In addition, Neisseria gonorrhoeae was shown to increase the expression of HD5 and HD6 in human vaginal epithelial cells, resulting in enhanced HIV infectivity. A loss of intramolecular disulfide linkages is associated with diminished HIV-enhancing effect of these defensins. Given that the function of defensins and the host-pathogen interaction are complicated topics, further studies on the antiviral activity of HD6 is required.

1.5 Human β-Defensins (HBDs)

Human β-defensin 1 (HBD1) is the first β-defensin that was isolated and characterized from human blood filtrate. β-Defensins are mainly produced by epithelial tissues either constitutively (HBD1) or in response to infection (HDB2-4). In contrast to the six human α-defensins, HBDs are not stored in granules. For example, HBD2 is packaged as a mature peptide in lamellar bodies of the skin. The expression level of
HBDs varies in some diseases. The amount of HBD2 and HBD3 increases on the skin of patients with psoriasis, whereas lower expression is found in atopic dermatitis. The expression of HBDs in the intestinal epithelium is reduced in patients suffering from Crohn’s disease.

β-Defensins are structurally different from α-defensins. HBDs possess an N-terminal α-helix linked via three disulfide linkages to a triple-stranded β-sheet fold (Figure 1.4). Similar to α-defensins, the glycine in a (GXC) motif involved in the formation of the β-bulge is conserved; however, the salt bridge between arginine and glutamate residues is missing in β-defensins. HBDs can also form dimers by aligning the second β-sheet of each monomer. Moreover, the crystal structure of HBD2 revealed the formation of octamers of the peptide.

HBDs exhibit broad-spectrum antimicrobial activity against bacteria and fungi. The oxidized form of HBD1 possesses the least antimicrobial activity among the characterized HBDs and the antimicrobial activity of HBD1 is sensitive to salt concentration. The activity of HBD1 is attenuated in cystic fibrosis, where the airway surface liquid contains high salt concentration. However, Wehkamp and coworkers reported that reduction of disulfide bonds in HBD1 unmasks its potent antimicrobial activity. HBD2 was found to cause mislocalization of sortase and associated secretion enzymes in the cell membrane of Enterococcus faecalis, resulting in disruption of its virulence factor secretion and assembly process. HBD2 can also cause local unfolding of bacterial toxins. The antimicrobial activity of HBD3 was shown to be less salt-sensitive and active against both Gram-positive and Gram-negative bacteria. The transcriptional response pattern of HBD3-treated S. aureus
resembled that of vancomycin-treated cells, suggesting that inhibition of cell wall biosynthesis is a major component of the killing process of HBD3. HBDs also play a role in sperm function, including maturation and motility by interacting with cell membrane receptors. In addition, HBDs may promote adaptive immune responses by recruiting immune cells to the site of microbial invasion.

1.6 Other Microbe-Binding Molecules at the Intestinal Mucosa

1.6.1 Mucins

Mucins are the main structural components of mucus, which is found throughout the gastrointestinal (GI) tract and plays a multifaceted role in the interactions between microbes and the epithelium. The GI tract exhibits the highest expression level and diversity of mucin in the body. In humans, more than 20 genes encoding mucins have been identified. Mucins are high-molecular-weight glycoproteins containing a tandem repeat region (TRR, Figure 1.7) that is rich in proline, threonine, and serine residues (called PTS sequences). The serine and threonine residues in the TRR are heavily glycosylated with O-linked (O-glycans) and less common N-linked (N-glycans) oligosaccharides, which results in large, flexible, rod-like molecules of mucins with viscoelastic properties.
Figure 1.7. Structures of mucins. The structure of mucin 1 (MUC1) represents the typical structure of membrane-bound mucins in the GI tract. The extracellular TRR is heavily O-glycosylated, and the protein is N-glycosylated near the SEA domain. The cytoplasmic domain of MUC1 is involved in intracellular transduction. Mucin 2 (MUC2) is a major component of the secreted mucus barrier in the intestine. The TRRs are heavily O-glycosylated and the N- and C-terminal D domains are involved in homo-oligomerization.

Mucins can be categorized into two main groups: membrane-bound or secreted proteins (Figure 1.7). Membrane-bound mucins, including MUC1, MUC3A/B, MUC4, MUC11-13, MUC15-17, MUC20, and MUC21, are essential components of the glycocalyx of mucosal surfaces and play important roles in cell-cell and cell-matrix interactions as well as in cell signaling. These mucins may be shed from the surface and subsequently integrate into the overlying mucus layer. Secreted mucins are further divided into two subfamilies, namely gel-forming (MUC2, MUC5AC, MUC5B, MUC6, and MUC19) and non-gel-forming (MUC7) mucins. Secreted mucins are the core structural components of the mucus gel. In the GI tract, mucins are produced and secreted by the goblet cells of the small intestine and colon, and the surface mucus cells of the stomach. The oligomerized mucins form homo-oligomers via intermolecular disulfide linkages that are formed between the cysteine-rich D domains.
found in the N- and C-termini of these mucins.\textsuperscript{153} N-glycosylation and C-terminal
dimerization take place in the endoplasmic reticulum, followed by O-glycosylation in the
Golgi, and mucins are packaged into granules prior to secretion.\textsuperscript{153}

In the small intestine, mucin 2 (MUC2) is the major component of mucus.\textsuperscript{154} Within
the endoplasmic reticulum (ER), newly synthesized MUC2 peptides dimerize via the
formation of disulfide bonds and the dimers are transported to the Golgi apparatus,\textsuperscript{155}
where the TRR domains of the mucin dimers are glycosylated before further assembly
into trimers in the trans-Golgi network\textsuperscript{156} and packaging into goblet cell granules in a
pH- and Ca\textsuperscript{2+}-dependent manner.\textsuperscript{157} As a monomer, fully glycosylated MUC2 exhibits a
size of 2.5 MDa, whereas extensive polymerization may lead to sizes greater than 100
MDa.\textsuperscript{158} Following secretion of the mucin granules at the mucosa, the densely packed
mucins are hydrated and expand into extensive polymers, resulting in a physical barrier
at the epithelium to entrap invading microorganisms.\textsuperscript{159} Moreover, the mucus gel
provides a matrix for antimicrobial molecules, which are mainly produced by Paneth
cells. Direct interactions with mucins can facilitate the diffusion of these antimicrobial
molecules.\textsuperscript{145} In addition to their protective and lubricating properties, mucins are a
nutritional source for microorganisms. Some commensal intestinal bacteria, including
Bacteroidetes, are mucolytic and can use mucin glycoproteins as an energy source.
Moreover, these bacteria provide substrates for other bacteria in the outer mucus layer
by degrading the mucins.\textsuperscript{160,161} Therefore, mucins have been proposed to play an
important role in shaping microbial communities at the intestinal mucosa. Recent
studies suggest the correlation between changes in mucin glycosylation profile and
deviations of overall microbial community ecology as well as altered abundances of specific microbes.\textsuperscript{162,163}

1.6.2 \textit{C-type Lectins}

Lectin is a carbohydrate-binding protein, which is another player in mucosal innate immunity. REG3\textgreek{3}\textsuperscript{\alpha} (also known as hepatocarcinoma intestine-pancreas/pancreatic associated proteins, HIP/PAP) and its murine homolog REG3\textgreek{3}\textgreek{3}\textsuperscript{\gamma} are soluble lectins found throughout the small intestinal epithelium.\textsuperscript{27,164} REG3 lectins are secreted apically into the lumen, where they interact with intestinal microbes.\textsuperscript{165} The expression of REG3\textgreek{3}\textsuperscript{\gamma} is also induced by the skin injury\textsuperscript{166} and by inflammatory signals in upper-respiratory tract epithelial cells of mice.\textsuperscript{165}

The interactions between REG3\textgreek{3}\textsuperscript{\alpha} and its bacterial targets are initiated by binding to peptidoglycan in the bacterial cell wall.\textsuperscript{167,168} Therefore, the antibacterial activity of REG3 is selective for Gram-positive bacteria because the peptidoglycan is readily accessible on the outer surfaces of these organisms.\textsuperscript{169} In contrast, peptidoglycan in Gram-negative bacteria is shielded by the outer membrane. Moreover, REG3\textgreek{3}\textsuperscript{\alpha} recognition of peptidoglycan involves high-affinity binding to the extended carbohydrate chains on the bacterial surfaces, but not to shorter, soluble peptidoglycan chains. This property of lectins allows selective binding to the bacterial surfaces while avoiding shorter peptidoglycan chains that are shed by bacteria.\textsuperscript{168}

Binding of REG3\textgreek{3}\textsuperscript{\alpha} to peptidoglycan is the first step in its antibacterial mechanism. After binding, REG3\textgreek{3}\textsuperscript{\alpha} permeabilizes the bacterial membrane by utilizing its cationic residues to interact with the negatively charged bacterial membrane.\textsuperscript{170} Upon contact
with lipids, REG3α oligomerizes to form hexameric transmembrane pores. Therefore, both bacterial binding and membrane permeabilization are essential for the bactericidal activity of REG3α.

1.6.3 Secretory Immunoglobulin A (sIgA)

Secretory IgA (sIgA) is the antibody that dominates humoral immunity at the mucosa. Due to its polymeric nature and hence multivalency, sIgA primarily protects mucosal surfaces by non-covalently cross-linking microorganisms or macromolecules, and thereby blocking their adhesins, sterically hindering their interactions with the epithelium, or inhibiting their motility and facilitating their entrapment in mucus. Eventually, sIgA promotes the clearance of these microbes by peristalsis or mucociliary movement. The overall process is known as immune exclusion. In the lamina propria, polymeric IgA also binds and excretes antigens back to the lumen using polymeric Ig receptor-mediated transcytosis across epithelial cells. In addition, sIgA responses are not only specific to pathogens, but also to commensal microorganisms residing in the gut. It has been proposed that coating of commensal bacteria by sIgA may lead to a different recognition by epithelial cells and dendritic cells as compared to pathogens, and hence facilitate the host to discriminate between symbionts and pathogens. Neutralization of bacterial toxins is another function of IgA. For example, binding of a murine IgA monoclonal antibodies to the O antigen of Shigella flexneri suppressed the activity of the bacterial type 3 secretion system that is necessary for S. flexneri to invade into intestinal epithelial cells. SLgA can also communicate across the epithelium to other immune cells and display immunomodulatory function.
1.7 Goals and Organization of the HD6-Related Projects

The goal of this dissertation project is to understand the fundamental properties of HD6 and gain more insight into its contribution to innate immunity. HD6 exhibits a unique host-defense mechanism involving its self-assembly and bacterial entrapment instead of directly killing them. The lessons we learn from the studies of HD6 structure and function, in the long term, may provide us with novel therapeutic approaches for the treatment of microbial infection. Moreover, although the self-assembly and biological function of HD6 have been reported, there are many questions building upon this seminal work. How is HD6 stored in the granules of Paneth cells? What makes HD6 behave differently from HD5 and other characterized defensins? Is the bacterial entrapment just one layer of protection provided by HD6? Are there any other consequences on microbes after being entrapped by HD6? To enable the studies on HD6 to address these questions, we first developed robust protocols for obtaining native and mutant HD6 in high purity from overexpression in E. coli.

In Chapter 2, we present molecular-level understanding for why HD6 functions differently from other human defensins. Native HD6 readily self-assembles into elongated fibrils observable by transmission electron microscopy, agglutinates both Gram-negative and -positive bacteria, and prevents Listeria monocytogenes from invading cultured mammalian cells. Mutation of hydrophobic residues (F2A, I22T, V25T, F29A) perturbs HD6 self-assembly and results in attenuated biological activity. In particular, the F2A and F29A mutants do not form fibrils under our experimental conditions and cannot agglutinate bacteria or prevent L. monocytogenes invasion. In total, our results demonstrate that the hydrophobic effect is essential for promoting HD6...
self-assembly and innate immune function. This work provides a description of how variations in amino acid sequence confer diverse physiological functions to members of the defensin family.

In Chapter 3, we elucidate critical steps in the self-assembly pathway of HD6. We demonstrate that HD6 is stored in the granules of Paneth cells as an 81-residue propeptide and is recovered from ileal lumen as a 32-residue mature peptide. The propeptide neither forms higher-order oligomers, nor agglutinates bacteria, nor prevents the human gastrointestinal pathogen *L. monocytogenes* invasion into epithelial cells. The Paneth cell granules also contain the protease trypsin, and trypsin-catalyzed hydrolysis of proHD6 liberates mature HD6, unmasking its latent activities.

In Chapter 4, we present exploratory studies that address the effect of HD6 on *Candida albicans* virulence traits. *C. albicans* is an opportunistic fungal pathogen that resides as a part of the gut microbiota in the intestine. Our work indicates that HD6 suppresses two *C. albicans* virulence traits, namely invasion into human epithelial cells and biofilm formation, by preventing the fungi from adhering to the surfaces. In addition, our preliminary data obtained from light microscopy suggest that HD6 may suppress *Candida* transition from non-virulent yeast to virulent hyphae. To date, most studies about HD6 focus on its effect on bacteria. Our results expand the scope of HD6 host-defense function to fungi.

In Appendix A, we present the synthesis, purification, and preliminary functional characterization of the Met(O)23-HD6. We demonstrate that the oxidation of Met23 in HD6 is catalyzed by acid. Our preliminary biophysical studies reveal that the methionine oxidation impairs the HD6 self-assembly possibly due to an increase in polarity of Met23.
These results are in agreement with our work with the HD6 variants described in Chapter 2.

In Appendix B, we present the preparation, purification, and functional studies of RALGSTR-HD6 (proHD6$_{62-100}$). In Chapter 3, we show that the pro region of proHD6 temporally and spatially controls the HD6 self-assembly. Subsequently, we question why proHD6 contains a long flexible pro region. Our results indicate that a 7-residue N-terminal extension is sufficient to suppress the HD6 self-assembly and biological function. Therefore, we speculate that there are additional roles of the pro region besides controlling the HD6 self-assembly. Further investigation is required to address this notion.

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Chapter 2

Molecular Basis for Self-Assembly of a Human Host-Defense Peptide that Entraps Bacterial Pathogens

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2.1 Introduction

The innate immune system provides the first line of defense for the detection of and response to microbial invasion. One of the key contributors to innate immunity is a family of small (2-5 kDa), cysteine-rich host-defense peptides called defensins. These biomolecules are typically described as antimicrobial peptides with broad-spectrum microbicidal activity. In the oxidized forms, mammalian defensins contain three conserved and regiospecific intramolecular disulfide bonds that stabilize a three-stranded β-sheet fold. The regiospecific disulfide-bond patterns divide these defensins into three subclasses called α-, β-, and θ-defensins. Humans produce and utilize α- and β-defensins in the battles against invading microbial pathogens.

The six α-defensins identified in humans are human neutrophil peptides 1-4 (HNP1-4) and human enteric defensins 5 and 6 (HD5 and HD6). These peptides exhibit Cys¹—Cysvi, Cysi—Cysiv, Cysiii—Cysv linkages following oxidative folding. HNP1-4 are stored in the azurophilic granules of neutrophils, human monocytes, and natural killer cells. HD5 and HD6 are abundant in the granules of Paneth cells, a type of secretory cell located at the bases of the crypts of Lieberkühn in the small intestine. Paneth cells protect the intestinal epithelium against infection and colonization of opportunistic and pathogenic microbes by secreting a mixture of antimicrobial peptides and proteins that includes HD5 and HD6.

Although the six human α-defensins display a common tertiary structure that results from conserved cysteine positioning and the regiospecific disulfide array, the primary sequences are highly variable and several recent studies demonstrated that α-defensins possess different structural and functional attributes. A comparison of the
32-residue Paneth cell defensins HD5 and HD6 exemplifies this notion.\textsuperscript{14-19} HD5 forms dimers and tetramers in aqueous solutions\textsuperscript{14,20} and provides broad-spectrum antibacterial activity \textit{in vitro}.\textsuperscript{22,23} The HD5 transgenic mouse exhibits resistance to oral \textit{Salmonella enterica} serovar Typhimurium challenge.\textsuperscript{18} In contrast, HD6 displays negligible antibacterial activity \textit{in vitro};\textsuperscript{15,16} however, studies of the HD6 transgenic mouse revealed that this peptide provides defense against \textit{Salmonella} challenge by an unprecedented mechanism.\textsuperscript{15} In the current working model, HD6 forms higher-order structures described as "nanonets" that entrap bacteria in the intestinal lumen and thereby prevent bacterial invasion of the host epithelium and subsequent dissemination to other organ systems.\textsuperscript{15} This remarkable observation gives rise to a number of questions about HD6 at the molecular level. Why does HD6 function differently from other human \(\alpha\)-defensins, including its Paneth cell congener HD5? What is the molecular basis for HD6 self-assembly that affords "nanonets" from 32-residue monomeric units?

Several studies support the importance of the hydrophobic effect for the \textit{in vitro} biological activities of \(\alpha\)-defensins, including HNP1, HD5, and murine cryptdin-4.\textsuperscript{20-23} The amino acid sequence alignment of the six human \(\alpha\)-defensins reveals that the distribution of hydrophobic residues in the HD6 primary sequence is distinct from those in HD5 and the HNPs (\textbf{Figure 2.1}). Prior crystal structural studies reveal that several hydrophobic residues of HD5 reside on the \(\beta\)-sheets and facilitate side-to-side dimerization (PDB: 1ZMP,\textsuperscript{19} \textbf{Figure 2.2}) whereas the hydrophobic residues of HD6 (PDB: 1ZMQ,\textsuperscript{19} \textbf{Figure 2.2}) are located in the loop (V22, M23, I25) and in the N- (F2) and C-terminal regions (F29, L32). Moreover, the hydrophobic residues of HD6 define
HNP1  ACYCRPACIAGERRYGTQIYQGRLWAFCC
HNP2  CDCYCRPACIAGERRYGTQIYQGRLWAFCC
HNP3  DCYCRPACIAGERRYGTQIYQGRLWAFCC
HNP4  VCSRLVFCRRTELVRGNCAGGVSIGFTYCTRVQ
HD5  ATCYCTGRCATRESLSGVEIISGRLYRLLCR
HD6  AFTCHCRSCYSTESYGTCTVMGINHRFCCL

Figure 2.1. Amino acid sequence alignment of human α-defensins with the hydrophobic residues labeled in red. The sequences of human neutrophil α-defensins (HNP1-4) and human Paneth cell α-defensins (HD5 and HD6) are aligned in a single-letter notation using the Cys1 residue as the reference point. A dash ("-"") in the HD6 sequence is used to maintain alignment of all Cys residues.

A hydrophobic pocket that forms at the interface between four monomers. In each hydrophobic pocket, two monomers each contribute F2, F29, and L32, and the other two monomers each contribute V22, M23, and I25. Although each HD6 monomer exhibits the canonical α-defensin fold in the solid state, the monomers assemble to form

Figure 2.2. Previously reported crystal structures of HD5 (PDB: 1ZMP, cyan) and HD6 (PDB: 1ZMQ, purple) showing hydrophobic residues in orange.
an elongated fibril-like chain that is unique among structurally characterized defensins (Figure 2.3a). We reasoned that formation of this hydrophobic pocket allows HD6 monomers to form the elongated fibril-like structures observed crystallographically (Figure 2.3), and hypothesized that the hydrophobic effect also contributes to formation of the nanonet structures observed in biological systems.

Figure 2.3. (a) Previously reported extended crystal structure of HD6 (PDB: 1ZMQ). Hydrophobic residues are shown in orange. Inset: a HD6 monomer unit illustrates the three-stranded β-sheet fold. The disulfide bonds are shown in yellow. (b) Close-up view of the hydrophobic pocket located among HD6 monomers. Individual HD6 monomers are labeled A-D.

In the present work, we describe biochemical and biophysical studies designed to investigate the self-assembly and biological function of HD6. We report that native HD6 forms micron-sized fibril structures in aqueous solution, agglutinates bacteria, and prevents *Listeria monocytogenes* invasion into cultured human cells. We demonstrate that hydrophobic residues, especially F2 and F29, are essential for self-assembly under the experimental conditions utilized in this work and provide HD6 with the ability to entrap bacteria and prevent invasive microbes from entering human cells. In total, our investigations provide important new molecular-level insight into a host-defense peptide with an unprecedented physiological activity and support a model whereby the
disposition of hydrophobic residues along the canonical α-defensin fold tunes innate immune function. Moreover, the gut is a primary site for *L. monocytogenes* infection and our results suggest that HD6 may confer host defense against this pathogen in the gut.

### 2.2 Experimental Section

#### 2.2.1 General Materials and Methods

All solvents, reagents, and chemicals were purchased from commercial suppliers and used as received unless noted otherwise. HD5 was overexpressed and purified as previously described. All buffers, aqueous solutions, and peptide/oligonucleotide stock solutions were prepared in Milli-Q water (18.2 MΩ•cm) after it was passed through a 0.22-μm filter. Oligonucleotide primers were synthesized by Integrated DNA Technologies and used as received (standard desalting protocol). A Biorad MyCycler thermocycler was employed for all polymerase chain reactions (PCR). Chemically-competent *E. coli* TOP10 and BL21(DE3) cells were prepared in-house via standard protocols. PfuTurbo DNA polymerase was purchased from Agilent Technologies. T4 DNA ligase and all restriction enzymes were purchased from New England Biolabs. DNA sequencing was performed by dedicated staff in the MIT Biopolymers Laboratory.

#### 2.2.2 Instrumentation

An Agilent 1200 series instrument equipped with a thermostatted column compartment set at 20 °C, and a multi-wavelength detector set at 220 and 280 nm (500 nm reference wavelength with 100 nm bandwidth), was used to perform analytical and semi-preparative high-performance liquid chromatography (HPLC). An Agilent PrepStar 218
instrument outfitted with an Agilent ProStar 325 dual-wavelength detector set at 220 and 280 nm was used to perform preparative HPLC. A Cliepus C18 column (5-\(\mu\)m pore, 4.6 x 250 mm, Higgins Analytical, Inc.) set at a flow rate of 1 mL/min was employed for all analytical HPLC experiments. A ZORBAX C18 column (5-\(\mu\)m pore, 9.4 x 250 mm, Agilent Technologies) set at a flow rate of 5 mL/min was employed for all semi-preparative HPLC purification. A Luna 100 Å C18 LC column (10-\(\mu\)m pore, 21.2 x 250 mm, Phenomenex) set at a flow rate of 10 mL/min was used for all preparative HPLC purification. HPLC-grade acetonitrile (MeCN) and trifluoroacetic acid (TFA) were routinely purchased from EMD and Alfa Aesar, respectively. For all HPLC runs, solvent A was 0.1% TFA/H\(_2\)O and solvent B was 0.1% TFA/MeCN.

An Agilent 1260 series LC system equipped with an Agilent 6230 TOF system housing an Agilent Jetstream ESI source was employed to perform high-resolution mass spectrometry. A Poroshell 120 EC-C18 column (2.7-\(\mu\)m particle size, 2.1 x 100 mm, Agilent Technologies) set at a flow rate of 0.4 mL/min was used for all LC-MS analyses. The samples were analyzed by using a solvent gradient of 5–95% B over 5 min with 0.1% formic acid/H\(_2\)O as solvent A, and 0.1% formic acid/MeCN (LC-MS grade, Sigma-Aldrich). The MS profiles were analyzed and deconvoluted by using Agilent Technologies Quantitative Analysis 2009 software version B.03.02.

All routine optical absorption measurements and the agglutination assays were performed by using a Beckman Coulter DU 800 UV-visible spectrophotometer maintained at ambient temperature. Extinction coefficients (280 nm) for native HD6 and its variants were calculated by using ExPASy ProtParam and are listed in Table 2.4.
Solution and buffer pH values were verified by using a Mettler Toledo S20 SevenEasy pH meter or a HANNA Instruments HI 9124 pH meter equipped with a microelectrode.

2.2.3 Design of the Synthetic Gene for PreproHD6

A synthetic gene for human preproHD6 was ordered from DNA 2.0 and optimized for *E. coli* codon usage. The synthetic gene was designed to include a N-terminal *Ndel* restriction site (N-terminal Met residue encoded by the *Ndel* site) and a C-terminal stop codon followed by a *Xhol* restriction site. It contains an N-terminal TEV protease cleavage site (ENLYFQG) followed by the 19-residue signal peptide sequence ("pre") and 81-residue proHD6. The TEV cleavage sequence and the "pre" region were not utilized in this work. The synthetic gene was obtained in pJ201 from DNA 2.0 and this pJ201-*preproHD6* was employed as a template for PCR amplification of proHD6.

In the nucleotide and amino acid sequences below, the sequences corresponding to the TEV site is in italics and the *Ndel* and *Xhol* codons and corresponding amino acid are in bold.

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2.2.4 Subcloning, Overexpression, and Purification of His\textsubscript{6}-proHD6

An \textit{E. coli}-optimized synthetic gene encoding preproHD6 was obtained from DNA 2.0 in the pJ201 vector. The proHD6 nucleotide (333 bp) was PCR amplified using pJ201-preproHD6 as a template and the forward and reverse primers 5'-GAATTCCATATGGAGCCGCTGCAAGCAG-3' and 5'-GATCCTCGAGTTACAGACAACAAAAGCGATG-3', respectively (restriction site, underlined; stop codon, bold). The PCR reactions were analyzed by 1\% (w/v) agarose gel and a GE Healthcare illu\textsuperscript{stra}TM GFX\textsuperscript{TM} PC DNA and Gel Band Purification Kit was employed to purify the PCR products. The products were subsequently digested with \textit{Ndel} and \textit{Xhol}. The resulting fragments were purified by 1\% (w/v) agarose gel and ligated into the \textit{Ndel} and \textit{Xhol} sites of pET28b using T4 DNA ligase (2 h, room temperature). The resulting plasmids were transformed into chemically-competent \textit{E. coli} TOP10 cells and the pET28b-proHD6 plasmid was isolated by using a QIAprep spin miniprep kit (Qiagen). The plasmid identity was confirmed by DNA sequencing.

The overexpression and purification of His\textsubscript{6}-proHD6 were modified from the literature.\textsuperscript{24} The pET28b-proHD6 plasmid was transformed into chemically-competent \textit{E. coli} BL21(DE3) cells. Overnight cultures were prepared by inoculating lysogeny broth (LB) medium containing 50 \(\mu\)g/mL of kanamycin with single colonies. These cultures were grown to saturation (37 °C, 150 rpm, 16-18 h) and used to prepare glycerol freezer stocks. The freezer stocks containing a 1:1 ratio of the overnight culture and sterile-filtered 50\% glycerol in Milli-Q water were stored at -80 °C. For a given His\textsubscript{6}-proHD6 overexpression, 50 mL of LB medium containing 50 \(\mu\)g/mL of kanamycin in a 250-mL baffled flask was inoculated with the freezer stock and grown to saturation (37 °C, 150
rpm, 16-18 h). The resulting culture was diluted 1:100 into 2 L of fresh LB medium containing 50 μg/mL of kanamycin in a 4-L baffled flask, and the culture and incubated at 37 °C, 150 rpm until OD_{600} reached ~0.6. A 400-μL aliquot of 0.5-mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to the 2-L culture and the culture was incubated for an additional 3-4 h (37 °C, 150 rpm) until an OD_{600} of 1.2-1.5 was achieved. The culture was centrifuged (3000 rpm x 15 min, 4 °C) and the cell pellets were collected. Typically, overexpression of His_{6}-proHD6 was performed on a 12-L scale and the cell pellets from 6 L of culture were combined in pre-weighed 50-mL polypropylene tubes (~2 g/L wet cell weight), flash frozen in liquid N_{2}, and stored at -80 °C for a period of 1-2 months.

For purification of His_{6}-proHD6, each 6-L cell pellet was thawed on ice and resuspended in 30 mL of cold lysis buffer (100 mM Tris-HCl, 6 M GuHCl, pH 8.0). Subsequently, a 1-mL aliquot of phenylmethyl sulfonyl fluoride (PMSF, 100 mM in EtOH) was added and the resuspension was transferred to a pre-chilled stainless steel beaker and lysed by two rounds of sonication (10% amplitude with pulse on for 1 s and pulse off for 4 s for 1 min, on ice, Branson sonicator). A second aliquot of PMSF (1 mL, 100 mM in EtOH) was added and the cell lysate was clarified by centrifugation (13000 rpm x 30 min, 4 °C). The resulting supernatant was incubated with pre-washed Ni-NTA resin (Qiagen, from 9 mL of Ni-NTA slurry that was pre-washed with Milli-Q water (3 x 30 mL)) with gentle shaking for 1.5 h at 4 °C. The resulting mixture was loaded onto a fritted column and the resin was washed with 40 mL of cold wash buffer (20 mM Tris-HCl, 300 mM NaCl, 6 M GuHCl, pH 8.0). His_{6}-proHD6 was eluted with 30 mL of cold elution buffer (10 mM Tris-HCl, 300 mM Na_{2}PO_{4}, 200 mM NaCl, 1 M imidazole, 6 M
GuHCl, pH 6.5). The eluent was transferred into a dialysis bag (3500 MWCO, Spectrum Laboratories) and dialyzed against 5% acetic acid (4 L x 12 h, 4 °C) and then 0.1% acetic acid (4 L x 12 h, 4 °C). The dialyzed solution was subsequently lyophilized to dryness to afford His₆-proHD₆ as a white fluffy powder, which was stored at -20 °C. The yield was 26 mg/L culture. The purity of His₆-proHD₆ was routinely determined by analytical HPLC (10-60% B over 30 min, 1 mL/min). The HPLC retention time and results from LC-MS are shown in Table 2.4.

2.2.5 Oxidative Folding

His₆-proHD₆ was folded by a modified literature procedure.²⁴,²⁵ A 120-mg portion of His₆-proHD₆ was dissolved in 15 mL of 8 M GuHCl containing 3 mM of glutathione and 0.3 mM of glutathione disulfide. Then, 45 mL of 250 mM NaHCO₃ was added to the solution to adjust the pH to ~8.3 and afford a final peptide concentration of ~2 mg/mL. The solution was incubated at room temperature overnight. The resulting solution was analyzed by HPLC and mass spectrometry to ensure that His₆-proHD₆ was completely folded. The solution was transferred into a dialysis bag (3500 MWCO) and dialyzed against 10 mM Tris-HCl pH 8.2 (4 L x 12 h, 4 °C) and then 100 mM Tris-HCl pH 8.2 containing 20 mM CaCl₂ (4 L x 12 h, 4 °C). The dialyzed solution was adjusted to a concentration of 1 mg/mL with 100 mM Tris-HCl pH 8.2 containing 20 mM CaCl₂ and was then transferred to 50-mL polypropylene centrifuge tubes and subjected to trypsin-catalyzed cleavage without further purification.
2.2.6 Preparation and Purification of Native HD6

An aliquot of a 1-mg/mL stock solution of TPCK-treated trypsin (Worthington) in Milli-Q water was added to the solution of His$_6$-proHD6 (1 mg/mL), which was obtained from oxidative folding as described above, to afford a final trypsin concentration of 0.01 mg/mL. The reaction was incubated at room temperature for 15 min and subsequently quenched by addition of 6% TFA/H$_2$O (10% v/v). The resulting solution was immediately vortexed, flash frozen in liquid N$_2$, and lyophilized to dryness. The resulting powder was resuspended in 25 mL of 6 M GuHCl for 15 min and passed through a 0.22-μm filter. HD6 was purified by preparative HPLC using a solvent gradient of 25–33% B over 18 min at 10 mL/min. HD6 eluted at 13.4 min and the corresponding fractions were lyophilized to dryness to provide a white fluffy powder. The yield was 1.9 mg/L culture. The HPLC retention time, and results from LC-MS are listed in Table 2.4.

2.2.7 Site-Directed Mutagenesis and Purification of HD6 Variants

The HD6 variants (Table 2.3) were generated by employing a modified Quick-Change site-directed mutagenesis protocol (Stratagene). The pET28b-proHD6 plasmid was used as the template. The forward and reverse primers are listed in Table 2.1 and the primer pairings are listed in Table 2.2.
### Table 2.1. Primers employed for subcloning and site-directed mutagenesis.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD6</td>
<td>5'-GAATTCCATATGGAGCCGCTGCAAGCAG-3'</td>
</tr>
<tr>
<td>HD6-R</td>
<td>5'-GATCCTCGAGTTACAGACAACAAAAGCGATG-3'</td>
</tr>
<tr>
<td>F2A</td>
<td>5'-GTAGCAGCGTGACAGCGACTTGCCACTGC-3'</td>
</tr>
<tr>
<td>F2A-R</td>
<td>5'-GCAGTGGAAGTCGGCTGACACGCGGTGCTAC-3'</td>
</tr>
<tr>
<td>V22T</td>
<td>5'-GCTATGGCACCCTGCAACCAGATGGGCAATTC-3'</td>
</tr>
<tr>
<td>V22T-R</td>
<td>5'-GATTAATGCCCAGTGCTGCGAGTGCCATAGC-3'</td>
</tr>
<tr>
<td>I25T</td>
<td>5'-CACGCGTATGGGGACCAATCGATGCTTTTG-3'</td>
</tr>
<tr>
<td>I25T-R</td>
<td>5'-CAAAAGCGATGATTGGTGCCCATCACCCTG-3'</td>
</tr>
<tr>
<td>H27A</td>
<td>5'-GGTGATGGGCAATATGCAGCTTTGGTTGTCTG-3'</td>
</tr>
<tr>
<td>H27A-R</td>
<td>5'-CAGACAACAAAAAGCGCCAGATATGCCCATCACC-3'</td>
</tr>
<tr>
<td>H27W</td>
<td>5'-GGTGATGGGCAATATTGCAGCTTTGGTTGTCTG-3'</td>
</tr>
<tr>
<td>H27W-R</td>
<td>5'-CAGACAACAAAAAGCGCAGATATGCCCATCACC-3'</td>
</tr>
<tr>
<td>F29A</td>
<td>5'-GCATTAATCATCGCGGGGGGATGATTGTCTGTAACACTCG-3'</td>
</tr>
<tr>
<td>F92A-R</td>
<td>5'-CGAGTTACAGACCAACACGCGCGATGATTATGC-3'</td>
</tr>
</tbody>
</table>

* Underlined black codons indicate restriction sites. Bold codons indicate stop codons. The codons containing the mutations are underlined and highlighted in red.

### Table 2.2. Templates and primer pairings employed in site-directed mutagenesis.

<table>
<thead>
<tr>
<th>Template</th>
<th>Product</th>
<th>Primers Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET28b-proHD6</td>
<td>pET28b-proHD6(F2A)</td>
<td>F2A, F2A-R</td>
</tr>
<tr>
<td>pET28b-proHD6</td>
<td>pET28b-proHD6(V22T)</td>
<td>V22T, V22T-R</td>
</tr>
<tr>
<td>pET28b-proHD6</td>
<td>pET28b-proHD6(H27A)</td>
<td>H27A, H27A-R</td>
</tr>
<tr>
<td>pET28b-proHD6</td>
<td>pET28b-proHD6(F29A)</td>
<td>F29A, F29A-R</td>
</tr>
</tbody>
</table>
For all mutagenesis reactions, the PCR conditions were 95 °C for 30 min (1x); 55 °C for 1 min and 68 °C for 17 min (25x); 4 °C hold. Following PCR amplification using PfuTurbo DNA polymerase, a 1.5-µL aliquot of DpnI was added to a 50-µL PCR reaction and it was incubated at 37 °C for 1.5 h. A second 1.5-µL aliquot of DpnI was then added to the PCR reaction and it was incubated at 37 °C for another 1.5 h. The DpnI digests were transformed into chemically-competent E. coli TOP10 cells. Single colonies were selected and grown in 5 mL of LB media containing 50 µg/mL of kanamycin (37 °C, 16-18 h). The plasmids were isolated by using a QIAprep spin miniprep kit and their identities were verified by DNA sequencing. The overexpression and purification of His6-proHD6 variants, and preparation and purification of HD6 variants, were conducted as described for native HD6. During the trypsin-catalyzed cleavage reaction, degradation of the 32-residue F29A product was observed by analytical HPLC following incubation times >15 min. The peptide yields ranged from 1.8 mg/mL (F2A) to 4.5 mg/mL (H27W). The HPLC retention time, and results from LC-MS for these variants are presented in Table 2.4. All experiments were performed with at least two independently prepared batches of each peptide.

2.2.8 Thiol Quantification Assays
The assays were conducted by modifying a reported literature protocol.24 To obtain a reduced-glutathione (GSH) standard curve, 0, 3, 6, 9, 12, or 15 µL of GSH (3 mM in 10 mM HCl) was added to Milli-Q water to achieve a volume of 15 µL and then added to 943 µL of the assay buffer (100 mM sodium phosphate, 200 µM EDTA, pH 7.0; degassed for at least 1 h by bubbling Ar). A 42-µL aliquot of 2,2'-dithiodipyridine (DTDP,
4 mM in 24 mM HCl) was added to the solution to achieve the final volume of 1 mL. The resulting solution was incubated at room temperature for 15 min and the absorbance at 341 nm was measured. The GSH standard curve was obtained by plotting corrected absorbance versus GSH concentration.

For quantifying the number of free thiol residues present in each peptide sample, peptide stock solutions were freshly prepared in Milli-Q water and the concentrations were determined by optical absorption spectroscopy. Then, aliquots of the peptide stock solutions were diluted with the assay buffer and an aliquot of DTDP was added to each sample as described above to achieve final peptide concentrations of 6 μM. The solutions were incubated at room temperature for 15 min and the absorbance at 341 nm was measured. The number of free thiol residues in each sample was determined by using the GSH standard curve. These assays were conducted with at least two independently prepared and purified samples of each peptide and in three independent trials.

2.2.9 Disulfide Bond Connectivity Determination

The canonical α-disulfide bond connectivity (Cys⁴—Cys³¹, Cys⁶—Cys²⁰, Cys¹⁰—Cys³⁰) for each of the six HD6 variants presented in this work was confirmed by employing trypsin digestion followed by four cycles of manual Edman degradation (Figure 2.4, Table 2.5). Each HD6 variant (1 mg) was incubated in 1.0 mL of 100 mM Tris-HCl pH 8.0, 20 mM CaCl₂, 1 M urea, and 0.1 mg/mL of trypsin at 37 °C. F2A, V22T, and F29A were incubated for 3 h and I25T, H27A, and H27W were incubated for 11 h before 100 μL of 6% TFA was added to quench the reaction. The digested peptides were purified
by semi-preparative HPLC (20–40% B over 20 min at 4 mL/min) and lyophilized to dryness.

Figure 2.4. Determination of disulfide bond connectivity of F2A by employing trypsin digestion and manual Edman degradation. This step-by-step summary applies for all HD6 variants analyzed in this work. The calculated and observed masses of all HD6 variant fragments are listed in Table 2.5.
Each purified peptide product containing three disulfide bonds (Figure 2.4) was then subjected to four cycles of manual Edman degradation. In each cycle, the peptide was resuspended in 200 µL of 5% phenylisothiocyanate (PITC, Thermo Scientific) in 50% pyridine/H₂O, and incubated at 50 °C for 30 min. The reaction was then dried at 60 °C under vacuum, resuspended in 100 µL of anhydrous TFA, and incubated at 45 °C for 10 min. The TFA was removed by flushing with nitrogen and the remaining crude material was dried at 45 °C under vacuum. Then, 200 µL of pre-chilled 5% pyridine/H₂O was added to the crude product and the resulting cloudy solution was extracted with 250 µL of pre-chilled n-butyl acetate three times. The aqueous layer was dried at 60 °C under vacuum to obtain the peptide for LC-MS analysis and the next cycle of Edman degradation. The results from LC-MS are listed in Table 2.5.

2.2.10 Negative-Staining Transmission Electron Microscopy

For each sample, a 5-µL aliquot of peptide solution (20 µM in 10 mM sodium phosphate pH 7.4 or 10 mM Tris-maleate pH 6.4) was placed onto the carbon-coated surface of a copper grid (400 square mesh, Electron Microscopy Sciences). After 1 min, the grid was stained with a 5-µL aliquot of 2% uranyl acetate (UA, Electron Microscopy Sciences) in Milli-Q water three times and air-dried for at least 15 min before imaging. A FEI Technai Spirit Transmission Electron Microscope was employed to collect all transmission electron micrographs (W.M. Keck Microscopy Facility, Whitehead Institute, Cambridge, MA). TEM images were obtained with at least two independent batches of each peptide and representative images are presented.
2.2.11 Sedimentation Velocity Experiments

Sedimentation velocity (SV) experiments were performed to determine the sedimentation coefficients of the F2A and F29A variants. A Beckman XL-I Analytical Ultracentrifuge equipped with an An-50 Ti rotor was used for all SV experiments. The rotor housed double-sector charcoal-filled Epon centerpieces within the sample cells and contained quartz windows. All SV sample cells contained either 410 μL of buffer reference or 400 μL of peptide solution. In one set of experiments, peptide stock solutions in Milli-Q water were lyophilized to dryness, dissolved in 400 μL of 10 mM sodium phosphate pH 7.4 or 10 mM Tris-maleate pH 6.4 to achieve the desired concentrations (40, 50, 100, and 160 μM), and transferred to SV sample cells. The pH of each solution was measured to confirm that it remained unchanged. The samples were centrifuged at 42,000 rpm and 20 °C until sedimentation was complete. The absorption wavelength used for optical detection was 280 nm. All SV experiments were conducted with at least two independently prepared and purified samples of each peptide and in two independent trials.

The details of data analysis are reported elsewhere. The buffer viscosity (η), buffer density (ρ), and the partial specific volume (v̅) values of F2A and F29A at 20 °C were calculated by employing SEDNTERP. The reported HD6 crystal structure (PDB: 1ZMQ) was used in HYDROPRO hydrodynamic modeling to calculate sedimentation coefficients of the HD6 monomer, dimer, and tetramer (Table 2.6). It was assumed that the F2A and F29A mutations would have negligible impact on the calculated sedimentation coefficients. The buffer viscosity (η) and buffer density (ρ) values for water at 20 °C, and a partial specific volume (v̅) value of 0.6994 mL/g for HD6 were
used in all HYDROPRO calculations. The experimental sedimentation coefficients of the HD6 variants were calculated by fitting the time derivative of the sedimentation velocity ($-dc/dt$) data by using DCDT$^{+}$.\textsuperscript{29,30} The $-dc/dt$ distribution was generated from 26 to 34 scans with a peak-broadening limit of 50 kDa by using DCDT$^{+}$. The results are reported in Tables 2.7, 2.8.

2.2.12 Circular Dichroism Spectroscopy

For all circular dichroism (CD) measurements, peptide solutions (20 $\mu$M, 300 $\mu$L) were prepared in 10 mM sodium phosphate pH 7.4 and transferred to a 1-mm path-length quartz CD cell (Hellma). The CD spectra were collected from 260-190 nm at 1-nm intervals (5 s averaging time, three independent scans per wavelength) by using an Aviv Model 202 CD spectrometer operated at room temperature. The data obtained from the three scans were averaged and the resulting averaged spectra are reported. The CD spectra were collected from at least two independently prepared and purified samples of each peptide and in two independent trials.

2.2.13 Antimicrobial Activity Assays

Bacteria from freezer stocks were grown to saturation with shaking (37 °C, 16 h) in 5 mL of the indicated medium (\textit{L. monocytogenes} ATCC 19115 in Brain Heart Infusion medium (BHI); \textit{S. aureus} ATCC 25923, \textit{E. coli} ATCC 25922, or \textit{S. Typhimurium} ATCC 14028 in Tryptic Soy Broth medium (TSB) without dextrose). The overnight culture was diluted 1:100 into 5 mL of fresh BHI or TSB and incubated at 37 °C until $OD_{600}$ of $\approx$0.6 was achieved. The resulting culture was centrifuged (3500 rpm x 10 min, 4 °C) and the
supernatant was removed. The bacterial pellet was resuspended in 5 mL of AMA buffer (10 mM sodium phosphate, 1% TSB, pH 7.4). The cell suspension was centrifuged (3500 rpm x 10 min, 4 °C) and the supernatant was discarded. The resulting cell pellet was resuspended in 5 mL of AMA buffer and diluted with AMA buffer to obtain an OD$_{600}$ value of 0.5 for _L. monocytogenes_, _E. coli_, and _S. Typhimurium_ or 0.6 for _S. aureus_. For _L. monocytogenes_ ATCC 19115, the bacterial suspension was further diluted 1:500 with AMA buffer in three steps (1:10 x 1:10 x 1:5). For _S. aureus_ ATCC 25923, the bacterial suspension was further diluted 1:100 with AMA buffer in two steps (1:10 x 1:10). For _E. coli_ ATCC 25922, the bacterial suspension was further diluted 1:250 with AMA buffer in three steps (1:10 x 1:10 x 1:2.5). For _S. Typhimurium_ ATCC 14028, the bacterial suspension was further diluted 1:250 with AMA buffer in three steps (1:10 x 1:10 x 1:2.5). The diluted cultures were used immediately.

The assays were conducted in 96-well plates. To each well was added 10 μL of a 10x concentrated aqueous peptide solution (200 μM) or sterile Milli-Q water as a no-peptide control. A 90-μL aliquot of the diluted bacterial culture was added to each well and the plate was incubated for 1 h (37 °C, 150 rpm). A 50-μL aliquot from each well was subsequently added to 450 μL of AMA buffer (10$^{-1}$ dilution). The resulting solution was vortexed gently and further diluted serially from 10$^{-2}$ to 10$^{-4}$ in 10-fold increments by adding a 100-μL aliquot from each dilution to 900 μL of AMA buffer. A 100-μL aliquot from each dilution was manually plated on BHI agar plates for _L. monocytogenes_ or TSB agar plates for _S. aureus_, _E. coli_, and _S. Typhimurium_. The plates were then incubated at 37 °C for 15 h for _E. coli_ and _S. Typhimurium_ or 20 h for _L. monocytogenes_ and _S. aureus_. The number of colony forming units obtained for each peptide sample...
was determined by colony counting. Only plates with 30-200 colonies were considered in these assays. These assays were performed with at least two independently prepared and purified samples of each peptide and in three independent trials. The resulting averages and standard deviations are reported.

2.2.14 Bacterial Agglutination Assays

A 5-mL portion of BHI medium for *L. monocytogenes* ATCC 19115 or Luria Broth (LB) for *E. coli* ATCC 25922 was inoculated with the bacteria from freezer stocks and grown to saturation with shaking (37 °C, 16 h). The overnight culture was diluted 1:100 into 10 mL of fresh BHI or LB medium and incubated at 37 °C until OD$_{600}$ reached =0.6. The resulting culture was centrifuged (3500 rpm × 10 min, 4 °C) and the supernatant was removed. The bacterial pellet was resuspended in 10 mL of 50% Mueller-Hinton Broth pH 7.4 (MHB, Fluka). The cell suspension was centrifuged (3500 rpm × 10 min, 4 °C) and the supernatant was discarded. The resulting bacterial pellet was resuspended in 10 mL of 50% MHB and further diluted with 50% MHB to obtain an OD$_{600}$ value of 0.25. The diluted bacterial cultures were immediately employed for the agglutination assays.

For all agglutination assays, a 450-μL aliquot of the bacterial culture (OD$_{600}$ = 0.25) was added to 50 μL of a 10x concentrated aqueous peptide solution (50, 100, or 200 μM) or sterile Milli-Q water as a no-peptide control and immediately transferred to an EtOH-sterilized two-sided disposable polystyrene cuvette (VWR International). The OD$_{600}$ values were measured at the indicated time points and plotted versus time to afford a sedimentation curve. After 6 h, a 100-μL aliquot from each cuvette containing 20 μM of each peptide was diluted with 900 μL of 11 mM sodium phosphate pH 7.4,
vortexed gently, and serially diluted from $10^{-2}$ to $10^{-7}$ in 10-fold increments by adding a 100-$\mu$L aliquot from each dilution to 900 $\mu$L of the buffer. If there was bacterial sedimentation in the cuvette, a 100-$\mu$L aliquot was taken from the supernatant for serial dilutions. Then, 100 $\mu$L of fresh 50% MHB was added to the cuvette and the bacterial aggregate was resuspended. Another 100-$\mu$L aliquot was subsequently taken from the resulting suspension for serial dilutions. To determine the number of colony forming units for each peptide treatment, a 100-$\mu$L aliquot from each dilution was manually plated on BHI agar plates for *L. monocytogenes* or LB agar plates for *E. coli* and incubated at 37 °C for 20 h or 15 h, respectively. Only plates with 30-200 colonies were considered in these assays. All assays were performed with at least two independently prepared and purified samples of each peptide and in three independent trials. The resulting averages with standard deviations are reported.

2.2.15 General Cell Culture Methods

Human colon epithelial T84 cells (ATCC CCL-248) were obtained from the American Type Culture Collection (ATCC) and grown in 1:1 Dulbecco’s modified Eagle medium (DMEM) and Ham’s F12 medium containing 2.5 mM L-glutamine, 15 mM HEPES, 0.5 mM sodium pyruvate, and 1.2 g/L sodium bicarbonate, and supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were cultured at 37 °C and 5% CO$_2$ according to the instructions from ATCC. The growth medium and all supplements were purchased from ATCC.
2.2.16 Listeria Invasion Assays

These assays were conducted by modifying reported literature protocols.\textsuperscript{15,31,32} \textit{L. monocytogenes} ATCC 19115 from freezer stocks were grown overnight with shaking (37 °C, 16 h) in 5 mL of BHI medium. The overnight culture was diluted 1:100 into 5 mL of fresh BHI media and incubated at 37 °C until OD\textsubscript{600} of ≈0.6 was achieved. The resulting culture was centrifuged (3500 rpm x 10 min, 4 °C) and the supernatant was removed. The bacterial pellet was resuspended in 5 mL of serum- and antibiotic-free 1:1 DMEM/F12. The resulting suspension was centrifuged (3500 rpm x 10 min, 4 °C) and the supernatant was removed. The bacterial pellet was resuspended in 5 mL of serum- and antibiotic-free 1:1 DMEM/F12 and diluted with the media to afford an OD\textsubscript{600} value of 0.5. The resulting suspension was further diluted 1:150 into 3 mL of the fresh media. A 190 µL aliquot of the diluted bacterial culture was immediately added to 10 µL of a 20x concentrated aqueous peptide solution (50, 100, or 200 µM) or sterile Milli-Q water as a no-peptide control and incubated at room temperature for 30 min.

T84 cells between passages 59 and 73 were cultured in 75 cm\textsuperscript{2} rectangular canted neck cell culture flasks (Corning) to approximately 95% confluency and treated with 3 mL of trypsin-EDTA (Corning). A 12-mL portion of fresh media was added to the detached cells, and the T84 cell suspension was centrifuged (600 rpm x 5 min, 37 °C). The supernatant was discarded and the cell pellet was resuspended in 6 mL of fresh culture media. The concentration of cells was quantified by using a manual hemocytometer (VWR International) and adjusted to 2 × 10\textsuperscript{5} cells/mL. A 500-µL aliquot of T84 cells were then added to 24-well Costar tissue culture plates (Corning) and incubated at 37 °C and 5% CO\textsubscript{2} for 24 h. With this cell density, the monolayers were
~80% confluent at the time of the experiment. With cells at this confluency, the media was discarded and the cells were washed twice with 500 µL of phosphate buffered saline (PBS) without calcium or magnesium (ATCC) and bathed in 500 µL of serum- and antibiotic-free 1:1 DMEM/F12. After a 2-h equilibration in serum- and antibiotic-free media, the media was discarded. The T84 cells were washed twice with 500 µL of PBS and infected with 200 µL of peptide-treated *L. monocytogenes* (multiplicity of infection, MOI = 10) at 37 °C and 5% CO₂. MOI is a ratio of the number of bacterial cells to mammalian cells when infection is initiated. A 100-µL aliquot of the diluted bacterial culture was used to determine the number of inoculum by colony counting as described below. After 1.5 h of infection, the medium was removed and the T84 cells were washed twice with 500 µL of PBS. To kill any remaining extracellular bacteria, the T84 cells were subsequently incubated in 200 µL of serum-free 1:1 DMEM/F12 containing 100 µg/mL of gentamycin for 1.5 h at 37 °C and 5% CO₂. After 1.5 h, the media was removed and the T84 cells were washed twice with 500 µL of PBS. The T84 cells were then incubated in 200 µL of sterile-filtered 1% Triton X-100 (EMD) in PBS for 10 min at room temperature.

A 50-µL aliquot from each well was diluted with 450 µL of 11 mM sodium phosphate pH 7.4, vortexed gently, and serially diluted from 10⁻² to 10⁻⁴ in 10-fold increments by adding a 100-µL aliquot from each dilution to 900 µL of the fresh buffer. To determine the number of invading bacteria for each peptide treatment, a 100-µL aliquot from each dilution was manually plated on BHI agar plates and incubated at 37 °C for 20 h. Only plates with 30-200 colonies were considered in these assays. The
percentage of invasion represents the ratio of the number of invading bacteria to the number of inoculated bacteria. All assays were performed with at least two independently prepared and purified samples of each peptide and in three independent trials. The resulting averages and standard deviations are reported.

2.3 Results and Discussion

2.3.1 Design and Preparation of a HD6 Variant Family

We designed and prepared a six-membered HD6 variant family (Figure 2.5a, Table 2.3) to evaluate the role of select residues in HD6 self-assembly and host-defense function. The variants F2A, F29A, V22T, and I25T were prepared to probe the consequences of decreased hydrophobicity for each of these residues. We also evaluated H27W and H27A. This residue abuts the hydrophobic pocket and, in prior work, a role for H27 in self-assembly was proposed from evaluation of the H27W and H27A variants. The crystal structure of H27W (PDB: 3QTE) differs from that of HD6 and supports a dimer of dimers arrangement and the absence of elongated structures. An electrostatic interaction between H27 of one monomer (A or D, Figure 2.5c) and the C-terminal carboxylic acid of L32 of another monomer (B or C, Figure 2.5c) was hypothesized to enable formation of the extended oligomers observed for native HD6. The crystal structure of HD6 also suggests that a π-π stacking interaction between the side chains of F2 of one monomer (A or D) and H27 of another monomer (B or C) may occur and contribute to self-assembly (Figure 2.5c).
Figure 2.5. (a) Amino acid sequences of HD6 and variants evaluated in this work. The numbers represent amino acid position. The Cys residues in blue comprise the Cys$^4$—Cys$^{31}$, Cys$^8$—Cys$^{20}$, and Cys$^{10}$—Cys$^{30}$ disulfide linkages (solid lines). The salt-bridge between Arg$^7$ and Glu$^{14}$ is indicated as a dashed line. The mutated residues are shown in red. The secondary structure depiction is based on the crystal structure. (b) Analytical HPLC traces of purified HD6 and variants (oxidized forms) dissolved in Milli-Q water (30 μM x 80 μL). Absorbance at 220 nm was monitored with a reference wavelength of 500 nm. Method: 10–60% B over 30 min at 1 mL/min. (c) Previously reported crystal structure of HD6 (PDB: 1ZMQ) with F2, H27, and L32 shown. Individual HD6 monomers are labeled A-D. The electrostatic interaction between H27 of one monomer and carboxylate of a C-terminus of another monomer is indicated by a dashed line.
Table 2.3. Amino acid sequences of peptides employed in this work. a

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino Acid Sequence</th>
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<tr>
<td>HD6</td>
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</tr>
<tr>
<td>F2A</td>
<td>AATCHRRSCYSTEYSYGTCVMGINHRFCCL</td>
</tr>
<tr>
<td>V22T</td>
<td>AFTCHRRSCYSTEYSYGTCVMGINHRFCCL</td>
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<tr>
<td>I25T</td>
<td>AFTCHRRSCYSTEYSYGTCVMGINHRFCCL</td>
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<td>H27A</td>
<td>AFTCHRRSCYSTEYSYGTCVMGINARFCCL</td>
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<td>H27W</td>
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<tr>
<td>F29A</td>
<td>AFTCHRRSCYSTEYSYGTCVMGINHRACCL</td>
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</tbody>
</table>

*a Mutations are colored in red.

In prior studies of HD5, we overexpressed His6-Met-HD5 and employed cyanogen bromide to cleave the affinity tag and afford native HD5.24 HD6 contains a Met residue at position 23 and is not compatible with this approach. The HNPs and HD5 are transcribed and translated as prepropeptides that undergo maturation,33-35 and mRNA analysis indicates that HD6 is also translated as a prepropeptide (100-aa).8 We therefore expressed proHD6 (81-aa) harboring a N-terminal His6 tag and released native HD6 (oxidized form) by protease digestion. For peptide overexpression, an E. coli-optimized synthetic gene for proHD6 was ligated into the Ndel and Xhol sites of pET28b, and His6-proHD6 was overexpressed in E. coli BL21(DE3) cells and isolated by using Ni-NTA column chromatography. The oxidized form of His6-proHD6 was obtained by following an oxidative folding protocol,24 and native HD6 was isolated following treatment of His6-proHD6 with trypsin and preparative HPLC purification (Figure 2.6).
Figure 2.6. Analytical HPLC traces from a representative overexpression of His6-proHD6 and purification of HD6 (30 μM x 80 μL). Absorbance at 220 nm was monitored with a reference wavelength of 500 nm. Method: 10–60% B over 30 min at 1 mL/min.

We employed the same procedure to prepare the six HD6 variants. Analytical HPLC indicated that HD6 and its variants were obtained in high purity (Figure 2.5b). The peptide yields ranged from 1.8 (F2A) to 4.5 (H27W) mg/L of culture (Table 2.4). Thiol quantification indicated that each peptide contained no free thiol moieties (Table 2.4), and the peptide identities were confirmed by LC-MS (Table 2.4). For each variant, the canonical α-defensin disulfide bond pattern exhibited by native HD6 (Cys4—Cys31, Cys6—Cys20, Cys10—Cys30) was confirmed by manual Edman degradation (Figure 2.4, Table 2.5). All experiments were performed with the oxidized species and in the absence of reducing agents.
Table 2.4. Characterization of peptides employed in this work.

<table>
<thead>
<tr>
<th>Peptide a</th>
<th>Retention Time (min)b</th>
<th>Free Thiol c</th>
<th>Calculated ( m/z )</th>
<th>Observed ( m/z )</th>
<th>Yield (mg/L culture)</th>
<th>( e_{280} ) (M ( ^3 ) cm (^{-1} )) d</th>
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<tr>
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<td>3705.5</td>
<td>1.9</td>
<td>4845</td>
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<td>F2A</td>
<td>15.7</td>
<td>0.05 ± 0.06</td>
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<td>1.8</td>
<td>4845</td>
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<tr>
<td>V22T</td>
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<td>3707.5</td>
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<td>I25T</td>
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<td>H27A</td>
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<td>0.16 ± 0.12</td>
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a See Table 2.3 for amino acid sequences. The HD6 variants are all oxidized. b Retention times determined by using analytical RP-HPLC on a C18 column and a gradient of 10–60% B over 30 min at 1 mL/min. c Free thiol content determined by using the DTDP assay (mean ± SDM, \( n = 3 \)). d Extinction coefficients at 280 nm were calculated by using the on-line resource ExPASy ProtParam. e n.d. = not determined.
Table 2.5. MS analysis of peptides after each Edman degradation cycle.

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<tr>
<th>Peptide</th>
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<th>Observed m/z</th>
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<td>C_{92}H_{142}N_{30}O_{35}S_{3}</td>
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<td>2242.9</td>
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</table>

2.3.2 Biophysical Characterization Reveals that F2 and F29 Are Essential for HD6 Self-Assembly

Transmission electron microscopy (TEM) revealed that HD6 spontaneously self-assembles into higher-order oligomers in aqueous solution. When we incubated HD6 (2 or 20 µM) at pH 7.4 in 10 mM sodium phosphate buffer and stained the samples with
2% uranyl acetate, we observed elongated fibril-like structures with lengths on the micron scale (Figures 2.7, 2.8). HD6 also formed micron-sized fibrils at pH 6.4 in 10 mM Tris-maleate buffer (Figure 2.9). The Tris-maleate buffer was employed in a prior investigation where *S. Typhimurium* entrapped by HD6 nanonets *in vitro* was visualized by scanning electron microscopy (SEM).\(^{15}\) A comparison of the TEM images obtained in both buffers revealed a buffer effect on fibril morphology. In particular, the Tris-maleate buffer afforded HD6 fibrils that are relatively short and in relatively low abundance when compared to same experiment performed in sodium phosphate buffer.

![Figure 2.7. Transmission electron micrographs of 20 μM HD6 and its variants in 10 mM sodium phosphate pH 7.4. Scale bar = 200 nm.](image-url)
Figure 2.8. Transmission electron micrographs of HD6 in 10 mM sodium phosphate, pH 7.4. (a) 2 μM HD6 and (b) 20 μM HD6 at magnification of 30,000x (left) and 98,000x (right).

Figure 2.9. Transmission electron micrographs of 20 μM HD6 and its variants in 10 mM Tris-maleate pH 6.4. Scale bar = 100 nm.

TEM of the HD6 variants provided evidence for varying degrees of fibril formation under the same conditions (Figures 2.7, 2.9). Notably, mutation of hydrophobic residues that comprise the hydrophobic pocket had pronounced effects on fibril formation and morphology. No fibrils were observed for the F2A or F29A variants in
either buffer, and the V22T and I25T variants afforded shorter and thicker fibrils. These observations indicate that these hydrophobic residues are important for self-assembly, and that mutation of either F2 or F29 to Ala prevents fibril formation. H27A afforded an extensive fibril network characterized by both thick and thin fibrils whereas fewer and significantly shorter fibril-like structures were observed for H27W. The latter result is in general agreement with prior studies of H27W, which reported defective oligomerization properties for this variant as ascertained by surface plasmon resonance (SPR) and X-ray crystallography (PDB: 3QTE).\textsuperscript{15} Nonetheless, fibril formation for H27A contrasts with conclusions drawn from a SPR investigation of H27A where this variant was reported to exhibit the same behavior as H27W.\textsuperscript{15} From comparison of H27A and H27W by TEM under our experimental conditions, as well as in other experiments described below, we conclude that (i) the nature of the self-assembly varies from mutation to mutation and (ii) the presence of the bulky Trp residue at position 27 results in greater perturbation to self-assembly than loss of H27 and hence loss of either the electrostatic interaction between this residue and L32 or the putative $\pi-\pi$ stacking interaction with F2.

To further evaluate the quaternary structures for F2A and F29A, we employed analytical ultracentrifugation (AUC) and determined the sedimentation coefficients ($s_{20,w}$) for each peptide (Tables 2.6, 2.7). At pH 7.4 in 10 mM sodium phosphate buffer, single peaks at ca. 0.8 S and 1.2 S are observed for F2A (≤160 \textmu M) and F29A (≤160 \textmu M), respectively, over the range $s_{20,w} = 0$–2.5 S in the Gaussian fits of the $-dc/dt$ distributions obtained using DCDT+ (Figures 2.10, 2.11, Tables 2.6, 2.7). The Gaussian fits support the predominance of a single species of both F2A and F29A over a concentration range of 40–160 \textmu M at pH 7.4. Substitution of phosphate buffer with
Tris-maleate buffer at pH 6.4 had negligible effect on the s values for both F2A and F29A (Figures 2.10, 2.11, Tables 2.6, 2.7). HYDROPRO\textsuperscript{28} estimated the sedimentation coefficients of HD6 to be 0.75 S (monomer), 1.17 S (dimer), and 1.83 S (tetramer) using the crystal structure of HD6\textsuperscript{19} as a model (Table 2.8). A comparison between the experimental and estimated s values suggests that F2A and F29A predominantly exist as a monomer and a dimer, respectively, under the conditions employed for these experiments.

**Figure 2.10.** Sedimentation velocity experiments of F2A in (a) 10 mM sodium phosphate pH 7.4 and (b) 10 mM Tris-maleate pH 6.4 shown as single Gaussian fits (red line) of $-dc/dt$ data (blue dots) measured with absorbance at 280 nm. The peptide concentrations ranged from 40 \(\mu\text{M}\) to 160 \(\mu\text{M}\). The summaries of the fits are provided in Table 2.6.
Table 2.6. Calculated sedimentation coefficients of F2A.*

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>$S_{20,w}$ (S)</th>
<th>$D$ (F)</th>
<th>MW (kDa)</th>
<th>Partial Specific Volume (mL/g)</th>
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</table>

*The temperature was 20 °C. Data were obtained by analysis with the dc/dt method implemented in DCDT+ using 26-34 scans with 50 kDa diffusion broadening maximum. Sedimentation coefficients are $S_{20,w}$ values, adjusted with a solvent density ($\rho$) of 0.99967 g/mL, and a solvent viscosity ($\eta$) of 1.0061 cP. Viscosity units are in centipoise (cP) (1 Poise = 1 g cm$^{-1}$ s$^{-1}$). Sedimentation coefficients are in Svedbergs (1 Svedberg = 100 fs = 1 x 10$^{-13}$ s). Diffusion coefficients correspond to the best-fit molecular mass in Fick units (1 Fick = 1 x 10$^{-7}$ cm$^2$/s). $^b$The samples were in 10 mM sodium phosphate pH 7.4. $^c$The samples were in 10 mM Tris-maleate pH 6.4.
Figure 2.11. Sedimentation velocity experiments of F29A in (a) 10 mM sodium phosphate pH 7.4 and (b) 10 mM Tris-maleate pH 6.4 shown as single Gaussian fits (red line) of $-\frac{dc}{dt}$ data (blue dots) measured with absorbance at 280 nm. The peptide concentrations ranged from 50 \( \mu \text{M} \) to 160 \( \mu \text{M} \). The summaries of the fits are provided in Table 2.7.
Table 2.7. Calculated sedimentation coefficients of F29A.

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>S_{20,w} (S)</th>
<th>D (F)</th>
<th>MW (kDa)</th>
<th>Partial Specific Volume (mL/g)</th>
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</tbody>
</table>

* The temperature was 20 °C. Data were obtained by analysis with the dc/dt method implemented in DCDT+ using 26-34 scans with 50 kDa diffusion broadening maximum. Sedimentation coefficients are $S_{20,w}$ values, adjusted with a solvent density ($\rho$) of 0.99967 g/mL, and a solvent viscosity ($\eta$) of 1.0061 cP. Viscosity units are in centipoise (cP) (1 Poise = 1 g cm$^{-1}$ s$^{-1}$). Sedimentation coefficients are in Svedbergs (1 Svedberg = 100 fs = $1 \times 10^{-13}$ s). Diffusion coefficients correspond to the best-fit molecular mass in Fick units (1 Fick = $1 \times 10^{-7}$ cm$^2$/s). The samples were in 10 mM sodium phosphate pH 7.4. The samples were in 10 mM Tris-maleate pH 6.4.

Table 2.8. Sedimentation coefficient calculations using HYDROPRO.

<table>
<thead>
<tr>
<th>Species</th>
<th>$S_{20,w}$ (S)</th>
<th>Partial Specific Volume (mL/g)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD6 monomer</td>
<td>0.746</td>
<td>0.6994</td>
</tr>
<tr>
<td>HD6 dimer</td>
<td>1.17</td>
<td>0.6994</td>
</tr>
<tr>
<td>HD6 tetramer</td>
<td>1.83</td>
<td>0.6994</td>
</tr>
</tbody>
</table>

* Monomer, dimer, tetramer $\bar{\nu}$ values of HD6 were estimated by SEDNTERP. 27
Prior to TEM imaging of the HD6 fibrils, we attempted to perform a sedimentation velocity experiment with HD6. This experiment failed because the peptide sedimented within 15 min, even at the lowest possible rotor speed (3000 rpm), and formed a white coat on the AUC cell attributed to rapid peptide aggregation. In total, the AUC results support the findings from TEM and confirm that F2 and F29 are essential for HD6 self-assembly in aqueous buffer.

Structural differences between native HD6 and its variants are also apparent in the CD spectroscopic signatures (Figure 2.12). Native HD6 exhibits relatively intense features defined by a negative peak at ca. 190 nm and positive peaks centered at 205 nm and 230 nm. This CD spectrum differs from those reported for other defensins, including HD5.24 The HD6 variants exhibit less intense CD features than native HD6, and the CD spectra of some variants (e.g. F29A) resemble that of HD5. The relatively intense features in the CD spectrum of native HD6 may provide a fingerprint of high-order oligomerization and therefore be used to identify other defensins that display similar self-assembly.
Figure 2.12. CD spectra of HD5 and native HD6 and its variants in 10 mM sodium phosphate, pH 7.4. The peptide concentrations were 20 μM.
2.3.3 *Mutation of HD6 Does Not Confer Antibacterial Activity*

We questioned whether any of the HD6 variants employed in this work, and F2A and F29A in particular, exhibits antibacterial activity as a result of disrupted oligomerization that allows smaller, potentially bioactive, species to exist. We compared the antibacterial activity of HD6 and the six variants against four bacterial species. *L. monocytogenes* ATCC 19115 and *S. aureus* ATCC 25923 were chosen as representative Gram-positive organisms whereas *E. coli* ATCC 25922 and *S. Typhimurium* ATCC 14028 were selected as representative Gram-negative microbes. In these assays, HD5, which has broad-spectrum antibacterial activity, was employed as a positive control. The results presented in Figure 2.13 clearly delineate that neither HD6 nor the variants exhibit significant antibacterial activity against any of the evaluated strains at a concentration of 20 μM. Thus, disruption of quaternary structure that affords lower-order oligomers (e.g. F2A, F29A) of the HD6 scaffold does not “turn on” a potent bactericidal killing activity for HD6 against these microbes.

![Graph](image)

**Figure 2.13.** Antibacterial activity assays against *L. monocytogenes* ATCC 19115, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *S. Typhimurium* ATCC 14028 (mean ± SDM, n = 3). The peptide concentrations were 20 μM. The asterisks indicate no colony formation.
2.3.4 Mutation of Hydrophobic Residues Alters the Bacterial Agglutination by HD6

In seminal studies of HD6 host-defense function, SEM revealed that HD6 entangles S. Typhimurium.\textsuperscript{15} We reasoned that point mutations that disrupt HD6 self-assembly likely exhibit a reduced propensity to entrap and agglutinate bacteria. Thus, we established a simple bacterial agglutination assay based on solution turbidity and we employed this assay to probe the ability of HD6 and its variants to entrap bacteria and cause sedimentation (Figures 2.14-2.17). When we introduced 20 μM HD6 into a bacterial culture (10\textsuperscript{8} CFU/mL) housed in a sterile plastic cuvette, we observed bacterial clumping by the eye within 5 min of mixing. These clumps sedimented to the bottom of the cuvette within about 2 h and clarified the culture medium. This agglutination phenomenon was observed for both Gram-negative \textit{E. coli} and Gram-positive \textit{L. monocytogenes}. In contrast, agglutination and sedimentation were not observed for the untreated control; these cultures remained homogeneous over the course of the assay.

We defined the two phases of the heterogeneous mixture obtained from HD6 treatment as “supernatant” for the clarified medium solution in the top portion of the cuvette and “agglutinate” for the sedimented material at the bottom of the cuvette (Figure 2.14). We defined “resuspension” as the mixture that results from thoroughly agitating the heterogeneous mixture containing the sedimented material at the bottom of the cuvette. After incubating the bacteria and HD6 for 6 h, we determined the CFU/mL of the supernatant and observed a ca. 1.3-fold log reduction in CFU/mL relative to the bacteria-only control. Following agitation, the CFU/mL of the resuspension was comparable to that of the untreated control (Figure 2.17). As expected, the reduction of
CFU/mL in the supernatant is attributed to sedimentation of bacteria in the cuvette, and not to a bactericidal activity of HD6.

Figure 2.14. Representative image of an untreated *L. monocytogenes* culture and a culture after incubation with 20 μM HD6 for 6 h at room temperature. The mixture is separated into two phases: supernatant and agglutinate as indicated in the figure.

Figure 2.15. Agglutination assays of *L. monocytogenes* ATCC 19115 incubated with 0-20 μM HD6 or its variant (mean ± SDM, n = 3).
Figure 2.16. Agglutination assays of *E. coli* ATCC 25922 incubated with 0-20 μM HD6 or its variant (mean ± SDM, n = 3).

Figure 2.17. Plots of colony forming units (CFU/mL) of *L. monocytogenes* ATCC 19115 and *E. coli* ATCC 25922 after treatment with 20 μM peptide for 6 h (mean ± SDM, n = 3).
We evaluated the ability of the HD6 variants (0-20 \( \mu \text{M} \)) to agglutinate and sediment \textit{L. monocytogenes} and \textit{E. coli} over a 6-h period (Figures 2.15, 2.16). Based on the resulting sedimentation profiles, we defined three different types of behavior for native HD6 and its variant and grouped the peptides accordingly. One group is comprised of native HD6 and H27A, both of which afforded similar and relatively rapid bacterial agglutination with the OD\(_{600}\) values approaching zero in the presence of peptide. The second group includes variants that caused bacterial agglutination, but to a lesser degree than native HD6 and H27A (Figures 2.15, 2.16). These peptides are V22T, I25T, and H27W, which agglutinated the bacteria only at relatively high (\( \geq 10 \mu \text{M} \)) peptide concentrations. The oligomerization-deficient F2A and F29A variants define the third group. The bacterial cultures containing these peptides remained homogeneous after 6 h even at the highest peptide concentration evaluated, indicating that these variants cannot promote bacterial agglutination and sedimentation under these conditions. These profiles correlate with the results from TEM where HD6 and H27A formed elongated fibrils, V22T, I25T, and H27W formed fibrils that are smaller and with variable morphologies, and F2A and F29A did not form any observable oligomer.

To verify that the decreases in OD\(_{600}\) values observed for V22T, I25T, H27A, and H27W correlated with bacterial agglutination and sedimentation at the bottom of the cuvette and not to bacterial cell death, we ascertained the CFU count for the supernatant and resuspension of each culture treated with 20 \( \mu \text{M} \) peptide for 6 h (Figure 2.17). As expected based on the results of the antibacterial activity assays (Figure 2.13), this experiment confirmed that the bacteria remained alive over the course of the experiment. Moreover, the CFU/mL reductions observed for the
supernatants correlate with the differences in the OD\textsubscript{600} values before and after sedimentation took place. A smaller change in OD\textsubscript{600} as observed for V22T correlates with a smaller decrease in CFU/mL for the supernatant. For I25T, H27A, and H27W, each of which caused OD\textsubscript{600} to approach zero at 20 μM of peptide, a CFU/mL reduction similar in magnitude to that of HD6 was observed.

2.3.5 Hydrophobic Residues Are Needed for HD6 to Prevent Listeria Invasion of Human T84 Cells

\textit{L. monocytogenes} is a pathogenic Gram-positive bacterium that can colonize the human gastrointestinal tract and cause foodborne illness in adults as well as meningitis which is a serious threat to fetuses and newborns.\textsuperscript{36, 37} This species binds to and invades host cells. We performed a series of \textit{L. monocytogenes} invasion assays and determined that HD6 in the culture medium blocks \textit{Listeria} invasion into human T84 intestinal epithelial cells. The percentage of \textit{Listeria} invasion dropped from ca. 10% to <2% when ≥2.5 μM HD6 was included in the culture medium (\textbf{Figure 2.18}). HD6 was previously shown to block invasion of mammalian cells by two different Gram-negative species, \textit{S. Typhimurium} and \textit{Yersinia enterocolitica}.\textsuperscript{15} Taken together with the current results obtained for a Gram-positive organism, we conclude that the ability of HD6 to prevent bacterial invasion, at least \textit{in vitro}, is broad-spectrum with no apparent selectivity for Gram-negative or Gram-positive organisms. Moreover, our results indicate that HD6 has the capacity to provide host defense against the gastrointestinal pathogen \textit{L. monocytogenes}.
Figure 2.18. Invasion of human T84 colon epithelial cells by *L. monocytogenes* ATCC 19115 in the presence of native HD6 and its variants. The bacteria (2 x 10^6 CFU/mL) were incubated with the indicated peptides for 30 min prior to infection of the T84 cells (mean ± SDM, n = 3).

Like native HD6, H27A provided ≥4-fold reduction in *Listeria* invasion over the concentration range evaluated. In contrast, oligomerization-deficient HD6 variants F2A and F29A did not inhibit *Listeria* invasion over this concentration range. Moreover, the V22T, I25T and H27W variants exhibited attenuated abilities to prevent *Listeria* invasion compared to HD6. On the basis of the trends depicted in Figure 2.18, these variants may prevent *Listeria* invasion at higher peptide concentrations. Our invasion results for H27W are in a good agreement with previous work where this variant exhibited attenuated ability to prevent *Salmonella* and *Yersinia* invasion into mammalian cells.\textsuperscript{21}

We conclude that the hydrophobic residues and hence self-assembly are important for HD6 to prevent invasive microbes from entering mammalian cells.

2.4 Summary and Outlook

In this study, we demonstrate that hydrophobic residues in HD6 drive its self-assembly and afford innate immune function. Our results highlight the importance of primary
sequence for defensin function and, in particular, how variable amino acid sequences between α-defensin family members afford different biophysical properties and biological activity. Of the four hydrophobic residues evaluated in this work, we discovered that F2 and F29 are particularly important for both HD6 self-assembly and biological function.

The results from our solution and TEM experiments as well as prior X-ray crystallographic characterization establish that HD6 forms higher-order structures in the absence of bacteria or other biomolecules. How the structures observed by TEM relate to the structure and composition of the HD6 nanonets entangling bacteria observed both in vitro and in vivo requires further exploration. Along these lines, SEM studies of wild-type and mutant S. Typhimurium treated with HD6 indicated that certain cell surface proteins contribute to formation of HD6 nanonets in vitro. This observation suggests that bacterial surface proteins provide a nucleation site. It will be informative to ascertain whether different bacterial species and different bacterial proteins affect the morphology and network of HD6 nanonets, as well as precisely how HD6 interacts with such proteins. Nanonet maturation as well as the physiological fate of the nanonet and the entrapped microbes are additional questions that must be addressed. From a functional standpoint, we reason that entrapment of bacterial pathogens by HD6 in the lumen not only prevents bacterial species that potentially reside and proliferate inside host cells from reaching this destination and causing infection, but also allows for other host-defense factors that operate in the intestinal lumen, such as other Paneth cell antimicrobials (e.g. HD5) and recruited neutrophils, to kill them in the extracellular space.
In closing, the hydrophobic effect plays a crucial role in biological processes that include cell membrane formation, protein folding and stabilization, and blood coagulation. It also contributes to pathologies associated with protein misfolding as exemplified by the Aβ-peptide (Alzheimer’s disease) and α-synuclein (Parkinson’s disease). HD6 provides a novel example of how Nature employs hydrophobicity for a beneficial outcome. Indeed, creating a biomolecular self-assembly from a 32-residue cysteine-rich defensin peptide to capture pathogens is a remarkable strategy for combating infection.

2.5 Acknowledgements

We gratefully acknowledge the NIH (Grant DP2OD007045 from the Office of the Director) and the Royal Thai Government (PC) for financial support. The Biophysical Instrumentation Facility for the Study of Complex Macromolecular Systems at MIT is supported by Grant NSF-007031. We thank Professor E. M. Nolan for preparing the His-proHD6 plasmid. We thank Ms. D. Pheasant and Dr. A. J. Wommack for assistance with the AUC experiments and data analysis, and Dr. E. J. Brignole for helpful advice on TEM sample preparation and data collection.

2.6 References


Chapter 3

Proteolysis Triggers Self-Assembly and Unmasks Innate Immune Function of a Human α-Defensin Peptide

This Chapter is published in Chem. Sci. 7, 1738-1752 (2016).
3.1 Contributions

Dr. B. Shen at the Cleveland Clinic Foundation for providing human samples. Professor C. L. Bevins, Dr. H. Chu, and Dr. P. Castillo at UC Davis conducted ex vivo analysis of HD6 isoforms and scanning electron microscopy of bacterial agglutination by HD6.

3.2 Introduction

The innate immune system mediates homeostasis at mucosal surfaces, in part by providing protection from microbial invasion. Host-defense peptides are abundant and important players in the interplay between host and microbe at mucosal surfaces. The intestine harbors the largest reservoir of colonizing microbes, termed the microbiota, and this diverse community is mostly comprised of microorganisms that are beneficial to the host. Nevertheless, some resident members of this community, as well as many transient microbes, can invade the epithelium and thus pose a significant challenge for the immune system to effectively protect the host and maintain intestinal homeostasis. Paneth cells, secretory cells located at the bases of the crypts of Lieberkühn in the small intestine, contribute to mucosal innate immunity by releasing a cocktail of host-defense peptides and proteins in response to microbial stimuli. In humans, Paneth cells express two α-defensins, human defensins 5 and 6 (HD5 and HD6). Defensins are small (2-5 kDa) cysteine-rich host-defense peptides expressed by epithelial cells and neutrophils. These peptides typically exhibit broad-spectrum antimicrobial activity. α-Defensins exhibit three regiospecific disulfide bonds (CysI–CysVI, CysII–CysIV, CysIII–CysV) in the oxidized form, which stabilize a three-stranded β-sheet fold and confer protease resistance. The oxidized form of HD6, in contrast to HD5
and other characterized α-defensins, exhibits negligible in vitro antimicrobial activity.\textsuperscript{12,15-18} Based on in vivo model studies and in vitro characterization, HD6 operates by an unprecedented host-defense mechanism involving its unusual self-assembly properties.\textsuperscript{16,17} HD6 monomers oligomerize into extended structures termed "nanonets" and thereby entrap bacteria in the small intestinal lumen. This capture mechanism prevents bacterial invasion into host epithelial cells and subsequent dissemination to other organs.\textsuperscript{16,17} The HD6 nanonets have been observed in vivo and in vitro, with the former studies employing HD6 transgenic mice infected with the enteric pathogen \textit{Salmonella enterica} serovar Typhimurium (S. Typhimurium).\textsuperscript{16}

A number of fundamental chemical and biological questions about HD6 arise from prior studies. First, our understanding of HD6 is limited because the peptide has not been isolated and characterized from human intestine. Current assumptions about mature HD6 are based on one study in which the peptide was detected in urine specimens obtained from bladder cancer patients with surgically created ileal neobladders.\textsuperscript{19} There are also uncertainties related to HD6 storage and maturation. In particular, how do Paneth cells package and deploy a self-assembling peptide from granules, and how is formation of nanonets regulated? Analysis of human mRNA indicated that HD6 is translated as a 100-residue prepropeptide.\textsuperscript{7} This prepropeptide is predicted to contain a 19-residue N-terminal signal sequence that targets the peptide to the secretory pathway, and an 81-residue C-terminal region that corresponds to a putative mature HD6, and an intervening acidic propeptide domain.\textsuperscript{7} The HD6 propeptide has not been detected in a human specimen or characterized to date. Based on prior studies of α-defensins in humans\textsuperscript{20-24} and mice,\textsuperscript{25,26} whether HD6 is stored as
mature peptide or as a propeptide is unclear because both cases are observed for other human α-defensins, and mice store α-defensins in their Paneth cells as mature peptides.

Guided by the biophysical properties of HD6, the oxidatively folded regioisomer with CysI-CysVI, CysII-CysIV, CysIII-CysV bonds that is the focus of the current work, we reasoned that the quaternary structure of a proHD6 and mature HD6 differ. The HD6 crystal structure reveals that mature 32-residue HD6 monomers are arranged as a chain of tetramers where the N- and C-termini from four monomers form a hydrophobic pocket. On the basis of this structure and our studies of HD6 variants that have defective self-assembly properties, we hypothesized that the N-terminal pro sequence of proHD6 would interrupt the alignment of HD6 monomers and prevent the formation of higher-order oligomers. We therefore hypothesized that storage of HD6 as the propeptide in Paneth cells would prevent HD6 nanonets from forming in the granules. Moreover, the amino acid sequence of proHD6 (Figure 3.1a) reveals that Argprovides a potential trypsin cleavage site, which is likely relevant because trypsin is expressed and released by human Paneth cells. Taken together, these observations provided us with a testable model whereby Paneth cells would package HD6 as an inactive propeptide, and that proteolytic processing by trypsin unleashes mature HD6 in the intestinal lumen.

Herein, we combine ex vivo analyses of human intestinal specimens with biophysical characterization and in vitro functional studies of HD6 and its propeptide to interrogate this model and characterize the HD6 maturation pathway. We report, for the first time, the detection and analysis of HD6 from samples of human intestinal tissue.
and luminal fluid. We demonstrate that an 81-residue proHD6 isoform exhibiting an N-terminal extension is found in ileal tissue and 32-residue mature HD6 is found in luminal fluid. We establish that proHD6 is an inactive isoform. The N-terminal region of proHD6 suppresses self-assembly and renders proHD6 unable to agglutinate bacteria and protect human epithelial cells from bacterial invasion. Moreover, we show that proHD6 is a substrate for trypsin, and that trypsin-catalyzed hydrolysis of proHD6 yields the 32-residue mature HD6 found in the lumen. In this protease-triggered cascade, trypsin-catalyzed release of HD6 unMASKS latent biological activity by enabling peptide self-assembly to form the nanonets that can entrap bacterial invaders.

3.3 Experimental Section

3.3.1 Materials and General Methods

All solvents, reagents, and chemicals were purchased from commercial suppliers and used as received unless noted otherwise. His$_6$-proHD5 and the mature 32-residue forms of HD5 and HD6 were recombinantly overexpressed and purified as previously described.$^{14,17}$ These procedures involve oxidative folding, which allows for isolation of the native Cys$^I$—Cys$^V$, Cys$^{II}$—Cys$^V$, Cys$^{III}$—Cys$^V$ regioisomers following purification. All functional and biophysical studies were performed with oxidatively folded peptides. Milli-Q water (18.2 mΩ·cm) was passed through a 0.22-μm filter before it was used to prepare all buffers, aqueous solutions, and peptide/oligonucleotide stock solutions. Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA) and used as received (standard desalting protocol). A Biorad MyCycler thermocycler was employed for all polymerase chain reactions (PCR). Chemically-competent
*Escherichia coli* TOP10 and BL21(DE3) cells were prepared in-house via standard protocols. PfuTurbo DNA polymerase was purchased from Agilent Technologies. T4 DNA ligase and all restriction enzymes were purchased from New England Biolabs. DNA sequencing was performed by staff in the Biopolymers Facility at the Massachusetts Institute of Technology.

### 3.3.2 Instrumentation

Unless noted otherwise, analytical and semi-preparative high-performance liquid chromatography (HPLC) were performed on an Agilent 1200 instrument equipped with a thermostatted autosampler set at 4 °C and thermostatted column compartment generally set at 20 °C, and a multi-wavelength detector set at 220 and 280 nm (500-nm reference wavelength with 100-nm bandwidth). Preparative HPLC was performed using an Agilent PrepStar 218 instrument outfitted with an Agilent ProStar 325 UV-visible dual-wavelength detector set at 220 and 280 nm. A C18 column (5-μm particle size, 4.6 x 250 mm, Higgins Analytical, Inc.) set at a flow rate of 1 mL/min was employed for all analytical HPLC experiments. A ZORBAX C18 column (5-μm particle size, 9.4 x 250 mm, Agilent Technologies, Inc.) set at a flow rate of 5 mL/min was employed for all semi-preparative-scale HPLC purification. A Luna 100 A C18 LC column (10-μm particle size, 21.2 x 250 mm, Phenomenex) set at a flow rate of 10 mL/min was utilized for all preparative-scale HPLC purification. HPLC-grade acetonitrile (MeCN) and HPLC-grade trifluoroacetic acid (TFA) were routinely purchased from EMD and Alfa Aesar, respectively. For all HPLC runs, solvent A was 0.1% TFA/H₂O and solvent B was 0.1% TFA/MeCN.
High-resolution mass spectrometry was performed by using an Agilent LC-MS system comprised of an Agilent 1260 series LC system outfitted with an Agilent 6230 TOF system housing an Agilent Jetstream ESI source. For all LC-MS analyses, solvent A was 0.1% formic acid/H₂O (LC-MS grade, Sigma-Aldrich) and solvent B was 0.1% formic acid/MeCN (LC-MS grade, Sigma-Aldrich). A Poroshell 120 EC-C18 column (2.7-μm particle size, 2.1 x 100 mm, Agilent Technologies, Inc.) set at a flow rate of 0.4 mL/min was employed for all LC-MS analyses of HD6. The samples were analyzed by using a gradient of 5–95% B over 5 min. A Poroshell 300SB C18 column (5-μm particle size, 2.1 x 75 mm, Agilent Technologies, Inc.) set at a flow rate of 0.2 mL/min was employed for all LC-MS analyses of proHD6. The samples were analyzed by using a gradient of 5–65% B over 30 min. The MS profiles were analyzed and deconvoluted by using Agilent Technologies Quantitative Analysis 2009 software version B.03.02.

A Beckman Coulter DU 800 UV–visible spectrophotometer was employed for all routine optical absorption measurements and agglutination assays. Extinction coefficients (280 nm) were calculated by using ExPASy ProtParam. The calculated extinction coefficients of native HD6, proHD6, and His₆-SUMO-proHD6 are 4845, 6335, and 7825 M⁻¹cm⁻¹, respectively. Peptide stock solutions were routinely prepared in Milli-Q water and concentrations were quantified by using the calculated extinction coefficients. Solution and buffer pH values were verified by using a Mettler Toledo S20 SevenEasy pH meter or a HANNA Instruments HI 9124 pH meter equipped with a microelectrode. An Aviv Model 202 circular dichroism spectrometer operated at room temperature was utilized to collect CD spectra.
3.3.3 Human Specimen Samples and Ethics Approval

The protocols for obtaining tissue specimens and luminal aspirates were reviewed and approved by the Institutional Review Board at the Cleveland Clinic Foundation (#06-673) and informed consent was obtained from all patients. All specimens were coded so that patient identifiers were removed and then handled as previously described.\textsuperscript{16,24,28} Nondiseased specimens of human distal small intestinal tissue were obtained from redundant surgically-resected tissue as described.\textsuperscript{24} Small intestinal lumen fluid aspirates were obtained from individuals undergoing colonoscopy for either routine colon polyp screening or for assessment of inflammatory bowel disease. Approximately 5–15 mL of ileal fluid was aspirated, immediately placed on dry ice, frozen, and stored at -80°C prior to use.

3.3.4 Immunogold Labeling and Transmission Electron Microscopy

Human ileal tissue specimens were fixed in 4% paraformaldehyde (EM Science, Hatfield, PA) overnight at room temperature. The tissue was then dehydrated in graded ethanol and thin-sectioned. Grids were incubated with rabbit polyclonal HD6 antiserum (1:1000),\textsuperscript{16} mouse monoclonal anti-HD5 IgG (HyCult, Plymouth Meeting, PA), or rabbit polyclonal trypsin antiserum (Abcam, Cambridge, MA) for 1 h at room temperature in a humidified chamber, washed with PBS, and incubated with 1:50 dilution of 5- or 15-nm gold-labeled goat-anti-rabbit or goat-anti-mouse antibody (EM Science, Hatfield, PA) for 30 min. Grids were washed with double-distilled water and visualized on a Philips CM120 Biotwin Lens (F.E.I. Company, Hillsboro, OR) with Gatan BioScan, model 792 (Pleasanton, CA).
3.3.5 HD6 Peptide Isolation From Intestinal Tissue

Protein extraction and fractionation from small intestinal tissue followed a published protocol,\textsuperscript{24} with some modifications. Briefly, approximately 1 g of human ileal tissue was homogenized with a Brinkmann Polytron homogenizer in ice-cold 20\% acetic acid (1:10 w/v) that contained 1:100 v/v Protease Inhibitor Cocktail III. The extract was stirred overnight at 4 °C, and then clarified by ultracentrifugation at 110,000x g (30 min, 4 °C). Ammonium sulfate was added (final concentration of 25\% w/v), and the mixture was stirred at room temperature for 1 h, and then clarified by ultracentrifugation (110,000x g x 30 min, 4 °C). The supernatant was then dialyzed against 5\% v/v acetic acid overnight at 4 °C using Spectra/Por dialysis membrane (1-kD MWCO, Spectrum Laboratories, Rancho Dominguez, CA). The resulting solution was passed through a strong cation exchange cartridge (Bio-Scale Mini Macro-Prep High Q, BioRad, Hercules, CA), washed with 5\% v/v acetic acid, and then eluted with 1 M NaCl. The eluate was further purified by RP-HPLC using a Waters 650E HPLC instrument with a variable wavelength detector (monitored at 214 nm and 280 nm) and a C18 column (Vydec) with a gradient of 5–80\% acetonitrile gradient in 0.1\% TFA. Fractions were collected at a flow rate of 1 ml/min and analyzed as described for the lumen aspirate specimens.

3.3.6 HD6 Peptide Isolation From Intestinal Lumen Fluid

The frozen ileal fluid aspirate was thawed on ice, and a protease inhibitor cocktail was added at a ratio of 1:100 v/v (Cocktail III, Calbiochem, La Jolla, CA). The resulting sample was immediately acidified with acetic acid (20\% v/v final). Clumps and particulates were dispersed with a Brinkmann Polytron homogenizer. The sample was
clarified by centrifugation at 29,000x g (2 x 30 min, 4 °C). The supernatant was diluted with water (1:1 v/v), flash frozen, and then lyophilized to dryness. The lyophilized product was dissolved in 1.5 ml of 5% v/v acetic acid, and filtered through a Millex 0.22-μm polyether sulfone filter (EMD-Millipore) to remove residual particulates. A portion of the sample was then fractionated by reverse phase HPLC (RP-HPLC) using a Waters 650E instrument outfitted with a C18 column (10-μm particle size, 4.6 x 250 mm, Vydac/Grace, Columbia MD) using a gradient of 5–62% B over 60 min at a flow rate of 1 mL/min. Fractions were collected at 1-min intervals and absorbance at 220 and 280 nm was monitored. Aliquots of each fraction were analyzed by MALDI-TOF mass spectrometry (2-μL aliquot) and for immunoreactivity (20-μL aliquot). Fractions positive for HD6 were then analyzed by Edman degradation as described below.

3.3.7 MALDI Mass Spectrometry
A 4700 MALDI TOF/TOF (Applied Biosystems, Foster City, CA) equipped with a pulsed Nd:YAG laser (337 nm) and a delayed extraction ion source was employed to screen the HPLC fractions for HD6 isoforms. An aliquot of each fraction (2 μL) was mixed with an equal volume of a saturated matrix solution (α-cyano-4-hydroxycinnamic acid in 0.1% TFA/50% MeCN). A 1-μL aliquot of this mixture was applied to the target plate. The mass spectra were acquired in linear mode and typically ≈1000 laser shots were used per sample.
3.3.8 Dot Blot Immunoreactivity

A dot blot method was used to screen HPLC sample fractions for HD6 immunoreactivity. A 20-μL aliquot of each fraction was spotted directly onto an Immobilon PSQ PVDF membrane (Millipore, Billerica, MA). The protein on the membrane was then fixed with 0.01% w/v glutaraldehyde (Sigma, St. Louis, MO) for 2.5 min, washed in Tris-buffered saline for 1 min, and the membrane surface was blocked with 5% w/v non-fat dry milk for 1 h. The membrane blots were probed with HD6 rabbit polyclonal antibody (1:7000) using a horseradish peroxidase (HRP)-conjugated goat-anti-rabbit secondary antibody (KPL, Gaithersburg Maryland). Signal was detected using Immobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA). The chemiluminescent signal was detected with a Biospectrum AC Imaging System (UVP, Upland, CA).

3.3.9 Edman Degradation

Sample fractions with the positive HD6 immunoreactivity and mass consistent with (pro)HD6 were analyzed by Edman degradation to determine N-terminal amino acid sequence. The analysis was performed with a Procise 494 microsequencer (Applied Biosystems) by staff at the UC Davis Molecular Structure Facility.

3.3.10 Western Blot Analysis

Acid urea-polyacrylamide gel electrophoresis (AU-PAGE) Western blot analysis was performed on small intestinal tissue and luminal fluid as previously described. Briefly, small intestinal ileal specimens (tissue and luminal aspirate fluid) were homogenized with a Brinkmann Polytron homogenizer in ice-cold 20% acetic acid (1:10 w/v) that
contained 1:100 v/v Protease Inhibitor Cocktail III. The resulting suspension was stirred overnight at 4 °C and clarified the following day by ultracentrifugation at 110,000x g (30 min, 4 °C). Aliquots of these specimens were diluted with 0.5 volume of loading buffer (9 M urea, 5% v/v acetic acid, 0.1 mg/mL methyl green (Sigma)) and then resolved on polyacrylamide gels (12.5% acrylamide/2% bis-acrylamide (Roche, Indianapolis, IN), 8 M deionized urea (Sigma), and 5% v/v acetic acid). Samples were run toward the cathode (reverse typical polarity) at 130 volts in 5% v/v acetic acid running buffer until the methyl green indicator dye reached the bottom of the gel (typically ≈1.5 h). Proteins were then transferred from the gels to Immobilon PSQ PVDF membranes in 5% v/v acetic acid using a semi-dry transfer apparatus (Fisher Scientific, Pittsburgh, PA) at 1.5 mA/cm² toward the cathode for 20 min. Each membrane was then fixed, washed, blocked, and probed as described for the dot blot analysis.

3.3.11 Subcloning, Overexpression, and Purification of His₆-SUMO-proHD6

The pET SUMO-proHD6 plasmid was prepared by using the Champion pET SUMO protein expression system (Invitrogen). The proHD6 nucleotide sequence (246 bp) was PCR amplified using pET28b-proHD6 as a template and the forward and reverse primers 5'-GAGCCGCTGCAAGCAGAG-3' and 5'-TTACAGACAACAAAAGCGATG-3', respectively (stop codon, underlined). The PCR products were analyzed by 1% (w/v) agarose gel and purified by using a QIAquick PCR purification kit (Qiagen). The PCR-amplified proHD6 gene was subsequently ligated into pET SUMO using T4 DNA ligase (9 ng of PCR product, 50 ng of linear pET SUMO, 1 µL of T4 DNA ligase; 16 h, 16 °C). A 1-µL aliquot of each ligation reaction was transformed into chemically-competent E.
coli Mach1™-T1R cells (Invitrogen). The plasmids were isolated by using QIAprep spin miniprep kit (Qiagen) and the plasmid identities were verified by DNA sequencing.

The overexpression and purification of His<sub>6</sub>-SUMO-proHD6 was modified from the procedure for obtaining His<sub>6</sub>-proHD6.\textsuperscript{17} pET SUMO-proHD6 was transformed into chemically-competent \textit{E. coli} BL21(DE3) cells. Overnight cultures were prepared by inoculating LB medium containing kanamycin (50 μg/mL) with single colonies. These cultures were grown to saturation (37 °C, 150 rpm, 16-18 h) and used to prepare freezer stocks. The freezer stocks, containing a 1:1 ratio of the overnight culture and sterile-filtered 50% glycerol in Milli-Q water, were stored at -80 °C. For a given His<sub>6</sub>-SUMO-proHD6 overexpression, 50 mL of LB medium containing 50 μg/mL kanamycin in a 250-mL baffled flask was inoculated from the freezer stock and grown to saturation (37 °C, 150 rpm, 16-18 h). The resulting culture was diluted 1:100 into 2 L of fresh LB medium containing 50 μg/mL of kanamycin in a 4-L baffled flask and incubated at 37 °C, 150 rpm until \( \text{OD}_{600} \) of ≈0.6 was achieved. Subsequently, a 400-μL aliquot of 0.5 M isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the 2-L culture and the culture was incubated for an additional 4-5 h until \( \text{OD}_{600} \) reached 1.2-1.5. The cells were centrifuged (3000 rpm x 15 min, 4 °C) and the cell pellets were collected. Overexpression of His<sub>6</sub>-SUMO-proHD6 was usually performed on a 12-L scale and the cell pellets from 6 L of culture were combined in pre-weighed 50-mL polypropylene centrifuge tubes (=2 g/L wet cell weight), flash frozen in liquid N<sub>2</sub>, and stored at -80 °C for a period of 1-2 months.

For purification of His<sub>6</sub>-SUMO-proHD6, each 6-L cell pellet was thawed on ice and resuspended in 40 mL of cold lysis buffer (6 M GuHCl, 100 mM Tris-HCl, pH 8.0). A 1-mL aliquot of phenylmethyl sulfonyl fluoride (PMSF, 100 mM in EtOH) was added to the
resuspension and the cells were transferred to a pre-chilled stainless steel beaker and lysed on ice by two rounds of sonication (10% amplitude with pulse on for 1 s and pulse off for 4 s for 1 min, on ice, Branson sonicator). A second 1-mL aliquot of PMSF (100 mM) was added to the cell lysate followed by centrifugation (13000 rpm x 30 min, 4 °C). The resulting supernatant was incubated with pre-washed Ni-NTA resin (Qiagen, from 9 mL of Ni-NTA slurry for a cell pellet from 6 L of culture that was pre-washed 3 x 30 mL with Milli-Q water) with gentle shaking for 1.5 h at 4 °C. The resulting mixture was then loaded onto a fritted glass column and the resin was washed with 40 mL of cold wash buffer (20 mM Tris-HCl, 300 mM NaCl, 6 M GuHCl, pH 8.0). The His<sub>6</sub>-SUMO-proHD6 fusion protein was eluted with 30 mL of cold elution buffer (10 mM Tris-HCl, 300 mM NaH<sub>2</sub>PO<sub>4</sub>, 200 mM NaCl, 1 M imidazole, 6 M GuHCl, pH 6.5). The eluent was diluted with 30 mL of Milli-Q water, transferred into a dialysis bag (3500 MWCO), and dialyzed (2 x 12 h) against refolding buffer (20 mM Tris-HCl, 150 mM NaCl, 10% v/v glycerol, 1% w/v CHAPS, pH 8.0). The solution of His<sub>6</sub>-SUMO-proHD6 was concentrated to 2 mg/mL, transferred to 50-mL polypropylene centrifuge tubes, flash frozen in liquid N<sub>2</sub> and stored at -80 °C. The average yield was 20 mg/L culture. The purity of His<sub>6</sub>-SUMO-proHD6 was routinely evaluated by SDS-PAGE (15% Tris-HCl gel). A representative gel is shown in Figure 3.4a.

3.3.12 Preparation and Purification of Native proHD6<sub>red</sub>

To a solution of His<sub>6</sub>-SUMO-proHD6, tris-(2-carboxylethyl)phosphine (TCEP, 100 mM stock solution in Milli-Q water) was added to achieve a final concentration of 2 mM and the solution pH was adjusted to pH 8.0 by drop-wise addition of 4 M NaOH. A 1-mg/mL
stock solution of Ulp1 in 20 mM Tris-HCl, 150 mM NaCl, pH 8.0 was added to the solution containing His$_6$-SUMO-proHD6 to afford a His$_6$-SUMO-proHD6:Ulp1 ratio of 100:1 (w/w). The reaction was incubated at room temperature for 2 h and quenched by addition of 6% aqueous TFA (10% v/v). The quenched reaction was immediately vortexed and incubated on ice for 10 min, and a precipitate formed. The mixture was centrifuged (3750 rpm x 15 min, 4 °C), and the supernatant was decanted and saved. The precipitate was resuspended in 20 mL of 6 M GuHCl and passed through a 0.22-μm filter. Analytical HPLC and LC-MS revealed that the majority of the precipitate was reduced 81-residue proHD6 (proHD6$_{\text{red}}$, Table 3.1). The supernatant portion, which was saved from the cleavage reaction, was dialyzed (3500 MWCO) against Milli-Q water (2 x 12 h), lyophilized, and resuspended in 75 mM HEPES, pH 8.0 containing 6 M GuHCl and 2 mM TCEP. After 15-min incubation at room temperature, the solution was acidified with 6% aqueous TFA (10% v/v). Analytical HPLC and LC-MS revealed that proHD6 in the supernatant portion was completely reduced. Subsequently, proHD6$_{\text{red}}$ from both supernatant and precipitate portions was purified by preparative HPLC using a solvent gradient of 33–38% B over 16 min at 10 mL/min. The desired product eluted at 14.2 min and was lyophilized to afford a white powder (=0.5 mg/L of culture, Table 3.1).

3.3.13 Oxidative Folding

Reduced proHD6 was oxidatively folded to afford a single regioisomer with the native S—S bonds by modifying a literature procedure.$^{17,29}$ A 6-mg portion of proHD6$_{\text{red}}$ was dissolved in 1 mL of 8 M GuHCl containing 3 mM glutathione and 0.3 mM glutathione.
disulfide. Then, 3 mL of 250 mM NaHCO₃ was added to the solution to raise the pH to ≈8.3 and afford a final peptide concentration of 1.5 mg/mL. The mixture was incubated at room temperature for 16 h. The resulting solution was analyzed by HPLC and LC-MS to confirm that proHD6_red was completely converted to a single oxidized species. The solution was centrifuged (3750 rpm x 10 min, 4 °C), passed through a 0.22-µm filter, and purified by preparative HPLC using a solvent gradient of 32–37% B over 16 min at 10 mL/min. The single regioisomer obtained by oxidative folding eluted at 13.1 min and was lyophilized, which afforded pure oxidized proHD6 as a white powder (~0.2 mg/L of culture). Analytical HPLC indicated that proHD6 was obtained in high purity (Figure 3.4b). Thiol quantification confirmed that proHD6 had no free Cys residues and LC-MS confirmed its identity (Table 3.1).

3.3.14 Antimicrobial Activity Assays

The assays were performed according to a literature procedure.¹⁷ Briefly, a 90-µL aliquot of the diluted bacterial culture was added to each well and then to each well was added 10 µL of a 10x concentrated aqueous peptide solution (500 µM) or sterile Milli-Q water as a no-peptide control. The plate was incubated for 1 h (37 °C, 150 rpm). The serial dilution for CFU counting was conducted as described in Chapter 2. These assays were performed with at least two independently prepared and purified samples of each peptide and in three independent trials. The resulting averages and standard deviations are reported.
3.3.15 Trypsin-Catalyzed Cleavage of proHD6

A solution of proHD6 (100 μM, 180 μL) was prepared in 100 mM Tris-HCl, 20 mM CaCl₂, pH 8.0. A 1-mg/mL stock solution of TPCK-treated trypsin (Worthington) in Milli-Q water was added to the solutions to achieve a final concentration of 0.01 mg/mL. The mixture was incubated at room temperature with gentle shaking. A 24-μL aliquot was taken at varying time points, diluted with 56 μL of Milli-Q water, and quenched with 8 μL of 6% aqueous TFA. The resulting samples were vortexed, centrifuged (13000 rpm x 10 min, 4 °C), and analyzed by HPLC (10–60% B over 30 min at 1 mL/min).

3.3.16 Negative-Staining Transmission Electron Microscopy

The samples for transmission electron microscopy (TEM) were prepared by following a literature protocol. For each sample, a 5-μL aliquot of peptide solution (20 μM in 10 mM sodium phosphate pH 7.4 or 10 mM Tris-maleate pH 6.4) was placed onto the carbon-coated surface of a copper grid (400 square mesh, Electron Microscopy Sciences). After 1 min, the grid was washed with a 5-μL aliquot of Milli-Q water, stained with a 5-μL aliquot of 2% uranyl acetate (UA, Electron Microscopy Sciences) in Milli-Q water three times, and air-dried for at least 15 min before imaging. A FEI Technai Spirit Transmission Electron Microscope was employed for all TEM imaging (W.M. Keck Microscopy Facility, Whitehead Institute, Cambridge, MA). TEM images were obtained with at least two independently prepared peptides and representative images are presented.
3.3.17 Scanning Electron Microscopy

Bacteria were grown aerobically at 37 °C overnight in LB media. Then, the strains were subcultured and grown to mid-logarithmic phase in LB media. An aliquot of the bacteria (0.5-2 x 10^7 CFU/mL) was removed, sedimented by centrifugation (7,000x g x 2 min, 4 °C), washed twice with 50 mM Tris-maleate buffer, pH 6.4, and then resuspended in 0.5 mL of the 50 mM Tris-maleate buffer. The bacterial suspension was incubated at room temperature for 30 min with buffer control, 0.4 μM APMSF-inactivated trypsin, 3 μM proHD6, 3 μM trypsin-cleaved proHD6, or 3 μM HD6. Trypsin-cleaved proHD6 was prepared by incubating 30 μM proHD6 with 4 μM trypsin (Affimetrix, Santa Clara, CA) for 1 h at room temperature in 50 mM Tris-maleate buffer, pH 6.4. At the end of incubation, the trypsin was inactivated by addition of APMSF (Millipore, 25 μg/mL), and the mixture was added to the bacterial suspension. The APMSF-inactivated trypsin was similarly prepared, except that proHD6 was omitted from the preincubation. After a 30-min incubation, the treated bacterial suspensions were sedimented by centrifugation (7,000x g x 5 min, 4 °C), and the buffer supernatant was carefully removed. The bacterial pellet was then resuspended in 100 μL of Karnosky fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 0.06 M Sorensen’s phosphate buffer (0.2 M sodium phosphate, pH 7.2)). Scanning electron microscopy was performed as previously described.16

3.3.18 Bacterial Agglutination Assays

Stock solutions (50 μL) of HD6 and proHD6 (0, 50, 100, and 200 μM; 10x concentrations) were prepared in sterile Milli-Q water. If trypsin was required, aliquots of
a 1-mg/mL stock solution of TPCK-treated trypsin (Worthington) prepared in sterile Milli-Q water were added to solutions of proHD6 to afford a final trypsin concentration of 0.01 mg/mL. The assays were then conducted following the reported procedure.\textsuperscript{17} All assays were conducted with at least two independently prepared and purified samples of each peptide and in three independent trials. The resulting averages with standard deviations are reported.

3.3.19 Listeria Invasion Assays

Concentrated (20x, 10 \( \mu \text{L} \)) solutions of HD6 and proHD6 were prepared in sterile Milli-Q water (0, 50, 100, and 200 \( \mu \text{M} \)). For conditions that required trypsin, a 1-mg/mL stock solution of TPCK-treated trypsin (Worthington) in sterile Milli-Q water was added to the 20x proHD6 solutions to yield a final trypsin concentration of 0.01 mg/mL. A 190-\( \mu \text{L} \) aliquot of the diluted bacterial culture was added to each 10x peptide solution and the resulting mixtures were incubated at room temperature for 30 min. The invasion assays were then performed by following a literature protocol.\textsuperscript{17,30} All invasion assays were conducted with at least two independently prepared and purified samples of each peptide and in three independent trials and the resulting averages with standard deviations are reported.

3.3.20 Nuclear Morphology Assays

Nuclear morphology assays were conducted to monitor T84 cell viability qualitatively following 1.5-h incubation with HD6 or proHD6 following a literature protocol with modification.\textsuperscript{31} T84 cells were passed and 500 mL of cells at the density of 2 x 10\textsuperscript{5}
cells/mL was added to each well of a 24-well plate, which contained 12-mm untreated glass coverslips 12-16 h before the assays. The 20-μM peptide solutions in 1:1 DMEM/F12 (200 μL) were added to the cells and the cells were incubated for 1.5 h at 37 °C, 5% CO₂. Then, the medium was removed and the cells were washed (1 x 500 mL) with PBS and fixed for 5 min with 500 μL of PBS containing 4% paraformaldehyde and 4% sucrose. The cells were subsequently washed with PBS (2 x 500 μL) and bathed in 500 μL of PBS containing 800 nM Hoechst 33258 (Sigma Aldrich) for 5 min. The Hoechst solution was then removed. The cells were washed with 500 μL of PBS, bathed in PBS, and mounted onto glass slides using the Vectashield antifading reagent (Vector Labs). The samples were examined using Zeiss LSM 710 NLO laser scanning confocal microscope (W.M. Keck Microscopy Facility, Whitehead Institute, Cambridge, MA) and 60-70 cells were scored for each sample. The images were processed using ImageJ.

3.3.21 Sedimentation Velocity Experiments
The experiments were set up as previously reported. In one set of experiments, 10x concentrated proHD6 solutions in Milli-Q water (≈40 μL) were transferred to microcentrifuge tubes and lyophilized to dryness. A 400-μL aliquot of 10 mM sodium phosphate buffer adjusted to pH 7.4 (pre-filtered, 0.22-μm filter) was added to each microcentrifuge tube to achieve the desired concentrations (30, 50, 100, and 140 μM), and transferred to AUC sample cells. The pH of each solution was measured to confirm that it remained unchanged. The samples were centrifuged at 42,000 rpm and 20 °C until sedimentation was complete. The absorption wavelength for optical detection was
280 nm. All SV experiments were conducted with at least two independently prepared and purified samples of each peptide and in at least two independent trials.

Additional SV experiments were conducted to evaluate the effect of buffer components on the sedimentation of proHD6. In all cases, the proHD6 samples (400 μL) were prepared as described above except that proHD6 was dissolved in 10 mM Tris-HCl or HEPES pH 7.4 to obtain a final peptide concentration of 50 μM.

The details of data analysis are reported elsewhere. SEDNTERP was employed to calculate the buffer viscosity (η), buffer density (ρ), and the partial specific volume (v) value of proHD6 at 20 °C. The theoretical sedimentation coefficients of the proHD6 monomer and dimer were calculated by employing Equation 1

$$s_{sphere} = 0.012 \frac{M^{2/3}(1-\bar{v}p)}{\bar{v}^{1/3}}$$

where $s_{sphere}$ is the sedimentation coefficient for an ideal sphere in S units, M is the molar mass of the molecule of interest in Dalton, $\bar{v}$ is in milliliter per gram, and $\rho$ is in gram per milliliter. These calculations assume that proHD6 behaves as a smooth, compact, and spherical peptide in water at 20 °C.

The experimental sedimentation coefficients were calculated by fitting the time derivative of the sedimentation velocity (−dc/dt) data using DCDT+. The −dc/dt distribution was generated from 22 to 28 scans with a peak broadening limit of 40 kDa by using DCDT+. The results are reported in Table 3.4.

3.3.22 Sedimentation Equilibrium Experiments

Sedimentation equilibrium (SE) experiments were performed to determine the molecular weight of proHD6. The proHD6 samples were prepared as described in the SV
experiments with modification. In a typical set of experiments, proHD6 solutions in Milli-Q water (≈100 μL) housed in microcentrifuge tubes were lyophilized to dryness. An aliquot (110-μL) of 10 mM sodium phosphate buffer adjusted to pH 7.4 (pre-filtered, 0.22-μm filter) was added to each tube to afford solutions with varying concentrations of proHD6 (30, 60, and 120 μM) and transferred to AUC sample cells. The pH of each solution was measured to confirm that each solution remained at pH 7.4. Based on the sedimentation coefficients obtained from the SV experiments, equilibrium profiles were obtained at rotor speeds of 30000, 36000, and 42000 rpm. Once equilibrium was established, two scans with five replicates were recorded. The absorption wavelength for optical detection was 280 nm, and the instrument was maintained at 20 °C.

The details of the experimental setup and data analysis are reported elsewhere. SEDNTERP was employed to calculate the buffer viscosity (η), buffer density (ρ), and the partial specific volume (v) value of proHD6 at 20 °C as described in the SV section. The molecular weight of proHD6 was determined by global fitting of the multispeed equilibrium data across all loading concentrations at pH 7.4 using SEDPHAT. The Species Analysis model with mass conservation was employed for data analysis. The bottom of the sample sector was assigned as a floating parameter. To further evaluate whether each least-squares curve-fitting procedure converged to a global minimum, the alternate methods of Simplex, Marquardt-Levenberg, and simulated annealing were employed to assess any change in the global reduced chi-squared value.
3.3.23 Circular Dichroism Spectroscopy

Peptide solutions (20 μM, 300 μL) were prepared in 10 mM sodium phosphate buffer, pH 7.4 and transferred to a 1-mm path-length quartz CD cell (Hellma) for all measurements. The CD spectra were collected from 260-190 nm at 1-nm intervals (5 s averaging time, three independent scans per wavelength). The data obtained from the three scans were averaged and the resulting averaged spectra are reported.

3.3.24 Thiol Quantification Assays

The assays were performed according to a literature procedure. Briefly, the peptide stock solutions were freshly prepared in Milli-Q water (for oxidized peptides) or 0.01 M HCl (for reduced peptides). The concentration of each stock solution was verified using the calculated extinction coefficients (Table 3.1). The samples were prepared by diluting an aliquot of each peptide stock solution with Ar-purged assay buffer and adding a 42-μL aliquot of 2,2'-dithiopyridine (DTDP) as previously described. The final peptide concentrations were 2-5 μM or 6-7 μM for the reduced proHD6 and oxidized HD6/proHD6, respectively. The resulting solutions were incubated at room temperature for at least 15 min and the absorbance at 341 nm was recorded. The number of free thiol residues in each peptide sample was determined by using a GSH standard curve. These assays were conducted with at least two independently prepared and purified samples of each peptide and in three independent trials. The resulting averages (±SDM) are reported.
3.4 Results

3.4.1 Identification of HD6 Isoforms in Human Intestinal Tissue and Luminal Fluid

Human Paneth cells express and release two α-defensins, HD5 and HD6.\(^6,7,16,38\) Immunogold transmission electron microscopy (TEM) of human ileal tissue using antibodies that selectively react with either HD5 or HD6 demonstrated that HD5 and HD6 are co-packaged in Paneth cell secretory granules (Figures 3.1b, 3.2a). Because preliminary dot blots revealed that the HD6 antibody (generated to the 32-residue C-terminal peptide\(^{16}\)) reacts with both recombinant proHD6 and mature HD6 (Figure 3.2a), the immunogold labeling did not elucidate which isoform(s) of HD6 was present in the Paneth cell granules, and prior work precludes prediction between these storage forms.

For example, mouse Paneth cell α-defensins contain an acidic propeptide (pI \(\approx\)4.5) and are stored as mature α-defensin peptides in the secretory granules.\(^{25,26}\) Similarly, human α-defensins 1-4 (also termed human neutrophil peptides 1-4, HNP1-4) have acidic propeptides (pI \(\approx\)5.5) and are stored in human neutrophil granules as the mature forms (29-30 amino acids).\(^{20-23}\) In contrast, HD5 has a cationic propeptide (pI \(\approx\)9.5) and is stored in Paneth cell granules as a propeptide.\(^{24}\) Therefore, uncertainty surrounded whether HD6, which has an acidic propeptide (pI \(\approx\)4.5), is stored in human Paneth cell granules as a mature peptide, like mouse Paneth cell α-defensins and human neutrophil α-defensins, or as a propeptide like HD5 despite its significantly different pI.
Figure 3.1. Identification and characterization of HD6 in intestinal tissue and fluid. (a) Left: primary amino acid sequences of proHD6 and HD6. The pro region is in red, the sequence of mature HD6 is in purple, and the disulfide linkages are depicted as black lines. The underlined amino acid sequences are matched with the results from Edman degradation shown in (c) and (d). Right: the crystal structure of mature HD6 (PDB ID: 1ZMQ).12 The disulfide bonds are shown in yellow. (b) Immunogold labeling transmission electron microscopy of human small intestinal tissue. (i) Left panel: low magnification of Paneth cell granules as a negative control where primary antisera was omitted, scale bar = 2 μm. (ii) Center-left panel: immunogold double-labeling of Paneth cell granules for HD5 (5-nm gold particles) and HD6 (15-nm gold particles), scale bar = 500 nm, (iii) Center-right panel: immunogold labeling of a single Paneth cell granule demonstrating co-packaging of HD5 (5-nm gold particles, open arrowheads) and HD6 (15-nm gold particles, filled arrowheads), scale bar = 200 nm. (iv) Right panel: immunogold double-labeling of Paneth cell granules for HD5 (5-nm gold particle, open arrowheads) and trypsin (15-nm gold particles, filled arrowheads), scale bar = 100 nm. (c) and (d) Analysis of HD6 in human small intestinal tissue (c) and luminal fluid (d) by Western blot, mass spectrometry, and N-terminal Edman degradation. Tissue extracts of human ileum and ileal fluid aspirates were resolved by AU-PAGE, transferred onto a PVDF membrane, and probed for HD6. Two separate gels with mobility normalized according to migration of tracking dye to gel bottom are shown and reveal HD6 immunoreactivity in both samples. The data presented in this figure were obtained by Dr. Chu and Dr. Castillo at UC Davis.
Figure 3.2. Characterization of HD6 in ileal fluid. (a) A dot blot showing specificity of the antibodies employed in this work. HD5 (0.14 nmol), HD6 (0.14 nmol), His6-proHD5 (0.16 nmol), and proHD6 (0.16 nmol) in Milli-Q water were spotted directly onto an Immobilon PSQ PVDF membrane. (b) Analysis of ileal lumen fluid aspirate. Ileal lumenal fluid, obtained by endoscopy aspiration, was acidified (20% acetic acid) clarified by centrifugation, and fractionated by analytical C18 RP-HPLC using a gradient of 5–62% acetonitrile over 60 min at 1 mL/min (280 nm absorbance). A 20-μL aliquot of each 1-mL fraction was analyzed for HD6 immunoreactivity by dot blot analysis (inset). A second aliquot (2 μL) was screened by MALDI-TOF MS analysis, to identify masses that potentially corresponded to HD6 isoforms. A single fraction (26, black arrow head) was positive for HD6. Recombinant mature HD6 (residues 69-100, 1 μg) was spotted as a positive control (open arrowhead). (c) MALDI-TOF MS analysis of fraction 26. The data presented in this figure were obtained by Dr. Castillo at UC Davis.

We therefore obtained human ileal tissue from surgical specimens and prepared protein extracts for analysis by Western blot, mass spectrometry, and Edman degradation. Western blot (AU-PAGE, acid urea-polyacrylamide gel electrophoresis) analysis of the protein extracts revealed a single band with HD6 immunoreactivity (Figure 3.1c). MALDI-TOF mass spectrometry revealed a m/z value of 8962.1, which is in agreement with the calculated m/z value of 8960.2 for the oxidized form of proHD6 (Table 3.1). Four rounds of Edman degradation afforded an N-terminal sequence of
EPLQ that is in agreement with the N-terminus of the propeptide predicted from mRNA analysis (Figure 3.1c). Data consistent with these findings were obtained from specimens of three individuals. These results established that HD6 is stored in Paneth cells as proHD6, an 81-residue propeptide, corresponding to residues 20-100 of the deduced preproHD6 sequence (Table 3.2). We found no evidence for the presence of mature HD6 in the tissue samples examined in this study.

Table 3.1. Characterization of peptides and protein employed in this work.

<table>
<thead>
<tr>
<th>Peptide/Protein</th>
<th>Retention Time (min)</th>
<th>Free Thiol</th>
<th>Calculated m/z</th>
<th>Observed m/z</th>
<th>Yield (mg/L culture)</th>
<th>ε²₈₀ (M⁻¹ cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD6</td>
<td>16.9</td>
<td>0.23 ± 0.08</td>
<td>3708.2</td>
<td>3707.4</td>
<td>1.9</td>
<td>4845</td>
</tr>
<tr>
<td>proHD6_red</td>
<td>22.4</td>
<td>6.31 ± 0.46</td>
<td>8966.7</td>
<td>8966.9</td>
<td>0.50</td>
<td>5960</td>
</tr>
<tr>
<td>proHD6</td>
<td>21.6</td>
<td>0.15 ± 0.20</td>
<td>8960.7</td>
<td>8961.2</td>
<td>0.20</td>
<td>6335</td>
</tr>
<tr>
<td>His₆-SUMO-proHD6</td>
<td>n.d.</td>
<td>n.d.</td>
<td>22227</td>
<td>22228</td>
<td>20</td>
<td>7825</td>
</tr>
</tbody>
</table>

See Table 3.2 for amino acid sequences. HD6 and proHD6 are the oxidatively folded peptides with three S-S bonds. Retention times determined by using analytical RP-HPLC on a C18 column and a gradient of 10–60% B over 30 min at 1 mL/min. Free thiol content determined by using the DTDP assay (mean ± SD, n = 3). Extinction coefficients at 280 nm were calculated by using the on-line resource ExPASy ProtParam. n.d. = not determined.

Table 3.2. Amino acid sequences.

<table>
<thead>
<tr>
<th>Peptide/Protein</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>preproHD6</td>
<td>MRTLTILTAVLQQAKAEPGLQAEDDPLQAKAYEADAEQREGANDQDFAVSFEDAASSLRALGSTRAFCHCRRSCYSTEYSYGTCVMGINHRFCCL</td>
</tr>
<tr>
<td>proHD6</td>
<td>EPLQAEDDPLQAKAYEADAQEQRGANDQDFAVSFAEDASSSLRALGSTRAFCHCRRSCYSTEYSYGTCVMGINHRFCCL</td>
</tr>
<tr>
<td>HD6</td>
<td>AFTCHCRRSCYSTEYSYGTCVMGINHRFCCL</td>
</tr>
<tr>
<td>His₆-SUMO-proHD6</td>
<td>GSSHHHHHHHGSGSASMSDSEVNQEAKPEVKEPETHINLKVDGSEIFKFKTPTRRLMFAEAFRQGKEMDSLRFLYDGIRIADQTPEDLDME DNIDIEAHREQQGEGPLQAEDDPLQAKAYEADAQEQRGANDQDFAVSFAEDASSSLRALGSTRAFCHCRRSCYSTEYSYGTCVMGINHRFCCL</td>
</tr>
</tbody>
</table>
Next, to ascertain which isoform(s) of HD6 is present in the small intestinal lumen, we analyzed intestinal luminal aspirates obtained by endoscopy. Western blot (AU-PAGE) of the luminal fluid revealed a single band of HD6 immunoreactivity (Figure 3.1d). We fractionated the luminal fluid by HPLC and screened the resulting fractions using HD6 immunoreactivity (dot blot) and MALDI-TOF mass spectrometry. We detected only one HD6 isoform characterized by a m/z value of 3709.9, which corresponds to the oxidized form of the 32-residue mature peptide (calculated m/z 3708.1 for residues 69-100 of the prepropeptide deduced from mRNA analysis) (Figures 3.1d, 3.2, Table 3.1). This assignment was confirmed by seven rounds of Edman degradation, which afforded the N-terminal sequence AFTCHCR (Figure 3.1d). Moreover, this HD6 isoform was the only one detected by MALDI-TOF mass spectrometry from luminal fluid specimens obtained from a total of six individuals.

A comparison of the N-terminal residues of the luminal HD6 peptide with the deduced cDNA sequence indicated that proteolytic processing of the propeptide occurred on the C-terminal end of Arg68 (Figure 3.1a). In agreement with our hypothesis, this cleavage site is consistent with trypsin-catalyzed hydrolysis of the amide bond linking Arg68 and Ala69. Previous studies reported that trypsin is expressed by human Paneth cells, and demonstrated that trypsin processes proHD5 to release the 32-residue mature form. A definitive experiment to show co-packaging of this protease and either of these α-defensins in Paneth cell granules has not been reported; therefore, we extended our immunogold co-labeling studies of human ileal tissue to include trypsin, and confirmed that human enteric α-defensins and trypsin are co-packaged in the granules (Figure 3.1b). In total, our analyses of HD6 in human ileal tissue and luminal
fluid support a model whereby the peptide is stored in the secretory granules of Paneth cells as an 81-residue propeptide. Either during or after granule release into the lumen, proHD6 is cleaved by trypsin to generate HD6, the mature 32-residue peptide found in the intestinal lumen (Figure 3.3).

3.4.2 Trypsin-Catalyzed Proteolysis of proHD6 Provides Mature HD6

To enable in vitro studies of proHD6, we obtained recombinant proHD6 following expression of His₆-SUMO-proHD6 in *E. coli*. This affinity tag allowed the first recombinant preparation and purification of native 81-residue proHD6. Ulp-1 digestion of His₆-SUMO-proHD6 afforded proHD6 as a mixture of species resulting from peptide oxidation and disulfide bond formation. A sample of the oxidized form, the regioisomer exhibiting the three native α-defensin disulfide linkages (Cys⁴—Cys¹⁹, Cys⁶—Cys¹⁰, Cys¹⁰—Cys³⁰), was obtained following chemical reduction of the peptide to yield the reduced form with six free thiols, oxidative folding, and purification (Figure 3.4, Table 3.1). We conducted antimicrobial activity assays with proHD6 (Figure 3.5) and determined that proHD6 does not exhibit antibacterial activity against *E. coli* ATCC 25922 or *L. monocytogenes* ATCC 19115, two strains employed in this work.
Figure 3.4. Preparation and purification of proHD6. (a) SDS-PAGE (15% Tris-HCl gel) of samples from a representative preparation of proHD6. His<sub>6</sub>-SUMO-proHD6 is 22.2 kDa, His<sub>6</sub>-SUMO is 13.3 kDa, and proHD6 is 8.96 kDa. Lane 1: P7711S pre-stained gel ladder (New England Biolabs). Lane 2: purified His<sub>6</sub>-SUMO-proHD6 from Ni-NTA chromatography. Lane 3: His<sub>6</sub>-SUMO-proHD6 after incubated with Ulp1 (1% m/m) for 2 h. Lane 4: proHD6. (b) Analytical HPLC traces of purified proHD6 (variable sample concentrations). Absorbance at 220 nm was monitored with a reference wavelength of 500 nm. Method: 10-60% B over 30 min at 1 mL/min.

Figure 3.5. Antibacterial activity assays against *E. coli* ATCC 25922 and *L. monocytogenes* ATCC 19115. The bacteria were treated with 50 µM of each peptide for 1 h at 37 °C in 10 mM sodium phosphate buffer containing 1% v/v TSB (mean ± SDM, n = 3). An asterisk indicates no colony formation.
To test our hypothesis that trypsin-catalyzed hydrolysis of proHD6 affords mature HD6, we conducted *in vitro* proteolysis assays (Figure 3.6). Under the conditions of this experiment (1:100 trypsin:proHD6 (w/w) in 100 mM Tris-HCl, 20 mM CaCl$_2$, pH 8.0), trypsin accepted proHD6 as a substrate, and cleavage of the propeptide was observed. Analytical HPLC and LC/MS of the product mixture revealed HD6 as well as a number of peptide fragments corresponding to fragments of the propeptide domain (Figure 3.7).

**Figure 3.6.** *In vitro* trypsin-catalyzed cleavage of proHD6. Analytical HPLC traces of trypsin-treated (a) proHD6 or (b) mature HD6 (30 nM x 80 µL) at indicated time points. The trypsin concentration was 0.01 mg/mL (1:100 trypsin:proHD6 (w/w)). Absorbance at 220 nm was monitored with a reference wavelength of 500 nm. Method: 10–60% B over 30 min at 1 mL/min.
Figure 3.7. Characterization of products from trypsin-catalyzed proteolysis of proHD6. Analytical HPLC trace of proHD6 (30 μM x 80 μL) incubated with 0.4 μM trypsin after 2 h (a 1:100 trypsin:proHD6 mass ratio in 100 mM Tris-HCl, 20 mM CaCl₂, pH 8.0) The starting polypeptide proHD6, which elutes at 21.6 min, was completely hydrolysed. Absorbance at 220 nm was monitored with a reference wavelength of 500 nm. Method: 10–60% B over 30 min at 1 mL/min. The HPLC retention times, m/z values, and amino acid sequences of the four isolated products are summarized in the table. The amino acid sequence for HD6 is given in Figure 3.1 and Table 3.2.

Moreover, these assays confirmed that the mature 32-residue HD6 is resistant to trypsin-catalyzed degradation (Figure 3.6). This observation is consistent with previous studies of other defensins, which demonstrated that the disulfide array confers protease resistance.¹³,¹⁴ These data, coupled with the presence of trypsin and HD6 in Paneth cells, implicate trypsin as the processing enzyme for HD6 maturation; however, we cannot exclude the possibility that other proteases contribute to proHD6 cleavage after secretion.
3.4.3 Trypsin Cleavage of proHD6 Unmasks Latent Bacterial Agglutination Activity

To probe whether proHD6 can self-assemble, we first imaged samples of proHD6 prepared in different buffers by transmission electron microscopy (TEM). The images of proHD6 were indistinguishable from the buffer-only controls, and we observed no evidence for the formation of higher-order oligomers (Figure 3.8). This property sharply contrasts with that of mature HD6, which forms extended fibrils that are >1 μm in length under the same experimental conditions (Figure 3.8). Moreover, when we added trypsin to proHD6 prior to TEM, we observed fibril-like features comparable to those observed for the mature HD6 peptide (Figure 3.8). Time-course experiments revealed that the fibril length increased with longer trypsin treatment (Figure 3.9). After a 5-min incubation with trypsin, relatively short fibrils were observed; however, the lengths of these fibrils were shorter than the HD6 fibrils at the 60-min time point. After 60 min, the fibrils closely resembled those of mature HD6. This experiment indicates that proHD6 cannot self-assemble into higher-order oligomers, but that trypsin-catalyzed hydrolysis of the propeptide affords this functional activity.

Figure 3.8. TEM analysis of HD6 self-assembly. Top row: TEM of 0 mM sodium phosphate pH 7.4 (control), 0.4 μM trypsin (control), 20 μM proHD6 in the absence and presence of 0.4 μM trypsin, and 20 μM HD6. Scale bar = 100 nm. Bottom row: TEM of 10 mM Tris-maleate pH 6.4 (control), 0.4 μM trypsin (control), 20 μM proHD6 in the absence and presence of 0.4 μM trypsin, and 20 μM HD6. Scale bar = 200 nm. All the samples were incubated at room temperature for 1 h.
Next, we investigated the bacterial agglutination properties of proHD6 using scanning electron microscopy (SEM). Previous investigations demonstrated that mature HD6 agglutinates Gram-negative\textsuperscript{16,17} and Gram-positive bacteria.\textsuperscript{17} Treatment of \textit{E. coli} ATCC 25922 or \textit{E. coli} Nissle with proHD6 (3 \textmu M) yielded no distinguishable agglutination. The bacteria appeared comparable to the buffer-only control and nanonets were not observed (Figures 3.10, 3.11). Treatment of proHD6 with trypsin prior to incubation with bacteria resulted in entangled and agglutinated \textit{E. coli} in the SEM images, indistinguishable from the agglutination observed for \textit{E. coli} treated with mature HD6 (Figures 3.10, 3.11). We found that treatment of \textit{S. Typhimurium}, another Gram-negative bacterium, with mature HD6 resulted in entangled and agglutinated bacteria (Figure 3.12), consistent with previous studies.\textsuperscript{16} As observed for \textit{E. coli}, proHD6 did not agglutinate \textit{S. Typhimurium} unless it was treated with trypsin prior to incubation with the bacteria (Figure 3.12). Finally, we observed the same trypsin-independent activity of proHD6 with the Gram-positive bacterium \textit{L. monocytogenes} (Figure 3.13). Thus, the SEM investigations provide evidence that mature HD6 readily agglutinates both Gram-negative and Gram-positive bacteria, whereas proHD6 lacks this activity until it is processed by a protease.
**E. coli ATCC 25922**

**Figure 3.10.** SEM analysis of *E. coli* ATCC 25922 agglutination by trypsin-cleaved proHD6. SEM of *E. coli* ATCC 25922 treated with buffer only, 0.4 μM APMSF-inactivated trypsin, 3 μM proHD6, 3 μM trypsin-cleaved proHD6, or 3 μM HD6 (50 mM Tris-maleate pH 6.4). Trypsin-cleaved proHD6 was prepared prior to incubation with the bacteria and the residual enzymatic activity was inhibited by APMSF as described in the Methods. Scale bar = 100 μm (left column), 20 μm (middle column), and 5 μm (right column). Dotted rectangles indicate field of view shown at higher magnifications. The data presented in this figure were obtained by Dr. Castillo at UC Davis.
Figure 3.11. SEM analysis of E. coli Nissle agglutination by trypsin-cleaved proHD6. SEM of E. coli Nissle treated with buffer only, 0.4 μM APMSF-inactivated trypsin, 3 μM proHD6, 3 μM trypsin-cleaved proHD6, or 3 μM HD6 (50 mM Tris-maleate pH 6.4). Trypsin-cleaved proHD6 was prepared prior to incubation with the bacteria and the residual enzymatic activity was inhibited by APMSF as described in the Methods. Scale bar = 100 μm (left column), 20 μm (middle column), and 5 μm (right column). Dotted rectangles indicate field of view shown at higher magnifications. The data presented in this figure were obtained by Dr. Castillo at UC Davis.
Figure 3.12. SEM analysis of *Salmonella enterica* serovar Typhimurium agglutination by trypsin-cleaved proHD6. SEM of *S. Typhimurium* treated with buffer only, 0.4 μM APMSF-inactivated trypsin, 3 μM proHD6, 3 μM trypsin-cleaved proHD6, or 3 μM HD6 (50 mM Tris-maleate, pH 6.4). Trypsin-cleaved proHD6 was prepared prior to incubation with the bacteria and the residual enzymatic activity was inhibited by APMSF as described in the Methods. Scale bar = 100 μm (left column), 20 μm (middle column), and 5 μm (right column). Dotted rectangles indicate field of view shown at higher magnifications. The data presented in this figure were obtained by Dr. Castillo at UC Davis.
Figure 3.13. SEM analysis of *Listeria* agglutination by trypsin-cleaved proHD6. SEM of *L. monocytogenes* treated with buffer only, 0.4 μM APMSF-inactivated trypsin, 3 μM proHD6, 3 μM trypsin-cleaved proHD6, or 3 μM HD6 (50 mM Tris-maleate pH 6.4). Trypsin-cleaved proHD6 was prepared prior to incubation with the bacteria and the residual enzymatic activity was inhibited by APMSF as described in the Methods. Scale bar = 100 μm (left column), 20 μm (middle column), and 5 μm (right column). Dotted rectangles indicate field of view shown at higher magnifications. The data presented in this figure were obtained by Dr. Castillo at UC Davis.
We previously reported a simple cuvette-based *in vitro* agglutination assay that enables time-dependent monitoring of agglutination of viable bacteria by HD6.\(^{17}\) We employed this assay to further characterize the trypsin-dependent activity of proHD6 on cultured bacterial cells (Figure 3.14a,b). When proHD6 was added to a suspension of either *E. coli* or *L. monocytogenes* (\(10^8\) CFU/mL), the cultures remained homogeneous for the 6-h duration of the assay, even at the highest concentration of proHD6 evaluated (20 \(\mu\)M). In contrast, bacterial agglutination and sedimentation occurred when a combination of proHD6 and trypsin was added to the bacterial cultures (Figure 3.14a,b), with kinetics comparable to those obtained for cultures treated with mature HD6. In these assays, we defined the "supernatant" as the culture solution in the top portion of the cuvette and the "re-suspension" as the mixture that results from agitating the entire culture after the 6-h incubation period.\(^ {17}\) We observed an \(\approx1.5\)-fold log reduction in CFU/mL for the "supernatant" from cultures treated with combination of trypsin and proHD6 relative to the no-peptide control (Figure 3.14c). Following "re-suspension," the total CFU/mL in the cuvette was indistinguishable from that of either the untreated control culture or the bacterial culture treated with proHD6. These results indicated that the reduction of CFU/mL in the "supernatant" for the cultures treated with proHD6 and trypsin was a result of bacterial agglutination and sedimentation rather than bacterial cell death.
Figure 3.14. Bacterial agglutination assays for native and cleaved proHD6. (a) Agglutination of *E. coli* ATCC 25922 and *L. monocytogenes* ATCC 19115 in 50% MHB treated with proHD6 in the absence and presence of 0.4 μM trypsin or HD6 (control). (b) Representative images of cuvettes containing *E. coli* ATCC 25922 or *L. monocytogenes* ATCC 19115 after incubation with 20 μM peptides for 2 h at room temperature. (c) Plots of colony forming units (CFU/mL) of *E. coli* ATCC 25922 and *L. monocytogenes* ATCC 19115 after treatment with 20 μM peptides for 6 h (mean ± SDM, n = 3). Supernatant is defined as the clarified medium solution in the top portion of the culture and resuspension is defined as the mixture after thoroughly agitating the heterogeneous solution.

### 3.4.4 Proteolysis of proHD6 Protects Human Epithelial Cells from Listeria Invasion

Consistent with our recent study, mature HD6 (≥2.5 μM) blocks *L. monocytogenes* invasion into human intestinal epithelial cells (Figure 3.15). In contrast to these findings with mature HD6, proHD6 (≤10 μM) did not impair the ability of *L. monocytogenes* to
invade intestinal epithelial cells (Figure 3.15). However, when proHD6 (≈2.5 μM) was treated with trypsin, the percentage of *L. monocytogenes* invasion decreased from ≈10% to <2%, similar to the decrease observed for equivalent concentrations of mature HD6 (Figure 3.15). Taken together with the agglutination studies, these results indicate that trypsin-catalyzed hydrolysis of proHD6 triggers *L. monocytogenes* entrapment, which prevents this pathogen from invading mammalian cells. In control experiments, nuclear morphology assays indicated that neither proHD6 nor HD6 exert cytotoxic effects on the epithelial cells over the course of these assays (Figure 3.16).

![Graph showing Listeria invasion assays for native and cleaved proHD6.](image)

**Figure 3.15.** *Listeria* invasion assays for native and cleaved proHD6. Invasion of human T84 colon epithelial cells by *L. monocytogenes* ATCC 19115 pre-treated with proHD6 in the absence and presence of 0.4 μM trypsin or HD6 (control). The bacteria (2 x 10⁶ CFU/mL) were incubated with the indicated peptides for 30 min prior to the infection of the T84 cells (mean ± SDM, n = 3).
Figure 3.16. Cytotoxicity studies of proHD6 and mature HD6 against human intestinal epithelial cells (T84 cells). Representative images of T84 cells after incubated with 20 μM peptides (t = 1.5 h, T = 37 °C, 5% CO₂). Excitation wavelength = 360 ± 20 nm. A scale bar = 10 μm.

3.4.5 Structural Determinants of proHD6 Impair Self-Assembly

The in vitro studies of proHD6 function obtained thus far suggest that this isoform is inactive as a result of suppressed self-assembly, leaving unanswered how the propeptide segment interferes with this process. Circular dichroism (CD) spectroscopy provides a fingerprint for mature HD6 oligomers because the CD signature of the HD6 self-assembly is characterized by a negative peak at 192 nm and positive peaks at 205 and 230 nm at pH 7.4 (Figure 3.17).17 Under the same conditions, the CD spectrum of proHD6 markedly differs with a negative peak at 197 nm, resembling the CD signatures of random-coils. Together with the initial TEM studies, these data provide biophysical evidence that the pro region interferes with the self-assembly process characteristic of mature HD6.
We employed analytical ultracentrifugation (AUC) to further evaluate the quaternary structure of proHD6. We determined the sedimentation coefficients of proHD6 prepared in different buffers (Table 3.4). When we prepared proHD6 (≤140 μM) at pH 7.4 in 10 mM sodium phosphate buffer, a single peak at ca. 1.8 S was obtained over the range $s_{20,w} = 0.5–3.5$ S in the Gaussian fits of the $-dc/dt$ distributions obtained using DCDT$^{+35,39}$ (Figure 3.18a). The Gaussian fits support the predominance of a single species of proHD6 over a concentration range of 30–140 μM at pH 7.4. Substitution of sodium phosphate with HEPES or Tris-HCl buffer at pH 7.4 had a negligible effect on the $s$ value for proHD6 (Figure 3.18b, Table 3.4). In contrast to proHD6, attempts to evaluate mature HD6 by AUC were not successful because HD6 sedimented too rapidly even at the lowest speed that AUC can provide (3000 rpm) and coated the AUC cell.$^{17}$ Nevertheless, AUC studies of mature HD6 variants that could not form fibrils observable by TEM yielded $s$ values that confirmed their limited ability to oligomerize.$^{17}$

Figure 3.17. CD spectra of 20 μM proHD6 (red) and 20 μM HD6 (purple) in 10 mM sodium phosphate buffer, pH 7.4.
Figure 3.18. Sedimentation velocity analysis of proHD6 under different conditions. (a) Analytical ultracentrifugation of proHD6 at concentrations ranging from 30 µM to 140 µM in 10 mM sodium phosphate buffer, pH 7.4. (b) Analytical ultracentrifugation of 50 µM proHD6 in 10 mM sodium phosphate pH 7.4 (red dots), HEPES pH 7.4 (blue dots), and Tris-HCl pH 7.4 (green dots). The colored dots are the −dc/dt data obtained from sedimentation velocity experiments (absorbance at 280 nm). The black lines are the single Gaussian fits obtained using DCDT+. The summary of the fits is provided in Table 3.4.

Table 3.3. Sedimentation coefficient calculations using the Svedberg-Stokes equation.\(^{34}\)

<table>
<thead>
<tr>
<th>Species</th>
<th>(S_{\text{sphere}}(S))^a</th>
<th>(S_{\text{sphere}}(S))^b</th>
<th>(S_{\text{sphere}}(S))^c</th>
<th>Partial Specific Volume (mL/g)</th>
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<tr>
<td>proHD6 monomer</td>
<td>1.743</td>
<td>1.746</td>
<td>1.748</td>
<td>0.7011</td>
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<td>proHD6 dimer</td>
<td>2.767</td>
<td>2.771</td>
<td>2.775</td>
<td>0.7011</td>
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</table>

\(^{a}\) Buffer conditions: 10 mM sodium phosphate buffer, pH 7.4, of which a solvent density (\(\rho\)) of 0.99967 g/mL and a solvent viscosity (\(\eta\)) of 1.0061 cP at 20 °C. Viscosity units are in centipoise (cP) (1 poise = 1 g·cm⁻¹·s⁻¹). Sedimentation coefficients are in svedbergs (1 svedberg = 100 fs = 1 x 10⁻¹⁵ s). \(^{b}\) Buffer conditions: 10 mM HEPES, pH 7.4, of which a solvent density (\(\rho\)) of 0.99901 g/mL, and a solvent viscosity (\(\eta\)) of 1.0104 cP at 20 °C. \(^{c}\) Buffer conditions: 10 mM Tris-HCl, pH 7.4, of which a solvent density (\(\rho\)) of 0.99851 g/mL, and a solvent viscosity (\(\eta\)) of 1.0037 cP at 20 °C. The solvent densities, viscosities, and the partial specific volume values of proHD6 monomer and dimer were predicted by SEDNTERP.
Table 3.4. Calculated sedimentation coefficients of proHD6.

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Buffer (10 µM)</th>
<th>pH</th>
<th>$s_{20,w}$ (S)</th>
<th>$D$ (F)</th>
<th>MW (kDa)</th>
<th>Partial Specific Volume (mL/g)</th>
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<tr>
<td>30</td>
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<td>1.727</td>
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<td>1.798</td>
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<td>12.8</td>
<td>0.7011</td>
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<tr>
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<td>NaP</td>
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<td>1.733</td>
<td>9.73</td>
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<td>1.844</td>
<td>10.4</td>
<td>14.4</td>
<td>0.7011</td>
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<tr>
<td>50</td>
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<td>1.865</td>
<td>10.3</td>
<td>14.8</td>
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<td>1.843</td>
<td>13.8</td>
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<td>9.94</td>
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</tr>
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<td>1.783</td>
<td>8.97</td>
<td>16.1</td>
<td>0.7011</td>
</tr>
</tbody>
</table>

All samples were prepared in the indicated buffer; NaP is sodium phosphate. The temperature was 20 °C. Data were obtained by analysis with the dc/dt method implemented in DCDT+ using 22-28 scans with 40 kDa diffusion broadening maximum. Sedimentation coefficients are $s_{20,w}$ values, adjusted with density and viscosity of the indicated solvent. NaP has a solvent density ($\rho$) of 0.99967 g/mL and a solvent viscosity ($\eta$) of 1.0061 cP. HEPES has a solvent density ($\rho$) of 0.99901 g/mL and a solvent viscosity ($\eta$) of 1.0104 cP at 20 °C. Tris-HCl has a solvent density ($\rho$) of 0.99851 g/mL and a solvent viscosity ($\eta$) of 1.0037 cP at 20 °C. Viscosity units are in centipoise (cP) (1 poise = 1 g•cm⁻¹•s⁻¹). Sedimentation coefficients are in svedbergs (1 svedberg = 100 fs = $1 \times 10^{-13}$ s). Diffusion coefficients correspond to the best-fit molecular mass in Fick units (1 Fick = $1 \times 10^{-7}$ cm²/s). The solvent densities, viscosities, and the partial specific volume values of proHD6 monomer and dimer were predicted by SEDNTERP.

Because this work includes the first isolation and biophysical evaluation of proHD6, there is no proHD6 crystal or solution structure available that can be used to calculate sedimentation coefficients. We therefore estimated the sedimentation coefficients of proHD6 (see Experimental Section) under the assumption that it behaves as a smooth, compact, spherical peptide in water at 20 °C, which afforded values of 1.7 S (monomer) and 2.8 S (dimer). A caveat to this analysis is that the 49-residue N-terminal pro domain of proHD6 is likely dynamic, and it is unlikely that proHD6 behaves as a spherical peptide in aqueous solution. Nevertheless, comparison of the
experimental and estimated s values suggests that proHD6 exists in monomer-dimer equilibrium with rapid association under these experimental conditions. We also conducted sedimentation equilibrium (SE) experiments to determine the molecular weight of proHD6 in solution (10 mM sodium phosphate, pH 7.4). Using SEDPHAT\textsuperscript{37} for a global analysis of the data, we obtained a molecular weight of 15549 Da with a standard deviation of ±230 Da. At the 95% confidence level, the interval ranged from 15059 Da to 15946 Da using a Monte Carlo analysis of the fit (Figure 3.19, Table 3.5). The molecular weights of a proHD6 monomer and dimer are 8961 and 17922 Da, respectively. Thus, the experimental molecular weight of 15549 Da indicates that the predominant proHD6 species under these conditions is a dimer and that monomer-dimer equilibrium occurs. Taken together, these data are consistent with a model where proHD6 monomers can reversibly associate to form dimers, whereas mature HD6 readily self-assembles to form higher-order complexes. Proteolysis therefore serves as a biochemical switch to induce these pivotal biophysical characteristics.

**Figure 3.19.** Sedimentation equilibrium analysis of proHD6. Representative sedimentation equilibrium profiles of proHD6 in 10 mM sodium phosphate buffer, pH 7.4 at high (a), medium (b), and low (c) concentrations. Best fits (black lines) of raw UV absorbance at 280 nm at rotor speeds of 30000 (red), 36000 (blue), and 42000 (green) rpm. The fits and calculated molecular weights are summarized in Table 3.5.
3.5 Discussion

In this work, we combined evaluation of HD6 localization and characterization in human intestinal specimens with in vitro functional studies and biophysical characterization to provide a model for the storage and maturation of this Paneth cell defensin. HD6 is an unusual defensin that lacks the typical broad-spectrum in vitro bactericidal activity of other defensins. It contributes to mucosal immunity by self-assembling into oligomeric structures, called nanonets, that entrap bacterial pathogens and block their invasion both in vitro and in vivo.\textsuperscript{16,17} This study illuminates a number of important facets pertaining to how HD6 is stored in the granules of Paneth cells and the mechanism of HD6 self-assembly in the intestinal lumen. First, our characterization of human ileal tissue demonstrates the co-packaging of HD5, HD6, and trypsin within the secretory granules of Paneth cells. Our analyses of ileal tissue protein extracts and luminal fluid enabled us to detect two distinct isoforms of HD6, the 81-residue propeptide that was only detected in the tissue samples and the 32-residue mature form that was only found in the luminal fluid. The N-terminal sequence of the mature HD6 peptide is consistent with cleavage of proHD6 by trypsin. On the basis of these observations and the primary sequence of proHD6, we proposed and tested a model whereby proHD6 is proteolytically cleaved to liberate mature HD6, which triggers its self-assembly in the gut. Our in vitro investigations show that trypsin-catalyzed hydrolysis of proHD6 generates the 32-residue defensin peptide and that this mature defensin is stable to further proteolysis. Moreover, our bacterial agglutination assays and biophysical characterization of proHD6 further inform this model by demonstrating that proHD6 cannot self-assemble into high-order oligomers or cause bacterial entrapment. Our work
thus establishes that Paneth cells package HD6 in an inactive form and that proteolysis triggers its innate immune activity in the intestine. This process provides spatial and temporal control of peptide self-assembly and thereby unveils the protective properties of this molecule at the intestinal mucosa.

The observed co-packing of defensin propeptides and trypsin in human Paneth cell granules indicates that trypsin is inactive at this biological site. The mechanism underlying this lack of trypsin activity is currently unknown and a topic for future investigation. Spatial and temporal control of protease activity can be achieved by alterations in local pH or calcium ion concentration, the presence of protease inhibitors, and storage as a zymogen. Along these lines, human Paneth cells produce α₁-antitrypsin, and this protein may inhibit trypsin packaged in the granules. We also reason that trypsin might be stored in the granules as a zymogen, trypsinogen, and activated by enterokinase or an enterokinase-like enzyme following release into the lumen. This model is based on the known processing of trypsinogen produced by pancreatic exocrine cells in the human intestine.

Whereas most α-defensins crystallize as dimers, the crystal structure of 32-residue HD6 revealed that a hydrophobic pocket occurs between four HD6 monomers (Figure 2.3). In each hydrophobic pocket, two monomers each contribute Phe2, Phe29 and Leu32, and the two other monomers each contribute Val22, Met23, and Ile25. The N- and C-termini of 32-residue HD6 are within close proximity, and residues at both the N- and C-terminal ends are constituents of this hydrophobic pocket. On the basis of the analytical ultracentrifugation studies presented here, the N-terminal pro-region prevents formation of this hydrophobic pocket and thereby self-assembly to
higher-order oligomers. Elucidating whether the pro-region merely provides a steric block to self-assembly or whether specific amino acid side-chains in the pro-region are needed for prevent HD6 oligomerization requires further biophysical investigations.

Our understanding of the roles of defensin propeptides and defensin maturation is limited, and the insights from the current work may also be considered in the context of reported observations about the proteolytic processing and maturation of other α-defensins.24-26,41,42 Although conversion from a propeptide to a mature form is common amongst α-defensins, the pro-regions of α-defensins exhibit variable primary sequences, and the maturation pathways and proteases involved in propeptide processing are oftentimes unknown. On the basis of characterized systems, some defensins are packaged as propeptides whereas others are stored in the mature forms, making it necessary to examine speciation on a case-by-case basis. A comparison of the human α-defensins illustrates this point. Previous studies24,43 and the current work demonstrate that the enteric α-defensins HD5 and HD6 are stored in Paneth cell granules as propeptides. In contrast, the human neutrophil peptides (HNP1-4) are stored in neutrophil granules as the mature, ≈30-residue peptides.20-22 The HD5 propeptide is highly cationic (pl ≈ 9.5) whereas the HD6 and HNP propeptides are anionic (pl ≈ 4.5 for HD6; pl ≈ 5.5 for HNPs). These comparisons indicate that the amino acid composition of the propeptide region does not provide a reliable predictor of whether a given peptide will be processed before or after packaging into granules, at least based on our current understanding. Moreover, prior studies of human ileal fluid detected several different isoforms of HD5 where the N-terminal proregion was truncated at different positions as a result of proteolytic processing at different sites, and all of these species displayed
antibacterial activity. This observation contrasts with the current results for HD6, where homogeneous immunoreactivity was observed by AU-PAGE and only the 32-residue mature HD6 was detected in ileal fluid. Lastly, mice also harbor enteric α-defensins that are packaged in Paneth cell granules. Like human α-defensins, the murine α-defensins are translated as propeptides. In contrast to HD5 and HD6, the murine α-defensins are packaged in Paneth cell granules as the mature peptide and matrix metalloproteinase 7 has been implicated as the processing enzyme. Taken together, nature has deployed a variety of strategies for defensin maturation even within a particular class for reasons that are as-yet undetermined and warrant exploration in future work.

At mucosal surfaces throughout the body, numerous host peptides and proteins boost barrier effectiveness of the epithelia. By inhibiting bacterial invasion, HD6 contributes to barrier function of the innate immune system at the intestinal mucosa, augmenting the contributions of other key biomolecules at this surface such as mucus and lectins. The adaptive immune system also contributes to barrier function by using immunoglobulin A, which is abundant in the intestine and other mucosal surfaces, to block bacterial invasion. Thus, the combined invasion-inhibiting activities of the innate and adaptive immune systems fortify the integrity of the intestinal barrier to mediate intestinal homeostasis. Further investigations are required to elucidate additional mechanistic details pertaining to how HD6 performs its host-defense function. In particular, how HD6 function integrates with other host-defense molecules deployed at the intestinal mucosa requires investigation, and whether HD6 affects the population dynamics of human gut commensal organisms under normal or pathological conditions.
is another important avenue for future work.

In closing, propeptides are employed as inactive precursors to suppress the biological functions of various peptides and proteins, and proteases are enlisted to unleash the activity of the mature isoforms on demand. The N-terminal extension from mature HD6 is a fascinating variation on this general theme, which allows for suppression of peptide self-assembly and storage of HD6 in Paneth cells as an inactive form. The protease-triggered self-assembly of HD6 is reminiscent of neuropeptides that form of higher-order oligomers following a protease cleavage event, including the prion protein and Aβ(1-42). In such cases, oligomer formation is associated with disease. Protease-triggered self-assembly of HD6, in contrast, affords a beneficial outcome to intestinal homeostasis by spatial and temporal control of its host-defense function entrapping bacterial invaders.

3.6 Acknowledgements

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3.7 References


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Chapter 4

Human Defensin 6 Suppresses

*Candida albicans* Virulence Traits
4.1 Introduction

4.1.1 Candida albicans is an Opportunistic Fungal Pathogen

Fungal infections have become a threat in modern medical care with the increase in the population of immunocompromised patients. Of more than 1.5 million known fungal species, approximately 600 species are human pathogens. These fungi can cause relatively mild infections of the skin (e.g. dermatophytes and Malassezia species), severe cutaneous infections (e.g. Sporotrix schenckii), and life-threatening systemic infections (e.g. Aspergillus fumigatus, Cryptococcus neoformans, Histoplasma capsulatum, and Candida albicans). Candida spp are the fourth most common cause of hospital-acquired systemic infections in the United States with a mortality rate of up to 50%. C. albicans is one of the most studied among the opportunistic fungal pathogens. It constitutes a part of the normal flora in healthy individuals and is usually confined to the skin and mucosal surfaces of the oral cavity, gastrointestinal tract, urogenital tract, and vagina. C. albicans can cause superficial infections in humans, such as oral, skin, and vaginal candidiasis. Oral candidiasis is extremely common among certain immunocompromised patients and it is a sentinel indicator for HIV disease progression before the appearance of more severe symptoms; however, superficial Candida infections are typically non-lethal. In contrast, systemic candidiasis is associated with a high mortality rate, even after treatment with antifungal drugs. Both neutropenia and damage of the gastrointestinal mucosa are risk factors for developing systemic infections. Moreover, central venous catheters, which allow fungi to directly access to the bloodstream, and the use of broad-spectrum antibiotics, which enable fungal overgrowth, also cause systemic candidiasis.
During both superficial and systemic infections, *C. albicans* employs a set of virulence factors and fitness traits. The morphological transition between yeast and hyphal forms,\(^8,13\) the expression of adhesins\(^14,15\) and invasins\(^16\) on the cell surface, thigmotropism (a movement in which an organism moves or grows in response to contact stimuli), the formation of biofilms, phenotypic switching, and the secretion of hydrolytic enzymes are virulence factors.\(^17\) The fitness traits include rapid adaptation to fluctuations in environmental pH, metabolic flexibility, robust nutrient acquisition systems, and stress response machineries.\(^18\) Some of virulence factors and fitness traits are discussed below.

**Polymorphism.** *C. albicans* is a polymorphic fungus that can grow either as ovoid-shaped budding yeast, as elongated ellipsoid pseudohyphal cells with constrictions at the septa, or as parallel-walled true hyphae.\(^13,19\) In addition, it can display white and opaque cells, formed during switching, and chlamydospores, which are thick-walled spore-like structures.\(^13\) Yeast and hyphae are regularly observed during infection and have defined properties, whereas the role of pseudohyphae and switching *in vivo* is not well understood and chlamydospores have not been observed in patients.\(^20,21\) A number of conditions, including starvation, the presence of serum or N-acetylglucosamine, and physiological temperature and CO\(_2\) promote hyphal formation.\(^8\) Quorum sensing is also shown to regulate morphogenesis of *C. albicans*,\(^22\) which mainly uses farnesol, tyrosol, and dodecanol.\(^23-25\) High cell densities favor yeast growth, whereas low cell densities promote hyphal formation. The hyphal form is more invasive than the yeast form.\(^19\) however, the yeast cells are primarily involved in dissemination.\(^26\) Generally, mutant
strains that cannot form hyphae under *in vitro* conditions are attenuated in their virulence.²⁷

**Adhesion and Invasion.** The adhesion to host cells and tissues is the initial and critical step in microbial infections. In candidiasis, *C. albicans* first colonizes and proliferates on the mucosa followed by invasion, dissemination, and tissue damage (*Figure 4.1*).²⁸ *C. albicans* employs a set of proteins called adhesins to mediate adhesion to other *Candida* cells, abiotic surfaces, and host cells. Adhesins are the agglutinin-like sequence (Als) proteins, Als1-7 and Als9. *ALS* genes encode glycosylphosphatidylinositol (GPI)-linked cell surface glycoproteins. Of the eight Als proteins, Als3 is highly important for adhesion.²⁹ *ALS3* gene expression is upregulated during infection of oral epithelial cells *in vitro* and vaginal infection *in vivo*.³¹ Another major adhesin of *C. albicans* is Hwp1, which is a hypha-associated GPI-linked protein. Hwp1 is a substrate for mammalian transglutaminases for covalently linking *C. albicans* hyphae to host cells.¹⁴ An *HWP1*⁺⁻ strain exhibited reduced adhesion to epithelial cells and displayed attenuated virulence in a mouse model of systemic candidiasis.¹⁴,³² Als3 and Hwp1 also contribute to biofilm formation by acting as complementary adhesins.³³
Figure 4.1. C. albicans invasion into host cells and tissues. The critical steps are adhesion to the epithelium, penetration and invasion by hyphae, vascular dissemination, which involves hyphal penetration of blood vessels and seeding of yeast cells into the bloodstream, and finally endothelial colonization and penetration during disseminated disease.

C. albicans uses two distinct mechanisms to invade into host cells: induced endocytosis and active penetration. For induced endocytosis, C. albicans expresses and utilizes specialized proteins on the cell surface called invasins (mainly Als3 and Ssa1) to mediate binding to host ligands, resulting in engulfment of the fungal cell into the host cell. Even dead hyphae are engulfed, indicating that such a process is passive and does not require the activities of viable fungal cells. Active penetration, in contrast, requires viable C. albicans; however, the mechanism is not clearly defined. Fungal adhesion and physical forces are thought to be essential for this second mode of Candida invasion.

Biofilm formation. Another important virulence factor of C. albicans is its ability to form biofilms on abiotic (catheters and dentures) and mucosa surfaces. Biofilms form
is a sequential process (Figure 4.2), which involves adhesion of yeast cells to the surface, proliferation of these cells, formation of hyphal cells, accumulation of extracellular matrix material, and dispersion of yeast cells from the mature biofilm. Mature biofilms confer the fungus resistance to antimicrobial substances and host immune factors. Several transcription factors, including Bcr1, Tec1, and Efg1, control biofilm formation. More recently, Nobile and coworkers identified additional key regulators of biofilm formation, which are Ndt80, Rob1, and Brg1. Deletion of any of these regulators resulted in defective biofilm formation in rat models. Contact sensing is found to be the key property that triggers invasion into the epithelium and biofilm formation of C. albicans. Upon contact to a surface, yeast cells switch to the hyphal form, leading to either invasion into host cells or biofilm formation.

![Candida albicans Biofilm Cycle](image)

**Figure 4.2.** C. albicans biofilm process involving adhesion to surfaces, formation of hyphal cells, accumulation of extracellular matrix material, and dispersion of yeast cells from the mature biofilm.

**Metal acquisition.** Trace metals are required for the growth of all living organisms, including fungi. To date, iron and its effect on C. albicans pathogenesis are the most
thoroughly studied among the transition metals. *C. albicans* acquires iron via several strategies: a reductive system, a siderophore uptake system, and a heme-iron uptake system.\(^{43}\) The reductive system involves the extracellular reduction prior to uptake of ferric salts, chelates, and proteins, such as host ferritin and transferrin by a ferrireductase.\(^{44}\) The resulting ferrous ion is imported via a high-affinity, ferrous-specific, permease system (Ftr). The adhesin and invasin Als3 was found to be the receptor for ferritin.\(^{45}\) Although *C. albicans* does not synthesize its own siderophores, the fungus expresses and utilizes a siderophore transporter named Sit1\(^{46}\) to uptake xenosiderophores, siderophores produced by other microorganisms. Deletion of *SIT1* in *C. albicans* impaired its capacity to damage *ex vivo* human keratinocyte tissue; however, the mutant still remained virulent in a mouse model of disseminated candidiasis.\(^{46}\) Lastly, the heme-iron uptake machinery mediates iron acquisition from hemoglobin and heme-proteins and is regulated by the heme-receptor genes, including *RB1, RBT5, CSA1, CSA2,* and *PGA7* (also known as *RBT6*).\(^{47}\)

Zinc is also essential for *C. albicans* growth and regulation of protein expression. The zinc cluster proteins are a family of zinc transcription factors that are abundant in fungi. They control the expression of virulence factors and play key roles in the development of antifungal drug resistance.\(^{48}\) *C. albicans* secretes the zinc-binding protein Pra1 (*pH*-regulated antigen 1), which acts as a zincophore by binding extracellular zinc.\(^{49}\) Re-association of Pra1 is mediated by the zinc transporter Zrt1.\(^{49}\) Although deletion of *PRA1* enhanced virulence of the mutant in mice, the mutant displayed significantly reduced capacity of *C. albicans* to damage endothelial cells *in vitro* in the absence of extracellular zinc, indicating that zinc acquisition plays an
important role only during certain steps of infection. Currently, the mechanisms by which *C. albicans* acquires manganese and copper are not clearly understood. Ccc1\(^{50}\) and Ctr1,\(^{51}\) which are transporters for manganese and copper, respectively, have been identified; however, their roles in virulence have not yet been investigated.

Classical antifungal drugs, such as amphotericin B and fluconazole, were designed to exert fungicidal properties, but they also exhibit strong adverse effects in patients. As a result, targeting specific virulence factors has become a new and promising antifungal strategy.\(^{52}\) Several virulence factors, including dimorphism and the expression of adhesins and invasins, are promising candidates for targets. The interplay between host-defense responses and fungi is another avenue that needs to be further explored to gain more insights on how the host suppresses *C. albicans* virulence factors at the mucosa, which may lead to development of novel therapeutic and diagnostic strategies.

### 4.1.2 HD6 and *C. albicans* Virulence Traits

Based on the ability of HD6 to block bacterial invasion as discussed in Chapter 2 and 3,\(^{53-55}\) we questioned whether HD6 is also able to prevent fungal invasion. Moreover, we questioned whether HD6 can suppress other fungal virulence traits, such as biofilm formation, which relies on initial cell adhesion to surfaces. Herein, we investigate the effect of HD6 on virulence factors of *C. albicans* by employing a combination of *in vitro* adhesion assays to human epithelial cells, *in vitro* biofilm assays, gene transcription profiling, and morphological studies by scanning electron microscopy and light microscopy. We hypothesized that HD6 may interact with the membrane of *C. albicans*
and hence prevent surface attachment of *C. albicans*, which is the first step in both invasion into the intestinal epithelial cells (Figure 4.1) and biofilm development (Figure 4.2). Our work demonstrates that HD6 prevents *C. albicans* adhesion, resulting in its decreased invasion into T84 epithelial cells and its reduced biofilm formation. We propose that HD6 contributes to host defense against *C. albicans* by suppressing virulence traits. This strategy may be a way of controlling *C. albicans* to live as a harmless commensal organism in the human gut.

4.2 Experimental Section

4.2.1 General Materials and Methods

All solvents, reagents, and chemicals were purchased from commercial suppliers and used as received unless noted otherwise. Native HD6 and F2A-HD6 were prepared and purified as previously described.\(^{53}\) All buffers, aqueous solutions, and peptide/oligonucleotide stock solutions were prepared in Milli-Q water (18.2 MΩ•cm) after it was passed through a 0.22-μm filter. Oligonucleotide primers were synthesized by Integrated DNA Technologies and used as received (standard desalting protocol). A Biorad MyCycler thermocycler was employed for all polymerase chain reactions (PCR).

4.2.2 Instrumentation

An Agilent 1200 series instrument equipped with a thermostatted column compartment set at 20 °C, and a multi-wavelength detector set at 220 and 280 nm (500 nm reference wavelength with 100 nm bandwidth), was used to perform analytical and semi-preparative high-performance liquid chromatography (HPLC). An Agilent PrepStar 218...
instrument outfitted with an Agilent ProStar 325 dual-wavelength detector set at 220 and 280 nm was used to perform preparative HPLC. A Clipeus C18 column (5-µm pore, 4.6 x 250 mm, Higgins Analytical, Inc.) set at a flow rate of 1 mL/min was employed for all analytical HPLC experiments. A ZORBAX C18 column (5-µm pore, 9.4 x 250 mm, Agilent Technologies) set at a flow rate of 5 mL/min was employed for all semi-preparative HPLC purification. A Luna 100 Å C18 LC column (10-µm pore, 21.2 x 250 mm, Phenomenex) set at a flow rate of 10 mL/min was used for all preparative HPLC purification. HPLC-grade acetonitrile (MeCN) and trifluoroacetic acid (TFA) were purchased from EMD and Alfa Aesar, respectively. For all HPLC runs, solvent A was 0.1% TFA/H₂O and solvent B was 0.1% TFA/MeCN.

An Agilent 1260 series LC system equipped with an Agilent 6230 TOF system housing an Agilent Jetstream ESI source was employed to perform high-resolution mass spectrometry. A Poroshell 120 EC-C18 column (2.7-µm particle size, 2.1 x 100 mm, Agilent Technologies) set at a flow rate of 0.4 mL/min was used for all LC-MS analyses. The samples were analyzed by using a solvent gradient of 5–95% B over 5 min with 0.1% formic acid/H₂O as solvent A, and 0.1% formic acid/MeCN (LC-MS grade, Sigma-Aldrich). The MS profiles were analyzed and deconvoluted by using Agilent Technologies Quantitative Analysis 2009 software version B.03.02.

All routine optical absorption measurements were performed by using a Beckman Coulter DU 800 UV-visible spectrophotometer maintained at ambient temperature. Extinction coefficients (280 nm) were calculated by using ExPASy ProtParam. The calculated extinction coefficients of native HD6 and F2A-HD6 both are 4845 M⁻¹cm⁻¹. Peptide stock solutions were routinely prepared in Milli-Q water and concentrations
were quantified by using the calculated extinction coefficients. Solution and buffer pH values were verified by using a Mettler Toledo S20 SevenEasy pH meter or a HANNA Instruments HI 9124 pH meter equipped with a microelectrode.

4.2.3 Strains and Growth Medium

The *Candida albicans* strains used in this study are listed in Table 4.1. Fungi from freezer stocks were streaked on Yeast extract-peptone-dextrose (YPD) agar plates (1% yeast extract, 2% peptone, 2% dextrose, and 2% agar) and incubated at 30 °C for at least 20 h. Single colonies were selected and grown in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) to saturation with shaking (30 °C, 175 rpm, 18-20 h) prior to each experiment. Roswell Park Memorial Institute (RPMI) medium was prepared by dissolving 10 g RPMI (Life Technologies) in 950 mL H₂O, and to this solution was added 30.3 g of 3-(N-morpholino)propanesulfonic acid (MOPS) to achieve a final concentration of 0.145 M. The pH was adjusted to 7.2 by using 4 M NaOH, and the final volume adjusted to 1 L with Milli-Q water. The RPMI medium was then sterile-filtered.

### Table 4.1. *C. albicans* strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC5314</td>
<td>Wild-type (clinical isolate)</td>
<td>Prof. S. Lindquist</td>
</tr>
<tr>
<td>SC5314-BFP</td>
<td>Wild-type expressing blue-fluorescent protein</td>
<td>Prof. S. Lindquist</td>
</tr>
<tr>
<td>16s1</td>
<td>Derived from SC5314 and one allele of <em>YWP1</em> is knocked out</td>
<td>Prof. B. Granger⁵⁶</td>
</tr>
<tr>
<td>4L1</td>
<td>Derived from SC5314 and both alleles of <em>YWP1</em> are knocked out</td>
<td>Prof. B. Granger⁵⁶</td>
</tr>
</tbody>
</table>
4.2.4 Stationary and Log-phase Antimicrobial Activity Assays (AMA)

For an overnight culture, a colony of *C. albicans* SC5314 was picked from the plate and grown in 20 mL of YPD in a 250-mL baffled flask with shaking (30 °C, 175 rpm, 18 – 20 h). For a log-phase culture, 400 μL of the overnight culture was added into 20 mL of fresh YPD medium (1:50 dilution) in a 250-mL baffled flask and the diluted culture was incubated at 37 °C with shaking until cells were at log-phase growth (OD_{600} ≈1.0). A 5-mL portion of the overnight culture at stationary phase or diluted culture at log-phase was centrifuged (3,600 rpm, 5 min, 4 °C) to pellet the cells. The supernatant was decanted and the cells were washed with 5 mL AMA buffer (10 mM sodium phosphate buffer, pH 7.2 supplemented with 1% YPD) and centrifuged (3,600 rpm, 5 min, 4 °C). The cells were resuspended in AMA buffer to obtain OD_{600} ≈2.0 for stationary-phase cultures or ≈1.0 for log-phase cultures. Another 1:250 dilution (stationary phase) or 1:40 (log-phase) of these cell suspensions were made with AMA buffer in three (1:10 x 1:5 x 1:5) and two (1:10 x 1:4) steps, respectively to obtain 10^6 CFU/mL as the initial inoculum. The assays were conducted in 96-well polystyrene plates. To each well was added 10 μL of a 10x concentrated aqueous peptide solution (200 μM), amphotericin B (AmpB, 100 μM in Milli-Q water, Enzo Life Sciences), or sterile Milli-Q water as an untreated control. A 90-μL aliquot of the diluted culture was added to each well and the plate was incubated for 1 h (37 °C, 150 rpm). The solutions were then serially diluted, and 10-μL drops were spotted on YPD-agar plates. These plates were incubated for 24 h at 37 °C. Colonies were counted and colony forming units/mL (CFU/mL) were calculated. These assays were performed with at least two independently prepared and
purified samples of each peptide and in three independent trials. The resulting averages and standard deviations are reported.

4.2.5 Candida Growth Studies in the Presence of HD6

An overnight culture of *C. albicans* SC5314 in YPD was prepared as described in the AMA section (*vide supra*) and the cultures were diluted 1:250 in three steps (1:10 × 1:5 × 1:5) in YPD. To each well of a 96-well plate (polystyrene, Corning) was added 10 μL of a 10x concentrated aqueous peptide solution (200 μM), amphotericin B (AmpB, 100 μM, Enzo Life Sciences), or sterile Milli-Q water as a no-peptide control. A 90-μL aliquot of the diluted fungal culture was added to each well and the plate was incubated with shaking (30 °C, 150 rpm). The absorbance at 600 nm (OD600) was recorded at 0, 2, 4, 6, 8, 10, and 24 h using a plate reader. These assays were conducted with at least two independently prepared and purified samples of each peptide and in three independent trials. The resulting averages and standard deviations are reported.

4.2.6 Candida Adhesion Assays

The adhesion assays were conducted following a literature protocol with modification. C. albicans adhesion to human colon epithelial T84 cells (ATCC CCL-248) was determined by using fluorescence microscopy. A colony of *C. albicans* SC5314-BFP (Table 4.1) were grown in 20 mL of YPD in a 250-mL baffled flask with shaking (30 °C, 175 rpm, 18–20 h). Then, 5 mL of the overnight culture was centrifuged (3,600 rpm, 5 min, 4 °C), washed with 5 mL of RPMI, and resuspended in fresh RPMI. The resulting suspension was then diluted 1:750 in four steps (1:10 × 1:5 × 1:5 × 1:3)
with fresh RPMI. A 290-µL aliquot of the diluted culture was immediately added to 10 µL of a 30x concentrated aqueous peptide solution (150, 300, or 600 µM) or sterile Milli-Q water as a no-peptide control and incubated at room temperature for 15 min.

T84 cells were routinely cultured in 1:1 Dulbecco’s modified Eagle medium (DMEM) and Ham’s F12 medium containing 2.5 mM L-glutamine, 15 mM HEPES, 0.5 mM sodium pyruvate, and 1.2 g/L sodium bicarbonate, and supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The detailed protocol for passing T84 cells is previously reported.53 A 500-µL aliquot of T84 cells (6 x 10⁵ cells/mL) was added to 24-well Costar tissue culture plates (Corning), each well of which contains a sterile 12.7-mm glass cover slip. The cells were incubated on the cover slips at 37 °C and 5% CO₂ for 24 h. Next, the T84 cells were washed twice with 500 µL of PBS and incubated with 300 µL of peptide-treated C. albicans in RPMI (the number of inoculum is ≈1 x 10⁵ CFU/mL) at 37 °C and 5% CO₂. After 2 or 4 h of incubation, the medium was removed and the T84 cells were washed three times with 500 µL of PBS to remove any non-adhered C. albicans and fixed for 10 min with 500 µL of PBS containing 4% paraformaldehyde and 4% sucrose. The cells were subsequently washed with PBS (2 x 500 µL) and bathed in 500 µL of PBS containing 20 µM SYTO 9 green fluorescent nucleic stain (S-34854, Thermo Fisher) at room temperature for 15 min. The stain solution was then removed. The cells were washed with 500 µL of PBS, bathed in PBS, and mounted onto glass slides.

The samples were examined using Zeiss AxioPlan2 upright microscope (W.M. Keck Microscopy Facility, Whitehead Institute, Cambridge, MA). DAPI Zeiss (filter 49) and GFP Chroma filter were used for blue and green channel, respectively. The number
of adhered *C. albicans* cells under each condition was counted from 10 different images, each of which contain ≈200 – 300 T84 cells. The percentage of adhesion represents the ratio of the number of adhered fungi per 100 T84 cells treated with peptide to the number of adhered fungi per 100 T84 cells of the untreated control after 4-h incubation. All adhesion assays were conducted with at least two independently prepared and purified samples of each peptide and in three independent trials and the resulting averages with standard deviations are reported.

### 4.2.7 Candida Biofilm Assays in the Presence of HD6

Cultures of *C. albicans* SC5314, 16s1, or 4L1 (Table 4.1) in 5 mL of RPMI were prepared from overnight cultures as described in the adhesion assays (*vide supra*) except that the overnight culture was diluted 1:250 in three steps (1:10 × 1:5 × 1:5). The biofilm formation assays were conducted following a literature protocol with modification.\(^{57,58}\) A 230-μL aliquot of the *C. albicans* culture was incubated with 10 μL of a 24× aqueous peptide solution (120, 240, or 480 μM) or sterile Milli-Q water as a no-peptide control and incubated at room temperature for 15 min. The resulting mixture was then added to two wells of a 24-well plate (100 μL per well) and incubated with gentle shaking (37 °C, 50 rpm). After 24 or 48-h incubation, the supernatant was removed by a sterile glass pipette and each well was washed with 100 μL of phosphate-buffered saline (PBS) pH 7.2 three times. An aliquot of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 100 μL, 1 mg/mL, pre-filtered, 0.22-μm filter) was added to each well and the plate was incubated for 4 h with gentle shaking (37 °C, 50 rpm). The supernatant was removed and 100 μL of
dimethylsulfoxide (DMSO) was added. The plate was incubated for 15 min with shaking (37 °C, 150 rpm). A 90-µL aliquot of the resulting solution was transferred to a new well and the absorbance at 550 nm was recorded.

For the assays in which hyphal C. albicans SC5314 were used, the overnight culture of C. albicans in YPD was centrifuged (3,600 rpm, 5 min, 4 °C), washed with 5 mL of RPMI, and resuspended in fresh RPMI. The resulting suspension (400 µL) was added into 20 mL of RPMI in a 250-mL baffled flask and the culture was incubated for 24 h with shaking (37 °C, 150 rpm) to induce hyphae form, which is confirmed by wide-field microscopy. Then, the biofilm formation assays were conducted using the overnight culture in RPMI and following the protocol described above.

4.2.8 Testing of Candida Biofilm Sensitivity to HD6

To test the sensitivity of C. albicans biofilms to HD6, 100 µL of the diluted fungal culture in RPMI was added to each well of a 24-well plate (100 µL for each) and incubated for 24 h with gentle shaking (37 °C, 50 rpm). The supernatant was removed and each well was washed with 100 µL of PBS three times. Then, 100 µL of fresh RPMI without or with 5, 10, 20 µM of HD6, or 20 µM F2A, or 5 µM AmpB was added to wells containing the biofilms. The plate was incubated for another 24 h with gentle shaking (37 °C, 50 rpm). Then, the amount of biofilm was quantified by an MTT assay (vide supra).

4.2.9 HD6-treated Candida Biofilm Formation after HD6 Is Removed

To investigate whether HD6-treated C. albicans regain their ability to form biofilm after HD6 is removed from the culture, 400 µL of C. albicans SC5314 (10^6 CFU/mL) in RPMI
was treated with 20 μM HD6 and each 100 μL of the mixture was added to four wells of a 96-well plate. The details of experimental setup are described in Section 4.2.7. After 24-h incubation, the supernatants from those wells were carefully combined in a sterile 1.7-mL microcentrifuge tube by using a sterile glass pipette. The resulting solution was divided into two tubes (200 μL each) and centrifuged (8,000 rpm, 10 min, 4 °C). The pelleted fungal cells were resuspended in RMPI and centrifuged (8,000 rpm, 10 min, 4 °C). Then, the first portion was resuspended in 200 μL of fresh RPMI, whereas the other portion was resuspended in 200 μL of fresh RPMI containing 20 μM HD6. Each suspension was incubated at room temperature for 15 min and 100 μL of each mixture was added to two wells of a 96-well plate. The plate was incubated for 24 h with gentle shaking (37 °C, 50 rpm). The MTT assays were then conducted following the protocol described in Section 4.2.7.

4.2.10 Extraction of RNA and Synthesis of cDNA

Cultures of *C. albicans* SC5314 in 5 mL of RPMI were prepared from overnight cultures as described in the adhesion assays (*vide supra*) except that the overnight culture was diluted 1:250 in three steps (1:10 × 1:5 × 1:5). A 950-μL aliquot of the *C. albicans* culture was incubated with 50 μL of a 20× concentrated aqueous peptide solution (400 μM) or sterile Milli-Q water as an untreated control and incubated at room temperature for 15 min. The resulting mixture was then added to each well of a 24-well plate and incubated for 24 h with gentle shaking (37 °C, 50 rpm). To analyze the effect of HD6 on biofilm-related gene expression in *C. albicans*, total RNA was extracted from *C. albicans* SC5314 biofilms (untreated or F2A-treated cells) or planktonic cells treated with native
HD6 with the MasterPure Yeast RNA Purification Kit (Epicentre). cDNA was generated from 1 μg of extracted RNA by using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher). cDNA samples were stored at -80 °C until use.

4.2.11 Quantitative Real-time PCR

All oligonucleotide primers used in these experiments are listed in Table 4.2. Real-time PCR was performed using the LightCycler® 480 SYBR Green I Master (Roche, Indianapolis, IN) and the Roche Lightcycler 480 II Real-time machine (MIT MicroBio Center) with the following run protocol: 50 °C (2 min), 95°C (3 min), and then 45 cycles of 95 °C (15 s), 50 °C (15 s), and 60 °C (1 min). Cycle threshold (C_T) values were obtained and used for analysis. The efficiency of each PCR reaction was calculated using Real-time PCR Miner online software (http://ewindup.info/miner/index.htm).^59^ Fold changes were calculated using the ΔΔ-C_T method in comparison to the average transcription levels of reference genes ACT1 and PMA1. The C_T values were obtained using two pairs of primers for each gene and at least two independently prepared and purified samples of each peptide. The experiments were conducted in four independent trials and the resulting averages with standard deviations are reported.

Table 4.2. Primers used in quantitative PCR.

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F represents forward primer. R represents reverse primer. The number refers to the set of primers.

4.2.12 Scanning Electron Microscopy

Cultures of *C. albicans* SC5314 in 5 mL of RPMI were prepared from overnight cultures as described in the adhesion assays (*vide supra*) except that the overnight culture was diluted 1:250 in three steps (1:10 × 1:5 × 1:5). A 480-μL aliquot of the diluted fungal culture was combined with 20 μL of a 25x concentrated aqueous peptide solution (500 μM) or sterile Milli-Q water as an untreated control, and incubated at room temperature for 15 min. The resulting mixture was then added to each well of a 24-well plate, which contained a sterile 12.7-mm glass cover slip (Electron Microscopy Sciences) and the
plate was and incubated for 24 h with gentle shaking (37 °C, 50 rpm). The supernatant was removed and the samples were fixed in 500 μL of Karnovsky fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.06 M Sorensen’s phosphate buffer, pH 7.2) at room temperature for at least 16 h. The samples were then prepared and SEM was performed as previously described.61

4.3 Results and Discussion
4.3.1 HD6 Exerts No Fungicidal Activity Against C. albicans

We conducted antimicrobial activity assays to investigate whether HD6 and F2A, the HD6 mutant which lacks an ability of self-assembly,53 provide antifungal activity against C. albicans in different growth states (log and stationary phases) because they have different metabolism depending on its growth phase. In these assays, amphotericin B (AmpB)62 was used as a positive control. AmpB, a secondary metabolite originally extracted from Streptomyces nodosus (a filamentous bacteria), exhibits potent antifungal activity by binding with ergosterol, a component of fungal cell membrane, and forming a transmembrane channel that leads to monovalent ion leakage.63 In agreement with a previous study by Schroeder et al.,64 HD6 did not exhibit antifungal activity against C. albicans, which were in either log or stationary phase (Figure 4.3a). Moreover, we monitored the growth of C. albicans in the presence of HD6 and F2A, and these peptides had negligible effect on its growth rate when the culture was incubated with shaking (Figure 4.3b).
Figure 4.3. HD6 is not fungicidal against C. albicans SC5314. (a) Antifungal activity of 20 μM HD6, 20 μM F2A, and 10 μM AmpB against log- and stationary-phase C. albicans SC5314. (b) Growth curves of C. albicans SC5314 in the presence of HD6 or F2A in YPD with shaking (mean ± SD, n = 3).

4.3.2 HD6 Protects Human T84 Cells from C. albicans Adhesion

Although C. albicans reside in the intestine as a commensal organism, this environment can be the origin of C. albicans systemic dissemination. C. albicans infections are initiated by fungal adhesion to the intestinal epithelial cells followed by invasion into these cells and blood vessels. Previous studies demonstrated that HD6 confers protection of host cells against invasion by both Gram-positive and Gram-negative bacteria. Therefore, we questioned whether HD6 could also protect host cells from adhesion and subsequent invasion by C. albicans. We performed a series of C. albicans adhesion assays by pre-treating BFP-expressing C. albicans with HD6 or F2A, and then adding the mixture to human T84 epithelial cells in 24-well plates. RPMI was used in these experiments to promote the hyphal growth of C. albicans. After 2 and 4 h of co-incubation and removal of non-adhered fungal cells, we employed fluorescent microscopy to quantify the number of adhered fungi to T84 cells. We observed that HD6
significantly decreased the number of attached *C. albicans* to the human T84 intestinal epithelial cells (Figures 4.4, 4.5) and this effect became more pronounced at longer incubation time (4 h). In contrast, there is no significant difference between the untreated and F2A-treated fungi attached to the T84 cells.

**Figure 4.4.** HD6 provides a protection of human colon epithelial cells (T84 cells) against adhesion of *C. albicans* SC5314. The fungi (10⁶ CFU in 300 µL RPMI) were incubated with the indicated peptides for 15 min before adding to the T84 cells. Representative images of T84 cells after incubated with different concentrations of peptides (t = 2 h, T = 37 °C, 5% CO₂). The nuclei of T84 cells are colored in green and *C. albicans* are pseudocolored in red. Scale bar = 20 µm.
Figure 4.5. HD6 provides a protection of human colon epithelial cells (T84 cells) against adhesion of *C. albicans* SC5314. The fungi (10⁵ CFU in 300 μL RPMI) were incubated with the indicated peptides for 15 min before adding to the T84 cells. Representative images of T84 cells after incubated with different concentrations of peptides (t = 4 h, T = 37 °C, 5% CO₂). The nuclei of T84 cells are colored in green and *C. albicans* are pseudocolored in red. Scale bar = 20 μm.

The quantitative data (Figure 4.6) reveals that the percentage of *Candida* adhesion dropped with an increase in the concentration of HD6. At the highest HD6 concentration (20 μM), the number of adhered cells decreased from 60% to 10% after 2-h incubation. In addition, we observed that more fungal cells were attached to the
epithelial cells at longer incubation time (4 h); however, the increase in the number of adhered cells was significantly lower when HD6 was present in the medium at high concentrations (10 and 20 \( \mu M \)). These data suggest that HD6 may provide protection against the adhesion and invasion of \( C. \text{albicans} \) in the intestine.

**Figure 4.6.** HD6 reduces the percentage of adhered fungal cells to human T84 colon epithelial cells. The fungi (10\(^5\) CFU in 300 \( \mu L \) RPMI) were incubated with the indicated peptides for 15 min before adding to the T84 cells. The number of adhered fungi was counted from the fluorescent images (e.g. Figures 4.4, 4.5) and the data were plotted in comparison to the untreated control after 4-h incubation (mean \( \pm \) SDM, \( n = 3 \)).

4.3.3 HD6 Suppresses Biofilm Formation of \( C. \text{albicans} \)

Another virulence trait of \( C. \text{albicans} \) is its ability to form biofilms on abiotic and biotic surfaces. Because biofilm formation relies on initial cell adhesion to the surface,\(^{38,39,67}\) we hypothesized that HD6 could also prevent biofilm formation of \( C. \text{albicans} \). To test our hypothesis, we conducted a standard biofilm assay (Section 4.2.7)\(^{57,58}\) in the presence of HD6, F2A, and AmpB. As shown in **Figure 4.7a**, the amount of biofilm was reduced with an increase in the HD6 concentration in the medium at both 24- and 48-h time points (~25% and ~20% biofilm formed in the presence of 20 \( \mu M \) HD6 after 24 and
48-h incubation, respectively). In contrast, F2A did not suppress biofilm formation of *C. albicans*. These observations suggest that self-assembly of HD6 is required for the peptide to prevent the formation of *C. albicans* biofilms. Because HD6 is not fungicidal against *C. albicans*, we expected that a decrease in biofilm formation is accompanied by more fungal cells in the supernatant. We determined the CFU/mL of the planktonic cells and this analysis revealed that the supernatant contained $\approx 10^6$ CFU/mL when *C. albicans* were pre-treated with HD6, whereas no colony was detected for the untreated or F2A-treated cultures (Figure 4.7b). Taken together, these results (Figure 4.7) indicate that the population of *C. albicans* is shifted from the biofilm to the supernatant in the present of HD6, resulting in reduced biofilm formation of *C. albicans*.

**Figure 4.7.** Self-assembly of HD6 decreases the amount of *C. albicans* biofilm formed after 24 and 48 h. (a) The amount of *C. albicans* biofilm in the presence of different concentrations of HD6. The absorbance at 550 nm from the MTT assay was reported. (b) The corresponding CFU/mL of *C. albicans* in the supernatant after treatment with HD6 (mean ± SDM, n = 3). An asterisk indicates that no colony was detected.
In order for HD6 to suppress biofilm formation of \textit{C. albicans}, we questioned whether HD6 has to be present in the initial stage of biofilm formation, which involves cell attachment to surfaces. We performed a set of assays to determine the effect of HD6 on pre-formed biofilms. We observed that HD6 did not disrupt the pre-formed biofilms (Figure 4.8). Thus, HD6 may suppress the biofilm formation of \textit{C. albicans} by preventing the initial cell attachment to surface, which is similar to what we observed in the adhesion assays (Figures 4.4-4.6).

![Figure 4.8](image)

**Figure 4.8.** The preformed biofilm of \textit{C. albicans} is resistant to HD6. The absorbance at 550 nm from the MTT assay was reported (mean ± SDM, n = 3).

We next investigated whether the inhibition of \textit{C. albicans} biofilm formation by HD6 is reversible. Does \textit{C. albicans} regain the ability to form biofilm if HD6 is removed from the supernatant? To address this question, we treated \textit{C. albicans} with HD6 for 24 h and then pelleted and resuspended the cells in fresh RPMI. The fungal suspension was then incubated for another 24 h. HD6-treated \textit{C. albicans} were able to form biofilm to a similar level to the untreated cells after HD6 was removed (Figure 4.9a). On the other
hand, if HD6 was added back into the supernatant, the amount of *C. albicans* biofilm remained at ~20% biofilm. These data (Figure 4.9) suggested that the reduction in biofilm formation of *C. albicans* by HD6 was reversible. Taken together, we conclude that HD6 has the capacity to suppress the biofilm formation of *C. albicans* by reducing the initial cell attachment to surfaces.

![Figure 4.9](attachment:image.png)

**Figure 4.9.** A reduction in biofilm formation of *C. albicans* by HD6 is temporary. The fungi form biofilm to the normal level once HD6 is removed from the supernatant. The concentration of HD6 is 20 μM. The absorbance at 550 nm from the MTT assay was reported (mean ± SDM, n = 3).

4.3.4 HD6 Affects Regulation of Biofilm-related Genes of *C. albicans*

To gain better understanding on how HD6 influences the biofilm formation of *C. albicans*, we conducted transcriptional profiling experiments by employing quantitative PCR (qPCR) to measure changes in the expression levels of selected virulence genes, which play a role in biofilm development of *C. albicans* (Table 4.3), upon treatment with HD6 or F2A.
Table 4.3. Biofilm-related genes evaluated in this study.\textsuperscript{39}

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<td>ALS3</td>
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\textsuperscript{a} ES = early stage, LS = late stage, RG = reference gene.

The qPCR analysis showed that the expression levels of 7 out of 17 genes, which involve in biofilm formation of \textit{C. albicans} at different stages, were altered in the presence of HD6 (\textbf{Figure 4.10}). \textit{YWP1} (4-fold), \textit{HWP2} (3.5-fold), \textit{RBT1} (3.5-fold), and \textit{PGA10} (2-fold) were up-regulated, whereas \textit{ALS1} (1.5-fold), \textit{PBR1} (1.5-fold), and \textit{TEC1}...
(1.5-fold) were down-regulated. These changes are not observed when *C. albicans* were grown in the presence of F2A.

![Graph](image)

**Figure 4.10.** Results of qPCR of *C. albicans* biofilm-related genes, comparing gene expression levels in RPMI in the presence of 20 μM HD6 or 20 μM F2A to their expression levels in the absence of the peptides. RNA was extracted from four independent biological replicates (mean ± SDM, n = 4). Data obtained from two sets of primers are in good agreement.

### 4.3.5 YWP1−/− C. albicans Form Biofilm in the Presence of HD6

Out of the seven biofilm-related genes that were up- or down-regulated upon treatment with HD6, the most striking change occurred for *YWP1*, which encodes a yeast cell protein that has an antiadhesive effect. It was reported that yeast cells that lack Ywp1 are more adhesive and more readily form
biofilm, whereas hyphae that are forced to express Ywp1 lose adhesion at least in vitro.\textsuperscript{56,68} We therefore hypothesized that HD6 may up-regulate the expression level of YWP1 to make \textit{C. albicans} less adhesive to surfaces and consequently, form less biofilm. We carried out another set of biofilm assays using YWP1 knockout \textit{C. albicans} strains 16s1 and 4L1 (Table 4.1), which were reported to express 42% and 1.5% of Ywp1 compared to the wild-type \textit{C. albicans}, respectively.\textsuperscript{56} Our results (Figure 4.11\textit{a,c,e}) indicated that there was no significant difference in the amount of biofilms formed among these three strains (wild-type, 16s1, and 4L1). These mutant strains behaved similarly to wt \textit{C. albicans} when treated with HD6.

**Figure 4.11.** The amount of biofilm formed by 16s1 and 4L1 strains of \textit{C. albicans}. (a, c, e) The amount of \textit{C. albicans} biofilm in the presence of different concentrations of HD6. The absorbance at 550 nm from the MTT assay was reported. (b, d, f) The corresponding CFU/mL of \textit{C. albicans} in the supernatant after treatment with HD6 (mean ± SDM, n = 3). An asterisk indicates that no colony was detected. Figure 4.11\textit{a,b} are the same as Figure 4.7\textit{a,b} and shown here to facilitate comparison among these strains.
We questioned whether HD6 suppresses the biofilm formation of *C. albicans* if they are already hyphae. We therefore conducted a series of biofilm assays using hyphae instead of yeast cells. *C. albicans* were grown in RPMI in a baffled flask for 24 h with rigorous shaking (37 °C, 150 rpm)\(^1\) and wide-field microscopy (Figure 4.12) confirmed that the fungi completely turned into true hyphae. These hyphae were subsequently used in the biofilm assays. We observed that the biofilm from hyphae was less robust than that from yeast cells. As a result, we had to be more careful with the washing step to remove any non-adhered cells and not to disrupt the biofilm.

![Figure 4.12](image.png)

**Figure 4.12.** Representative images of *C. albicans* SC5314 after growing in YPD (30 °C, 150 rpm, top row) and RPMI (37 °C, 150 rpm, bottom row) for 24 h. A scale bar = 10 μm.

The data in Figure 4.13a,c revealed that the amount of biofilm was reduced with an increase in the HD6 concentration in the medium at both 24 and 48-h time points; however, we observed slightly more biofilm over longer incubation time with HD6 (~20% and ~25% biofilm formed in the presence of 20 μM HD6 after 24- and 48-h incubation, respectively), which contrasted with the data obtained from yeast cells. We also observed that F2A did not suppress biofilm formation of the hyphae. In general, there were more cells in the supernatant and fewer cells in the biofilm when the concentration
of HD6 increased (Figure 4.13). Nonetheless, in contrast to the yeast CFU/mL data, we detected some colonies formed on YPD agar even in the supernatant of untreated and F2A-treated cultures, supporting that the hyphal biofilm was less robust than the yeast biofilm. Taken together, we conclude that HD6 reduces the amount of biofilm formed by hyphal C. albicans; therefore, the capacity of HD6 to suppress biofilm formation of C. albicans is independent on the initial form of C. albicans (yeast cells or hyphae).

Figure 4.13. The amount of biofilm formed by hyphal C. albicans SC5314. (a, c) The amount of C. albicans biofilm in the presence of different concentrations of HD6. The absorbance at 550 nm from the MTT assay was reported. (b, d) The corresponding CFU/mL of C. albicans in the supernatant after treatment with HD6 (mean ± SD, n = 3). An asterisk indicates that no colony was detected. Figure 4.13a,b are the same as Figure 4.7a,b and shown here to facilitate comparison between yeast and hyphal forms of C. albicans.
4.3.6 Morphology of C. albicans Biofilm Is Altered by HD6

*C. albicans* can grow into several forms, including yeast, pseudohyphae, and hyphae, depending on the environment.\(^{19}\) Hyphae is shown to be a virulent form of this organism, which is more adhesive and invasive.\(^{39}\) One possible question that arises from the qPCR results (Figure 4.10) is whether HD6 suppresses the *C. albicans* transition into hyphae. Does HD6 cause the fungi to remain in yeast form or pseudohyphae, and express Ywp1? To address this question, we employed scanning electron microscopy (SEM) to visualize the morphology of *C. albicans* cells with and without HD6, as well as the architecture of biofilms.

The morphology of *C. albicans* biofilm in the presence of HD6 was significantly different from that of untreated or F2A-treated samples (Figure 4.14). At the high concentrations of HD6 (\(\geq 10 \mu M\)), the fungal cells agglutinated instead of forming biofilm. In the presence of 5 \(\mu M\) of HD6, both biofilm and cell aggregate formed. The biofilms of untreated, 5 \(\mu M\) HD6-treated, and 20 \(\mu M\) F2A-treated cultures as well as cell aggregates of 10 and 20 \(\mu M\) HD6-treated cultures were subjected to analysis by SEM. During the sample preparation for SEM, we observed that those cell aggregates were less sticky to the cover slips in contrast to the biofilms. SEM revealed that *C. albicans* was in elongated form and densely packed in the biofilms in all three cases (untreated, 5 \(\mu M\) HD6-treated, and 20 \(\mu M\) F2A-treated cultures). *C. albicans* in the cell aggregates obtained from 10 and 20 \(\mu M\) HD6-treated cultures also demonstrated elongated structure; however, the lengths of these cells were significantly shorter than those of the fungal cells in biofilms. Taken together, our SEM data suggested that HD6 may interfere with *C. albicans* transition into hyphae by either slowing down the process or forcing
them to remain as pseudohyphae, resulting in reduced biofilm formation by *C. albicans*. Nonetheless, it was difficult to distinguish between pseudohyphae and hyphae of *C. albicans* by SEM.

![Figure 4.14. Morphological studies of *C. albicans* SC5314 with HD6 or F2A by SEM. For 10 and 20 μM HD6, cell aggregates instead of biofilms were mounted onto stubs for SEM analysis. Scale bar = 100 μm (left and middle columns) and 10 μm (right column).](image)

We next employed light microscopy for *C. albicans* morphological studies. Both untreated and F2A-treated cells in the biofilms exhibited similar morphology (Figure...
Both conditions promoted *C. albicans* hyphal growth. In contrast, there were few yeast cells present in the HD6-treated samples. These preliminary data suggested that HD6 may suppress the hyphal growth of *C. albicans*, resulting in its attenuated virulence traits (invasion and biofilm formation).

**Figure 4.15.** Morphological studies of *C. albicans* SC5314 with HD6 or F2A by wide-field microscopy. For 20 μM HD6, planktonic cells instead of biofilms were mounted onto coverslips. Scale bar = 20 μm.

### 4.4 Summary and Outlook

This work demonstrates that HD6, which is expressed in intestinal Paneth cells and plays a role in mucosal innate immunity, reduces two virulence traits of the opportunistic fungal pathogen *C. albicans*. These virulence traits include adhesion and invasion into epithelial cells, and biofilm formation, the latter of which provides *C. albicans* greater resistance to antimicrobial agents. Thus, our work built upon previous studies show that HD6 protects host cells from invasion by both Gram-positive and Gram-
negative bacteria, and allow us to generalize the model for the host-defense function of HD6 to include fungi. Recent work on the mucin MUC5AC, a glycoprotein which is expressed in the stomach and in the lungs, revealed that the mucin suppressed several virulence traits of *C. albicans*, which included filamentous growth and biofilm formation. Studies on MUC5AC and other mucins as well as our present work suggest that suppression of virulence traits of *C. albicans* by biopolymers (i.e., mucins and HD6) may be a general mechanism employed by innate immune system to protect mucosal surfaces.

Nonetheless, it remains unclear how HD6 affects these virulence traits of *C. albicans*. Prior studies by Bevins and coworkers suggest that HD6 interacts with certain surface proteins of *Salmonella enterica* serovara Typhimurium, which are type I fimbriae and flagella. Moreover, our SEM studies of *Listeria monocytogenes* and *Escherichia coli* indicate that the morphology of HD6 nanonets may differ for Gram-positive and Gram-negative bacteria. These observations may result from different compositions of membranes of Gram-positive and Gram-negative bacteria, although further studies are required to address this notion. We speculate that HD6 may interact with certain microbial membrane proteins, such as adhesins, and prevent these proteins from functioning. For example, adhesins may no longer bind hydrophobic surfaces after interacting with HD6. The identification of the moieties of HD6 that are recognized by these microbes, as well as the proteins and the regulatory pathways of *C. albicans* that are affected by HD6, may lead to new strategies for preventing and ameliorating *C. albicans* infections, especially at mucosal surfaces.
evaluate the importance of the hydrophobic residues (F2, V22, I25, and F29) on the self-assembly of HD6. Our biophysical studies employing transmission electron microscopy (TEM) and analytical ultracentrifugation (AUC) establish that HD6 forms higher-order oligomers in the absence of bacteria or other biomolecules, whereas the HD6 variants exhibit impaired ability to self-assemble. For example, the self-assembly of HD6 is completely diminished by either F2A or F29A mutation. The bacterial agglutination assays and *Listeria* invasion assays indicate that lack of ability to self-assembly causes attenuation in the biological function of HD6. F2A and F29A do not cause bacterial agglutination or block *Listeria* invasion into the epithelial cells. In this work, we demonstrate that the self-assembly of HD6 is driven by the hydrophobic residues. In addition, our results highlight the importance of primary sequence for defensin function and, particularly, how variable amino acid sequences among α-defensins afford different biophysical properties and biological function.

We questioned the storage of HD6 in intestinal Paneth cells. The analysis of human mRNA indicated that HD6 is translated as a prepropeptide, which contains a 19-residue signal peptide, a 49-residue pro region and the 32-residue mature HD6.75 Guided by this analysis, we elucidated the effect of the pro region on the self-assembly and biological activity of HD6 (Chapter 3). In collaboration with the Bevins Lab at UC Davis, we show that HD6 exists in two isoforms: proHD6 in the ileal tissue and mature HD6 in the luminal fluid. These results are also the first evidence for detecting proHD6 ex vivo. Our biophysical and biological studies reveal that the pro region suppresses the self-assembly and biological activity of HD6 and trypsin, which is also found in Paneth cells, is the processing enzyme that cleaves proHD6 to unleash the mature peptide and
4.5 Summary of the HD6-Related Projects

Prior to my dissertation project, there were only a few studies that focused on HD6, and the information on the biological function of HD6 was fairly limited in the literature. The contribution of HD6 to innate immunity had been enigmatic because several studies showed that HD6 exerts negligible antimicrobial activity.\textsuperscript{73,74} In 2012, Bevins and coworkers reported how HD6 promotes mucosal innate immune system and this activity of HD6 is unique among defensin peptides.\textsuperscript{55} Instead of killing bacteria, HD6 self-assembles into nanonets and these oligomers entrap the pathogens and prevent them from invading into the epithelium. This study also provided evidence that each defensin may exhibit unique mechanism of action to fight against microbes and the peptides should be studied individually.

This seminal work on HD6 had raised many more questions about this unusual peptide. Why does HD6 behave differently from other defensins given that the monomers of these peptides share several structural features? How are HD6 nanonets stored in the granules of intestinal Paneth cells? Moreover, most studies on HD6 have focused on the interaction between the peptide and bacteria. Are there any other consequences on bacteria after being entrapped by HD6? Is the entrapment and blockage of invasion by HD6 applicable to other organisms, such as fungi? We have conducted studies to address some of these questions.

We investigated the mechanistic details of HD6 unusual behavior by considering the primary sequence of this peptide (Chapter 2). The self-assembly of bioactive molecules is usually driven by hydrophobic residues. Analysis of the crystal structure of HD6 supported this notion. As a result, we prepared a family of HD6 variants to
triggers its oligomerization and biological activity. As a result, we propose the working model, in which HD6 is stored as an inactive propeptide proHD6 in the granules of intestinal Paneth cells and upon secretion into the lumen, trypsin cleaves proHD6 and activates the host-defense function of HD6. Our working model highlights the role of the pro domains in temporally and spatially controlling the biological activity of peptides.

In Chapter 4, we expand the scope of HD6 biological function to another organism, an opportunistic fungal pathogen *Candida albicans*. This pathogen exhibits several virulence traits, including transition into virulent hyphae, invasion into host cells, and biofilm formation. Some of these traits are essential for *C. albicans* to cause systemic infection and some traits make the treatment of *C. albicans* infection complicated, leading to a high mortality rate, especially in immunocompromised patients. *C. albicans* is a part of the gut microbiota and the mechanisms that trigger the virulence of *C. albicans* are currently not well documented. Because HD6 is also found in the small intestine, we questioned the contribution of HD6 to controlling *C. albicans* at mucosal surfaces. Our study demonstrate that HD6 suppresses two virulence traits of *C. albicans*, invasion and biofilm formation, by preventing initial adhesion of the cells to surfaces. Our preliminary morphological studies indicate that HD6 may prevent *C. albicans* transition into hyphae and thereby keeping the fungi avirulent. Further investigation is required to address how HD6 affects the hyphal growth of *C. albicans*. Taken together, these observations demonstrate that similar to other defensins, the biological activity of HD6 is broad-spectrum against bacteria and fungi.

To date, the mechanism of antimicrobial activity of defensins is not always clear; however, more recent studies support that each defensin possesses unique mechanism
of action and the peptides can have multiple host-defense functions. Our studies have provided more insights into the structural and functional properties of HD6. Nonetheless, there are many questions about this host-defense peptide. Our data indicate that apart from self-assembly and microbial entrapment, HD6 may exhibit other effects on microbes. For example, although HD6 entraps both Gram-positive and Gram-negative bacteria, differences in nanonet morphology are observed by SEM. These bacteria have different components on the membrane, which may cause HD6 to interact with the bacteria through varying mechanisms. In addition, how HD6 suppresses C. albicans virulence traits requires further studies. Some defensins, including human neutrophil peptides (HNPs), also function as signaling molecules. The study of HD6 and C. albicans may provide evidence of HD6 functioning as a signaling molecule to suppress the hyphal growth of this fungal pathogen. In closing, the current knowledge of HD6 and other defensins is still limited. Studies of these peptides will provide better understanding on how defensins contribute the innate immune system and we may apply the information we learn about these small peptides to the development of new therapeutic approaches for combating microbial infection.

4.6 Acknowledgements

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4.7 References


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Chapter 5

Introduction to Siderophore-Based Strategies to Target Gram-Negative Bacteria
5.1 Iron Homeostasis in Humans and Bacteria

Iron is an essential nutrient for almost all living organisms. One of the key properties of iron is its participation in redox reactions due to its two major oxidation states, $\text{Fe}^{2+}$ and $\text{Fe}^{3+}$. Therefore, iron plays several roles in biological systems. For example, it involves in oxygen transport and storage (hemoglobin and myoglobin), catalyzing syntheses of some metabolites (Fe-S clusters in hydrogenase, nitrogenase, and ribonucleotide reductase), and signaling (hepcidin). Nonetheless, iron exhibits low solubility in aqueous solution at physiological pH and enables Fenton chemistry (Figure 5.1), which yields reactive oxygen species that can cause damage to proteins, lipids, and DNA. As a result, the levels of “free” iron in biological systems (ca. $10^{-24}$ M in mammalian serum) are tightly regulated by homeostatic mechanisms, such as the expression of the iron transport and storage proteins transferrin and ferritin.

\[
\begin{align*}
\text{Fe}^{3+} + \text{O}_2^{-} & \rightarrow \text{Fe}^{2+} + \text{O}_2 \\
\text{Fe}^{2+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \cdot\text{OH} \\
\text{O}_2^{-} + \text{H}_2\text{O}_2 & \rightarrow \text{O}_2 + \cdot\text{OH} + \cdot\text{OH}
\end{align*}
\]

Figure 5.1. Iron catalyzes the Fenton reaction to generate a toxic hydroxyl radical.

5.1.1 Iron Homeostasis in Humans

In response to the toxicity and low solubility of iron under physiological conditions, almost all organisms have evolved efficient ways of conserving and regulating the levels of this metal ion. A healthy human body contains ≈5 g of iron, which is mostly stored in hemoglobin in red blood cells, and about 1-2 mg of iron is lost daily. The most efficient way for humans to uptake iron is from the heme-bound form found in red meat, poultry,
and seafood. Heme iron is absorbed by enterocytes in the duodenum, the first part of the small intestine. Heme-bound form is uptaken through the dedicated transporter (HCP1) and then degraded by heme oxygenase 1 (HO-1) to release ferrous ion. Non-heme iron is mostly found in plants; however, the absorption rate of non-heme iron is lower than that of heme iron. During digestion, iron may exist as ferric ion. This insoluble ion is then further reduced to ferrous ion by ferric reductases found on the membrane of enterocytes, and subsequently transported into the cytosol via a divalent metal transporter DMT1 (also known as NRAMP2).

Iron is exported from the enterocytes via the iron exporter ferroportin. Following the export, ferrous ion is oxidized by ferroxidase to afford ferric ion, which is immediately captured by transferrin (Tf) in the plasma to deliver iron to other sites of the body. Transferrin (Tf) is a 79-kDa glycosylated protein and it functions as the major iron carrier in the plasma. One Tf molecule binds to two ferric ions with a dissociation constant of $10^{-23}$ M at pH 7.4. The Fe-Tf complex is transported into target cells by binding to the Tf receptor (TfR) to initiate its internalization by the cells via endocytosis (Figure 5.2). The acidic conditions in the endosome triggers the release of ferric ion, which is reduced by ferrireductase STEAP3 and exported into the cytosol via DMT1. Both Tf and TfR are recycled to the extracellular space and the cell membrane, respectively. In addition to Tf, acetate, citrate, albumin can also bind to iron.
Figure 5.2. Transferrin receptor (TfR)-mediated iron uptake. The ferric Tf/TfR complex is acquired by endocytosis. The endosome is acidified to trigger the release of ferric ion from Tf. The ferric ion is then reduced by ferrireductase STEAP3 and exported into the cytosol via a divalent metal transporter DMT1. Tf and TfR are recycled to the membrane.

Ferrous ion is the main oxidation state present in the cytosol and its storage involves ferritin. Ferrous ion released from the endosome is oxidized by the ferroxidase domain of ferritin and stored as ferrihydrite or amorphous ferric phosphate in the center cavity of ferritin. Ferritin is a spherical hetero-oligomeric protein consisting of 24 units of heavy and light chains, resulting in a molecular weight of 450 kDa. This unique structure allows ferritin to store up to 4,500 iron atoms. 15

Hepcidin is a key regulator of iron release into the circulatory system in mammals. Hepcidin is a 25-residue cysteine-rich peptide that is synthesized and secreted by hepatocytes and circulates in the plasma. 16 HAMP, which encodes hepcidin, is
upregulated in the liver when the plasma concentration of Fe-Tf is high. Hepcidin interacts with ferroportin, the iron exporter on the cell membrane, to prevent further iron transport into the plasma. In this process, hepcidin-ferroportin complex induces its internalization via endocytosis. The complex is subsequently degraded in lysosomes, which reduces ferroportin levels and the levels of iron released into the plasma. On the other hand, some biological processes, such as the production of red blood cells in the bone marrow, downregulate \textit{HAMP}, which lowers hepcidin expression and allows more iron to be released into the plasma and delivered to the bone marrow. At the cellular level, iron regulatory protein 1 and 2 (IRP1 and IRP2) are the main contributors to cellular iron homeostasis. When the intracellular iron concentration is low, IRPs bind the iron-responsive element (IRE) in the ferritin mRNA and cause reduced translation rate. In contrast, binding to the multiple IREs in the Tf mRNA enhances the stability of the mRNA, resulting in increased expression level of Tf and hence elevated iron delivery to the cell.

5.1.2 \textit{Bacterial Iron Acquisition Systems}

Most microbial pathogens require micromolar concentrations of iron to colonize and cause disease. In response to microbial infection, the host withholds iron from the environment as one of the host-defense strategies to limit the colonization and growth of invading pathogens. Therefore, these pathogens have evolved efficient iron uptake machineries to overcome iron-limiting host-defense mechanisms.

Siderophore-mediated iron acquisition is one of the strategies that is broadly used among microorganisms. Siderophores are low-molecular-weight iron chelators, which
are biosynthesized and secreted by bacteria and fungi to sequester ferric iron from the environment. Siderophores display high affinity for ferric ion with dissociation constants ranging from $10^{-30}$ to $10^{-49}$ M. As a result, siderophores can sequester iron from host iron-containing proteins, including Tf.\textsuperscript{21} Once siderophores bind iron, the ferric-siderophore complexes are recognized and transported into the microbial cells by dedicated uptake machineries. In the cytosol, iron is released by reducing Fe\textsuperscript{3+} to Fe\textsuperscript{2+}, degrading the siderophores, or a combination of both approaches. In addition to producing their own siderophores, several microbes, such as \textit{Staphylococcus aureus}, \textit{Mycobacterium fortuitum}, \textit{Bacillus subtilis}, \textit{Vibrio cholerae}, and \textit{Pseudomonas aeruginosa}, express receptors to uptake xenosiderophores, which are siderophores produced by other microbes. For example, \textit{V. cholerae} expresses and utilizes VctA and IrgA to uptake enterobactin, a siderophore synthesized by other Gram-negative bacteria, including \textit{Escherichia coli}.\textsuperscript{22} Several siderophores are considered to be virulence factors because they play an important role in iron acquisition and promotion of microbial colonization and growth in the host.\textsuperscript{23-25} More details about siderophores are discussed in Section 5.2.

In addition to importing ferric iron via siderophore-mediated iron acquisition machineries, bacteria also express dedicated ferrous iron uptake systems. For example, \textit{E. coli} possesses \textit{feoABC}\textsuperscript{26} and \textit{efeUOB},\textsuperscript{27} which are upregulated under anaerobic and iron-deficient conditions. The Feo system is actually widely distributed among bacterial species, yet its physical structure and mechanism of iron transport are poorly understood. FeoB is a GTPase and is proposed to be the main energy-dependent Fe\textsuperscript{2+} transporter located in the inner membrane of \textit{E. coli}.\textsuperscript{28} Mutation of the \textit{feoB} gene
resulted in attenuated colonization of *E. coli* in the mouse intestine. FeoA is a cytoplasmic protein that may interact with FeoB; however, its function is not well understood. FeoC is proposed to be a transcriptional regulator due to its helix-turn-helix structure.

Bacterial pathogens also import heme by expressing dedicated heme uptake machineries or employing hemophore-dependent systems. Gram-positive bacteria generally use the direct heme uptake machinery, whereas Gram-negative bacteria employ both direct heme uptake machinery and hemophore-mediated system. For example, *S. aureus*, a Gram-positive pathogen, expresses the iron-regulated surface determinant system (Isd), which is encoded by the *isdABCDEFGHI* gene cluster (Figure 5.3). IsdB and IsdH are cell-surface receptors that recognize hemoglobin and haptoglobin, whereas, IsdA and IsdC are cell-wall shuttle proteins that transfer heme to the cell membrane. The ATP-binding cassette (ABC)-type transporter IsdDEF is responsible for internalizing heme into the cytosol and heme oxygenases IsdG and Isdl degrade heme to iron and staphylobilin. Heme is found to be the major iron source for *S. aureus* even though this species also employs other iron acquisition systems, including siderophore-mediated systems.
Figure 5.3. Heme uptake machinery in *S. aureus*. Heme is extracted from hemoglobin and transferred to the cell membrane by IsdA and IsdC. The ABC transporter IsdDEF delivers heme into the cytoplasm where IsdG and IsdH degrade heme to release ferrous ion.

On the other hand, *P. aeruginosa*, a Gram-negative pathogen, has the capacity to uptake heme form hemeproteins via the two systems (Figure 5.4), namely heme acquisition system (Has) and *Pseudomonas* heme uptake (Phu).\(^3\) In the Has system HasA(p), a hemophore protein, is secreted to extract heme from host hemeproteins and transfer heme to the outer membrane TonB-dependent receptor, HasR. In the Phu system, heme is directly extracted by the outer membrane TonB-dependent receptor, PhuR. Once in the periplasm, heme is bound by a periplasmic binding protein (PBP) and transported into the cytoplasm by an inner membrane ABC transporter. Then, heme is bound to a heme chaperone, PhuS, before being delivered to the heme oxygenase (HemO), which degrades heme into biliverdin, CO, and Fe\(^{2+}\).\(^4\)
Figure 5.4. Two heme uptake systems, Phu and Has, in *P. aeruginosa*. The TonB-dependent receptor PhuR binds directly to heme proteins and extracts heme, whereas the TonB-dependent HasR binds a complex between heme and a secreted hemophore protein HasA. In the periplasm, heme is bound to a periplasmic binding protein (PBP), which delivers it to an ABC transporter. In the cytoplasm, heme is directed to the heme oxygenase HemO by the PhuS chaperone. HemO degrades heme into biliverdin, CO, and ferrous ion.

Transferrin (Tf) is another iron source for certain pathogens. *Neisseria meningitidis*, a Gram-negative pathogen, expresses two transferrin transporters (TbpA and TbpB) on its outer membrane.\(^{35}\) The receptors extract ferric ion from Tf and a periplasm ferric-binding protein subsequently transfers iron to the inner membrane, in which an ABC transporter delivers iron into the cytosol. To extract ferric ion from Tf, TbpA inserts its helical element into the iron-binding C loop of Tf to induce the release of iron and transfer it to the receptor.\(^{36}\) *Haemophilus influenzae*, another Gram-negative pathogen, also sequesters iron from Tf as well as from lactoferrin, which is abundant in human neutrophils and contributes to the host innate immunity by withholding iron at the sites of infection.\(^{37}\)
5.1.3 Iron Homeostasis in Bacteria

Bacteria employ three proteins to store iron, which are ferritin, bacterioferritin, and a Dps protein. Similar to eukaryote ortholog (Section 5.1.1), bacterioferritin consists of 24 subunits and forms a spherical shape with a central cavity for iron storage. Furthermore, it contains 12 heme molecules per 24-mer, which are involved in the reductive release of iron from bacterioferritin. Dps is a 250-kDa protein comprised of 12 subunits that exhibits lower iron storage capacity than ferritin and bacterioferritin.

For regulating iron acquisition, bacteria mainly rely on iron-sensing proteins. The ferric uptake regulator (Fur) family is one of the major regulators in several Gram-negative and certain Gram-positive bacteria. The Fur family is a class of bacterial proteins used as repressors on the transcriptional level. Fur is a homodimer and each monomer binds one Fe²⁺ ion. Once Fur binds iron, its affinity for DNA increases by ~1,000 fold. Therefore, iron-bound Fur readily binds to DNA and consequently blocks the RNA polymerases and inhibits transcription. Fur controls several genes in E. coli, including iron uptake machineries and siderophore biosynthesis pathways. Furthermore, Fur displays an indirect regulatory effect on the transcription of many genes. For example, in E. coli, RyhB is a small regulatory RNA that binds the mRNAs encoding iron-utilizing proteins and triggers RNAse-mediated degradation of those mRNAs. As a result, expression levels of the proteins are decreased, and thereby iron usage in the cells is reduced. When intracellular iron concentrations are high, iron-bound Fur represses the expression of RyhB, leading to an increase in the expression levels of the iron-utilizing proteins. The Fur regulation system also plays a
role in oxidative stress responses because it controls the expression levels of several iron-containing enzymes that are involved in resistance to oxidative stress.45

5.1.4 Lipocalin 2 Is an Iron-Withholding Host-Defense Protein
In response to infection and during inflammation, the host limits iron availability in a process known as “nutritional immunity”.46 In this process, epithelial cells and neutrophils secrete the antimicrobial protein lipocalin-2 (LCN2), which belongs to a functionally diverse protein family called lipocalins. The common features of lipocalins are that they bind small hydrophobic molecules and exhibit conserved 8-stranded antiparallel β-barrel core structure (Figure 5.5). Goetz and coworkers demonstrated that LCN2 inhibits siderophore-mediated iron uptake by capturing ferric enterobactin (Ent).47 The dissociation constant \( (K_d) \) for ferric Ent of LCN2 is \( \sim 0.4 \) nM, suggesting that LCN2 can compete with the bacterial Ent receptor, FepA \( (K_d \text{ for ferric Ent } \sim 24 \text{ nM}) \).48 The crystal structure of LCN2 (PDB 1L6M) reveals that it contains three rigid binding pockets that fit the three catechol rings of Ent. The cation–π interactions from Arg81, Lys125, and Lys134 along with the hydrogen bonding from Trp79 are important for binding Ent.49 After sequestering ferric Ent from the bacteria, LCN2 is endocytosed into the host cells and the complex releases iron in the acidic endosome, possibly due to the hydrolysis of Ent.50,51 In addition to Ent, LCN2 also binds other siderophores, including bacillibactin produced by \( B. \text{ anthracis} \) and mycobactin produced by \( M. \text{ tuberculosis} \).52

In the battle for iron, invading pathogens have evolved strategies to overcome LCN2 by biosynthesizing and utilizing a different set of siderophores. For example, uropathogenic \( E. \text{ coli} \) and \( S. \text{ salmonella} \) spp. express salmochelins, which are Ent analogs
with glucose modifications on the catechol rings. The presence of glucose moieties confers steric clashes in the binding pocket of LCN2, resulting in reduced binding affinity ($K_d > 1 \mu M$). The \textit{in vitro} growth of salmochelin-producing bacteria is not impaired in the presence of LCN2 and the bacteria become more virulent \textit{in vivo} due to their ability to evade LCN2. \textit{E. coli} also uses aerobactin, which lacks the catechol groups and hence is not captured by LCN2.

Figure 5.5. Crystal structure of lipocalin-2 (LCN2) with [Fe(DHB)$_3$]$^-$ in the binding pocket. Left: overall structure of LCN2. Right: binding pocket with the four key amino acid side chains shown in pink. 2,3-Dihydroxybenzoic acid (DHB) is shown in yellow.

\section*{5.2 Bacterial Siderophores}

\subsection*{5.2.1 Iron Affinity, Selectivity, and Structures of Siderophores}

Over 500 siderophores have been identified since the first isolation of mycobactin P in 1949. Different organisms produce and utilize different siderophores. Even one microbial species can employ several siderophores; for example, there are 62 analogs of pyoverdin isolated from \textit{Pseudomonas}.\textsuperscript{21}
Siderophores exhibit higher binding affinities for Fe³⁺ than for Fe²⁺. The high ferric-binding affinity of siderophores is a result of employing proper donor atoms and appropriate structural design. Ferrous ion prefers donor atoms like nitrogen, whereas ferric ion, which is a hard Lewis acid, prefers stronger donor atoms like oxygen. There are three main types of chelating groups used by siderophores, namely catechol, hydroxamate, and α-hydroxycarboxylate (Figure 5.6). These chelating groups each contain two oxygen atoms that bind ferric ion. Other donor atoms such as nitrogen and sulfur are also found in some siderophores (Figure 5.7); however, these siderophores tend to exhibit lower affinity for Fe³⁺ ion.

![Figure 5.6. Structures of common iron-chelating moieties used by siderophores. The oxygen atoms involved in binding iron are shown in red.](image)

As shown in Figure 5.7, the structures of siderophores are highly diverse and the iron-binding mode of siderophores can range from bidentate, tridentate, tetradentate, and hexadentate. The bidentate and hexadentate siderophores usually bind ferric ion with a ratio of 1:1, resulting in an octahedral or pseudo-octahedral geometry. The tridentate and tetradentate siderophores are likely to form complexes with two or even more ferric ions. The backbone of siderophores can be either linear or cyclic. Capping N- and C-termini and cyclization may confer resistance to peptidase hydrolysis and thereby enhance the stability of siderophores.
Figure 5.7. Structures of representative siderophores. The atoms involved in binding iron are shown in red.

**Figure 5.7** illustrates representative siderophores. An example of tris-hydroxamate siderophores is desferrioxamine (DFO) B 1, which is produced by *Nocardia* spp. and *Sterptomyces* spp. Another example is ferrichrome 2, which is produced by several fungi. Tris-catecholate siderophores include enterobactin 3 and its glycosylated derivatives 4, and bacillibactin 5, which are produced by enterobacteria and *Bacilli* spp., respectively. Examples of tris-\(\alpha\)-hydroxycarboxylate siderophores are achromobactin 6 and staphyloferrin B 7, which are used by *Pectobacterium chrysanthemi* and *Staphylococcus* spp. Moreover, several siderophores (8-12, **Figure 5.7**) employ a combination of these three chelating groups and heterocycles.
5.2.2 Dedicated Uptake Machineries for Siderophore Transport

Gram-positive and Gram-negative bacteria have different cell wall structures and therefore utilize different mechanisms for transporting siderophores (Figure 5.8). The cell wall of Gram-positive bacteria consists of a single layer of phospholipid membrane covered by a thick peptidoglycan layer. The Gram-negative bacterial cell wall is comprised of one outer and one inner phospholipid membrane, and the periplasmic space between the two membrane layers contains a thin peptidoglycan. In addition, Gram-negative bacteria have lipopolysaccharide embedded in their outer membrane.

Figure 5.8. A cartoon represents the cell wall structures of Gram-positive and Gram-negative bacteria showing phospholipid layers and peptidoglycan.

The siderophore uptake machineries of Gram-positive bacteria are less studied compared to those of Gram-negative bacteria. The lipid-anchored extracellular proteins in Gram-positive bacteria are homologous to the periplasmic binding proteins in Gram-negative bacteria and these proteins can function as siderophore receptors. After
binding an iron-siderophore complex, these proteins pass the complex to ABC permeases, which are located in the membrane, and transport the complex into the cytosol of Gram-positive bacteria.

To uptake an iron-siderophore complex in Gram-negative bacteria, there are three key steps: outer membrane recognition, periplasmic transfer, and inner membrane transport. Gram-negative bacteria express a dedicated siderophore receptor on the outer membrane to recognize an iron-siderophore complex and transport the complex into the cytosol. The affinities of the receptors to iron-siderophore complexes are typically in the nanomolar range. Many characterized siderophore receptors, including the Ent receptor FepA (Figure 5.9), share a transmembrane β-barrel structure containing 22 strands. Furthermore, the receptors have an N-terminal globular domain, which is composed of α-helices and β-sheets and functions as a “plug” of the barrel. The mechanism of iron-siderophore recognition by the receptor is proposed to be that the iron-siderophore complex interacts with the extracellular loops of the receptor and induces the conformational change in the plug domain to allow the complex to travel through the transient channel. This uptake process is TonB-dependent. TonB forms a protein complex with ExbB and ExbD, which is located in the inner membrane of Gram-negative bacteria. TonB interacts with the N-terminal domain of the receptors and transmit the proton motive force from the inner membrane as the energy source for this process although the detailed mechanism is not determined.
Figure 5.9. Crystal structure of the Ent receptor FepA from *E. coli* (PDB 1FEP). The N-terminal plug domain is colored in purple and the β-barrel and extracellular loop are colored in grey.

Once the iron-siderophore complex is in the periplasm, a periplasmic binding protein, such as FepB in *E. coli* (Figure 5.10), captures the complex and deliver it to the inner membrane permeases. The inner membrane permeases are ABC transporters and the permease complex consists of transmembrane channel proteins and ATPases tethered to the cytoplasmic side of the membrane. ATP hydrolysis by ATPases provides the energy for the active transport of iron-siderophore complexes through the transmembrane channels.\(^6\) Figure 5.10 illustrates the Ent uptake machinery in *E. coli.*
Figure 5.10. Ent uptake system in *E. coli*. The outer membrane receptor FepA recognizes ferric Ent and delivers it into the periplasmic space in a TonB-dependent manner. Then, the periplasmic binding protein FepB binds and passes ferric Ent to the inner membrane ABC permease complex FepCDG, which delivers ferric Ent into the cytosol. The esterase Fes hydrolyzes the trilactone of Ent and the siderophore-interacting protein YqjH reduces Fe$^{3+}$ ion to Fe$^{2+}$, triggering the release of iron from the complex.

The most commonly used strategy to release iron from siderophores in the bacterial cytoplasm is to reduce ferric ion to ferrous ion because siderophores exhibit much lower binding affinity to ferrous ion than to ferric ion. The reductases usually require the cofactors nicotinamide adenine dinucleotide (NADH) and flavin mononucleotide (FMN). In addition, reductases containing an Fe-S cluster are also identified, such as FhuF in *E. coli*. Some apo siderophores are then recycled and secreted into the extracellular space. Some siderophores like Ent, which display extremely high affinity for iron and have too low redox potentials for intracellular reduction, require an additional mechanism for releasing iron, which involves degradation of the siderophores. For example, the esterase Fes in *E. coli* catalyzes hydrolysis of the trilactone ring of Ent, resulting in the change of a hexadentate ligand to
three bidentate ligands. Such a change increases the redox potential from -750 mV to -350 mV, allowing the reductase YqjH to reduce ferric ion to ferrous ion and facilitate the iron release.\textsuperscript{67}

5.2.3 Enterobactin and Salmochelins

The second part of this thesis focuses on Ent and its glycosylated derivatives, salmochelins (GlcEnt). These secondary metabolites are tris-catecholate siderophores that are used by Gram-negative Enterobacteriaceae, including \textit{E. coli}, \textit{Salmonella} spp., and \textit{Klebsiella} spp.\textsuperscript{2} Ent is also used as xenosiderophores by bacterial strains that include \textit{P. aeruginosa}\textsuperscript{68} and \textit{B. subtilis}.\textsuperscript{69} To date, Ent is considered to be the natural product with the highest affinity for ferric ion (\(K_d = 10^{-49}\) M at neutral pH),\textsuperscript{2} and both Ent and GlcEnt contribute to bacterial virulence.\textsuperscript{70-72} The \textit{fep-ent} gene cluster consists of \textit{entABCDEF} and \textit{fepABCDEG} and which are responsible for the biosynthesis and transport of Ent, respectively.\textsuperscript{2} The \textit{iroA} gene cluster encoding \textit{iroBCDEN} is responsible for the biosynthesis and transport of GlcEnt and this gene cluster is found in uropathogenic \textit{E. coli}, \textit{Salmonella}, and \textit{Klebsiella} strains.\textsuperscript{53} Both gene clusters are regulated by Fur.

Ent is synthesized in bacteria by the enterobactin synthetase, a non-ribosomal peptide synthetase (NRPS). Chorismic acid is a precursor for 2,3-dihydroxybenzoic acid (DHB) via three steps catalyzed by EntC, EntB, and EntA (\textbf{Figure 5.11}). The assembly of Ent from DHB and \textit{L}-serine requires the adenylation enzyme EntE, phosphopantetheinyl transferase EntD, aryl carrier domain of EntB, and the multi-domain enzyme EntF. Post-assembly line tailoring affords GlcEnt (\textit{vide infra}).
Figure 5.11. Biosynthesis of Ent by EntABCDEF. (a) Overview of the assembly line. PPTase = phosphopantetheinyl transferase. A = adenylation domain. ICL = isochorismate lyase domain. ArCP = aryl carrier protein. C = condensation domain. PCP = peptidyl carrier protein. TE = thioesterase domain. (b) The biosynthesis of DHB from chorismic acid is catalyzed by EntABC. (c) The assembly of Ent is achieved by EntBDEF. The aryl carrier domain of EntB and EntF is phosphopantetheinylated by EntD, and then acylated with activated DHB and serine by EntE. The formation of the amide bond is catalyzed by the condensation of EntF, which also catalyzes the cyclization and hydrolytic release of Ent.

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After Ent is synthesized, it is transported across the inner membrane into the periplasm via the efflux pump EntS, which is also under Fur regulation. The outer membrane channel protein ToIC is important for exporting Ent into the extracellular space. Nonetheless, Ent breakdown products were identified in the growth media of EntS- or ToIC-deficient strains, suggesting that there are other secretion pathways for these smaller molecules. The uptake machinery for ferric Ent was discussed in the previous section (Figure 5.10).

The biosynthesis and transport machinery of GlcEnt share some similarity with those of Ent as shown in Figure 5.12. GlcEnt results from post-assembly line tailoring of Ent and the C-glycosyltransferase IroB is responsible for attaching glucose moieties to the catechol rings. Figure 5.13 illustrates structures of GlcEnt derivatives, including salmochelin S2, the linearized form of diglycosated enterobactin (DGE), and salmochelin S4, the cyclic form of DGE. The outer membrane receptor IroN recognizes GlcEnt and its degradation products. The inner membrane Ent transporter FepCDG delivers GlcEnt into the cytosol. The ABC transporter IroC was proposed to be the inner membrane permease for GlcEnt and it may function as the exporter for GlcEnt. Two esterases, IroD and IroE, hydrolyze both Ent and GlcEnt. IroD is located in the cytoplasm, whereas IroE is proposed to be located in the periplasm with an N-terminal transmembrane domain. IroD was shown to prefer apo Ent and GlcEnt, whereas IroE hydrolyzes both apo and ferric GlcEnt with similar efficiency.
Figure 5.12. GlcEnt biosynthesis and uptake machinery. The C-glycosyltransferase IroB introduces glucose moieties to the C5 positions of the catechol rings. The esterase IroD may be involved in the synthesis of lineralized GlcEnt. IroN is the receptor for GlcEnt, which recognizes Ent and the degradation products of Ent and GlcEnt. The Ent receptor FepA and Cir can also recognize degradation products of GlcEnt. The inner membrane permease complex FepCDG is proposed to transport GlcEnt and its derivatives into the cytosol. The periplasmic esterase IroE and cytoplasmic esterase IroD play a role in the release of iron. GlcEnt = salmochelins. DBS = DHB-serine. GDBS = glycosylated DHB-serine.

Figure 5.13. Representative structures of salmochelins. The glucose moieties are shown in red.
Ent can be isolated from the culture supernatant of a mutant *E. coli* strain that lacks FepA\textsuperscript{78} or chemically synthesized. Corey and coworkers reported the first total synthesis of Ent, in which the trilactone ring was obtained through sequential coupling steps of the monomers and multiple protection and deprotection steps.\textsuperscript{79} Subsequently, Ramirez and coworkers reported an improved synthetic approach towards Ent, which involves a one-step synthesis of the trilactone ring.\textsuperscript{80} More recently, Nolan and coworkers reported the synthesis of monofunctionalized Ent, which contains a carboxylic acid group at C5 position of one of the catechol rings.\textsuperscript{81} This modification allows for the attachment of several types of molecules, including antibiotics and fluorophores, to Ent, which is shown to facilitate the delivery of these cargos into the cytosol of Gram-negative bacteria.\textsuperscript{81,82} In addition, due to the stability issue of the trilactone ring in Ent, several Ent analogs have been synthesized (Figure 5.14) to enable *in vitro* characterization of Ent and its uptake machineries. For example, the trilactone ring was replaced with tricyclic tripod (13),\textsuperscript{83} 1,3,5-trisubstituted phenyl ring (14, TRIMCAM, and 15, MECAM),\textsuperscript{84} tris(2-aminoethyl)amine (16, TRENCAM)\textsuperscript{85} to afford Ent analogs with enhanced stability.\textsuperscript{85} Nevertheless, these Ent analogs exhibit lower iron-binding affinities than Ent, indicating that the trilactone ring is essential for the high iron-binding affinity of Ent.

![Figure 5.14. Structures of synthetic Ent analogs.](image-url)
5.3 Targeting Virulence to Combat Bacterial Infections

The rapid increase in resistance to antibiotics combined with the slow progression of a new antibiotic development over the past decades has led to a looming public health crisis in combating microbial infections. An alternative to killing bacteria is to search for strategies that disarm bacteria and prevent them from causing disease. Targeting bacterial virulence offers several advantages, including reducing resistance development due to decreased selective pressure, potentially enhancing target specificity and thereby preserving the gut microbiota. There are many virulence pathways that are currently being targeted for the development of antivirulence drugs. These pathways include adhesion, secretion, and toxin production, which are discussed below.

5.3.1 Adhesion

An essential first step for bacteria to colonize is to adhere to host cells. Blocking this process may prevent bacteria from colonizing and causing infection. Bacterial adhesion is mediated by surface proteins, including adhesins, autotransporters, and multi-protein scaffolds (e.g. pilus) extending from bacteria that interact specifically with carbohydrates on the host cell surface. In Gram-positive bacteria, adhesion depends on sortases, a family of cysteine transpeptidases that covalently anchor adhesins to the bacterial cell wall. Antibacterial-drug screening processes against S. aureus and B. anthracis have identified hits that could potentially be developed into potent sortase inhibitors.
In contrast, cell adhesion and invasion in Gram-negative bacteria relies on the production of pilus. Two approaches have been developed to block pilus-mediated adhesion. One is the identification of pilicides, which are mimics of the normal pilin subunit that, when incorporated into the growing pilus, prevent elongation and the formation of functional pilus. Pilicides can also reduce production of several components of the chaperon-usher pathway in uropathogenic *E. coli*, including type I and P fimbriae proteins, Dr family adhesins, and curli. Some pilicides also prevent biofilm formation. The chaperone-usher pilus assembly system is present in many Gram-negative pathogens, such as *E. coli*, *Salmonella* spp., *Klebsiella* spp., *Yersinia* spp., *Pseudomonas* spp. Treatment with pilicides decreased the efficiency of colonization of uropathogenic *E. coli* isolates in the urinary tract.

The other strategy to inhibit pilus-based adhesion is to physically block the interaction between the adhesin and the host cell. Many carbohydrate derivatives and molecules mimicking mammalian glycans significantly reduced the adhesive property of bacterial pilus. For example, an inhibitor, which targets FimH, a type I fimbrial adhesin, prevented acute infection *in vivo* and also treated chronic cystitis caused by a multi-resistant *E. coli* in an animal model.

5.3.2 Specialized Bacterial Secretory Systems

Bacteria have evolved complex machineries to deliver proteins and toxins into a host cell and these systems play an important role in pathogenesis. Three systems have been identified: type III, IV, and VI. Each of these machineries has minor differences in structure and gene content, but they are all driven by ATP hydrolysis. Type III secretion
systems resemble syringes that inject effectors into the host cell. Type IV secretion systems use pilus-like structure and type VI secretion systems are tubular structures and are the most recently found. To date, the most studied system is the type III secretion system (TTSS). This syringe-like multiprotein complex punctures the host membrane and injects bacterial effector proteins and toxins directly into the host cytosol, which hijacks a wide range of host cellular processes. Many components of the TTSS are specific to prokaryotes and TTSS inhibitors have been shown as potential therapeutics. TTSS inhibitors either block the interaction between TTSS and the host cell or prevent the secretion of the effector molecules.

Several small-molecule TTSS inhibitors have been identified via high-throughput screening of small-molecule libraries. *Chlamydia* spp. are obligate intracellular bacteria and encode a TTSS that secretes peptides into the inclusion membrane of the infected host cell and thereby is essential for intracellular survival of this pathogen. A class of TTSS inhibitors called acylated hydrazones of salicylaldehydes (17-19, Figure 5.15) blocked secretion through the TTSS of *Chlamydia* spp. Moreover, these molecules impaired the developmental process of *Chlamydia* spp. by locking the bacteria into one phase and preventing the progression of disease. Another study identified small-molecule inhibitors, including INP0400 (20, Figure 5.15), which blocked the TTSS of *C. trachomatis* and *Y. pseudotuberculosis*. INP0400 inhibited TTSS secretion in the *C. trachomatis* infection and impaired the virulence of *C. trachomatis*. Nonetheless, neither of these studies probed the molecular target of TTSS inhibition by these molecules. Another example is enteric pathogens, which infect the human gastrointestinal tract. Small-molecule screens have identified TTSS inhibitors (21-23,
Figure 15.5) of enteropathogenic *E. coli*,<sup>110</sup> *Shigella* spp.,<sup>111</sup> *S. Typhimurium*, *P. syringae*, and *Y. enterocolytica*.<sup>112,113</sup> Although screens have been performed for a number of inhibitors, the precise mechanism of inhibition and molecular targets have not been determined.<sup>104</sup>

![Structures of antivirulence drugs.](image)

**Figure 5.15.** Structures of antivirulence drugs.

### 5.3.3 Toxins

Toxins are the primary virulence factors of many pathogens. Examples include botulinum and tetanus neurotoxins, cholera, anthrax, diphtheria, and Shiga toxins. These proteins are delivered into the host to cause mass cell destruction and tissue damage.<sup>114</sup> The cytotoxicity and critical role in pathogenesis of these toxins makes them potential targets for development of antivirulence antibacterials. Targeting toxin transcription and expression have shown to be promising antivirulence strategies. For example, a small-molecule inhibitor of toxin TcdA and TcdB expression in *Clostridium*
difficile exhibited antivirulence property in a hamster model of gastrointestinal
infection. Another example is virstatin, which inhibits the transcriptional factor that
regulates expression of cholera toxin. This molecule will be discussed further in the next
section.

In addition, antibodies have been developed to neutralize toxins and already
employed to treat certain bacterial infections, such as tetanus, diphtheria, and
botulism. There are other antibody-based therapies, which are still in the
developmental stage. For example, antibodies against Shiga toxin conferred protection
against Shiga toxin-producing E. coli in a piglet model of acute gastroenteritis.
Antibodies that inhibit anthrax toxins (e.g. ABthrax and Valortim) have provided
promising protection against B. anthracis infection in a range of animal models and are
in clinical development.

5.3.4 Virulence gene expression

V. cholerae is responsible for some of the largest irregular outbreaks (pandemics) of
diarrhoeal disease. Studies on inhibition of expression of virulence genes in V. cholerae
have been conducted to search for new strategies to prevent infection caused by this
organism. ToxRS and TcpPH, which are expressed on the membrane, control the
expression of the transcriptional factor ToxT. Dimerization of ToxT activates expression
of two essential virulence factors: cholera toxin (CT) and toxin co-regulated pilus
(TCP). A small-molecule screening of 50,000 compounds identified 15 molecules that
inhibited virulence gene expression, but did not kill V. cholerae. For example,
virstatin (24, Figure 5.15) directly inhibited dimerization of ToxT and thereby
suppressed expression of CT and TCP. It also blocked intestinal colonization of *V. cholerae* in an infant-mouse model.\textsuperscript{122}

5.3.5 *Cell-to-cell signaling*

Bacterial cell-to-cell communication is required for bacteria to adapt to changes in the environment and this communication is regulated by quorum-sensing (QS) systems. Bacteria use complex regulatory QS networks to sense their population densities and regulate the expression of virulence factors, allowing successful establishment of infection.\textsuperscript{124} Due to the importance of QS systems in bacterial pathogenesis, several efforts have focused on interfering with these systems.\textsuperscript{125} One advantage of interfering with bacterial QS systems is that they are specific to the pathogen of interest.\textsuperscript{104} QS pathways can be modulated at three different stages:

(i) Inhibiting signal generation, for example, by blocking synthesis of acyl homoserine lactones (AHLs) \textit{in vitro} using AHL analogues.\textsuperscript{126,127}

(ii) Degrading the signal molecule. For example, *Bacillus* spp. express lactonases that degrade the homoserine ring of AHLs. Transgenic plants expressing the *Bacillus* lactonase exhibited resistance to infection caused by bacteria that use AHLs as quorum-sensing molecules.\textsuperscript{128} Moreover, AHLs can be degraded by increasing the pH or inactivated with antibodies.\textsuperscript{129}

(iii) Blocking the interaction of the QS signal molecule with the receptor. Screening of natural and synthetic compounds has yielded potent antigonists of sensing receptors for many bacteria, including *Pseudomonas* spp. and *Staphylococci*.\textsuperscript{130-132} For example, the marine macroalga *Delisea pulchra* blocks virulence gene
expression that is dependent on quorum sensing by producing a halogenated furanone, which acts as a competitive inhibitor for the bacterial AHL-based quorum-sensing system. C-30 (25, Figure 5.15), a derivative of this furanone, inhibited quorum sensing and caused P. aeruginosa biofilms to be susceptible to clearance by detergent and antibiotics.

In summary, these antivirulence strategies have provided promising outcome in combating bacterial infections and are still on the process of development. Nevertheless, apart from antibodies that inactivate specific bacterial toxins, none of these compounds with new mechanisms of action has made it to the clinical trial. Therefore, it is difficult to determine whether antivirulence agents can be the next generation of therapeutic approach against bacterial infections.

5.4 Siderophore-Based Approaches Towards Treatment of Bacterial Infections
As discussed in Section 5.2, siderophores are classified as virulence factors of many pathogens, including Salmonella spp., Klebsiella spp., P. aeruginosa and S. aureus. Siderophores are essential for the pathogens to colonize and cause infection in the host and the bacteria produce dedicated uptake machineries for importing ferric-siderophore complex into the cytosol. Therefore, siderophores and their uptake systems have become potential targets for developing new therapeutic strategies against bacterial infections. Examples of these siderophore-based strategies are discussed below.
5.4.1 Siderophore-Antimicrobial Conjugates

The design and development of a “Trojan Horse” strategy employing the siderophore and its dedicated uptake machinery have been made for decades. Most studies focus on Gram-negative bacteria. Due to the presence of the outer membrane, which serves as a permeability barrier, Gram-negative bacterial infections are generally harder to treat compared to Gram-positive bacterial infections. This Trojan Horse strategy has been inspired by studies on sideromycins and microcins, natural siderophore-antimicrobial conjugates. Furthermore, the siderophore-mediated iron uptake machinery is a virulence factor and it is upregulated during infection. These facets make the siderophore-antimicrobial strategy a fascinating and promising solution to Gram-negative bacterial infections. Moreover, this approach provides specificity towards certain bacterial species; therefore, conjugating to siderophores can tune a broad-spectrum antibiotic into a narrow-spectrum one.

In 1977, the first synthetic siderophore-antibiotic conjugates were made by a Japanese pharmaceutical company. The conjugates were comprised of desferrioxamine B and ferricrocin linked to sulfonamide antibiotics. Since 1980s, the Miller group at University of Notre Dame have become one of the major contributors to this field. Other contributors to the field include the Braun group, the Budzikiewicz group, the Möllmann group, and the Mislin group. Most of studies have focused on catechol/hydroxamate siderophore analogs conjugated to β-lactams. β-Lactams covalently bind the transpeptidases, which are involved in the synthesis of peptidoglycan, an essential component of bacterial cell wall. Care is required in the design of a siderophore-antibiotic conjugate so that: the mechanism of siderophore
recognition and uptake is not hampered; a suitable linker is used, thus the conjugate is stable in extracellular environment, but the antibiotic often needs to be released intracellularly in either the periplasm or the cytosol to maximize the activity of the conjugate.\footnote{150}

One of the most successful siderophore-antibiotic conjugate is BAL30072 bearing a dihydroxypyridone (26, Figure 5.16) as the siderophore-mimicking small molecule and aztreonam as the drug. This conjugate provided promising results enough to enter the clinical trials. BAL30072 exhibited potent antibacterial activity against several strains of Gram-negative bacteria, including multidrug-resistant (MDR) species.\footnote{151} The conjugate also proved effective against 80\% of the \textit{Acinetobacter baumanii} strains tested in a rat soft-tissue infection model.\footnote{152} \textit{In vitro} combinations of BAL30072 and carbapenems were more effective than individual agents against MDR Gram-negative strains, including Enterobacteriaceae and \textit{P. aeruginosa}.\footnote{153}

As discussed above, many siderophores have been employed in the siderophore-antimicrobial conjugates. For example, Budzikiewicz and coworkers synthesized pyoverdin-ampicillin (Amp) conjugates (27 and 28, Figure 5.16) to target \textit{P. aeruginosa} and the conjugates displayed minimum inhibitory concentrations in a low micromolar range, whereas Amp was not active at all.\footnote{142} Miller and coworkers synthesized few catechol/hydroxamate-carbacepham conjugates\footnote{143,148} (29, Figure 5.16) and reported that these conjugates exhibited low antibacterial activities compared to the parent antibiotics; however, their uptake was shown to be dependent on the siderophore receptors.\footnote{154,155} More recently, Nolan and coworkers reported the synthesis of Ent-
Amp/Amx (30, Figure 5.16), which displayed up to 1,000-fold enhanced antibacterial activity against *E. coli* and *P. aeruginosa*.\(^2\)

Several siderophores, including staphyloferrin A, citrate, hydroxamate, pyoverdin, and pyochelin, have been attached to fluoroquinolones,\(^{156-159}\) which target DNA gyrases and topoisomerases IV and cause DNA fragmentation in bacteria.\(^{160}\) These antibiotics exhibit potent antimicrobial activities; however, they also display mild to moderate adverse effects in patients.\(^{161}\) Nevertheless, these conjugates showed much lower antibacterial activities compared to the parent drugs. One possible explanation for such low activities could be due to the improper design for the linker. This notion is exemplified by a recent work done by Miller and coworkers on siderophore-ciprofloxacin conjugates bearing a reducible linker (31, Figure 5.16).\(^{162}\) Two of these conjugates were more potent than their counterpart with a non-cleavable linker, suggesting that the drug release occurs inside the bacterial cells. Nevertheless, they were still weaker than the unmodified drug; therefore, more optimization on the siderophore recognition, the linker design, or both factors is required.

It should be noted that the Miller group synthesized a mycobactin-artemisinin conjugate (32, Figure 5.16).\(^{163}\) Artemisinin is reduced and cleaved by ferrous ion to generate toxic oxygen-reactive radicals.\(^{164}\) Although artemisinin has antimalarial activity, but no anti-tuberculosis property, coupling it to mycobactin led to a conjugate with displayed highly potent antibacterial activity against *M. tuberculosis*, including several MDR isolates. This high potency may be attributed to the release of iron inside the bacterial cells following the mycobactin uptake, which provides ferrous ion in close proximity to artemisinin.
In addition, siderophore analogs have been used to facilitate the synthesis and/or enhance the stability of siderophore-antimicrobial conjugates. For example, Miller and coworkers reported an Ent analog coupled to Amp and Amx (33, Figure 5.16), which exhibited significantly enhanced activity against \textit{P. aeruginosa} strains under iron-limited conditions; however, such enhancement was not observed against \textit{E. coli} and \textit{K. pneumoniae}.\textsuperscript{144} Another example is a series of siderophore-monobactam conjugates that were BAL30072 mimics. In these conjugates, pyridoxal isonicotinoyl hydrazine (PIH) was used as the siderophore-mimicking small molecule and covalently linked to aztreonams that were used as the antibiotics.\textsuperscript{165} Some of these conjugates exhibited enhanced antibacterial activities against \textit{E. coli} strains, including MDR ones.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{5.16}
\caption{Examples of siderophore-antibiotic conjugates. The siderophore parts are shown in red.}
\end{figure}
Taken together, although these studies provide mixed results in term of success of the strategy, they have provided few useful lessons for future studies. One of the important factors is the choice of siderophore used in the synthesis. Bacteria may utilize more than one siderophore-mediated iron uptake systems during infection. Hence, with the available information on the role of siderophores in bacterial virulence factors and infection, one should select proper siderophores based on their importance during infection when designing the synthesis of siderophore-antimicrobial conjugates. In addition, the relative expression levels of different siderophores and their receptors should also be taken into account.

5.4.2 Antibody-Based Approaches Against Siderophore-Mediated Iron Uptake System

Antibodies were naturally produced by the immune system to fight against invading pathogens and antibody-based therapy was used for the treatment of several infections even before the age of antibiotics. Passive immunization with hyper-immune serum was introduced in the 19th century by Behring and coworkers. Patients with diphtheria and tetanus received horse sera obtained from immunization with toxoids of Corynebacterium diphteriae and C. tetani, respectively. Since the pathogenesis of diphtheria and tetanus is driven by highly potent toxins, employing antibodies to neutralize these toxins resulted in effective treatment. Serum therapy was also applied to other infections caused by Streptococcus pneumoniae and S. aureus. For these two pathogens, serum therapy was less effective than the case of diphtheria and tetanus, likely due to a more complex disease pathogenesis, as well as higher heterogeneity of the pathogens. Antibacterial serum therapies were abandoned after the
introduction of antibiotics in 1940s. Nevertheless, after half a century, the use of antibiotics has caused widespread resistance in bacteria. To address the failing antibiotic pipeline, serum therapy in the form of monoclonal antibodies (mAbs) is now being reintroduced as one of the potential strategies to fight bacterial infections. To date, most mAbs target either bacterial endotoxins or bacterial surface components. For example, mAbs were produced against lipopolysaccharide, the outer membrane component in Gram-negative bacteria, and against lipoteichoic acid, a cell wall component in Gram-positive bacteria. Another example is Aurograb, which is an antibody against the GrfA surface protein of *S. aureus*. GrfA belongs to a family of ABC transporters, membrane-anchored proteins involved in transport of macromolecules across the membrane. Aurograb exhibited synergistic effect with vancomycin; however, it failed to show efficacy in the clinical phase III in 2004-2006. A few attempts to utilize mAbs that target siderophore and its uptake machinery were also made to inhibit siderophore-mediated iron acquisition of bacteria and thereby prevent them from causing diseases in the host. The details of these studies are discussed below.

To successfully acquire iron from the environment by employing siderophores, bacteria need to express specific receptors on the membrane to recognize and transport the ferric siderophore complexes back into the cytosol. Therefore, interfering with the recognition between the complexes and the receptors may be another potential strategy to develop new treatment for bacterial infection. For example, a few studies using antibodies to target the ferric Ent receptor FepA in *E. coli* were conducted. Smith and coworkers demonstrated that a murine monoclonal antibody that recognized FepA
exhibited an inhibitory effect on the growth of *E. coli* isolates from a bovine intramammary infection (IMI); however, they found that antigenic variation in the binding site of FepA resulted in a low percentage of *K. pneumoniae* isolates of which growth was inhibited by the monoclonal antibody.\textsuperscript{176} FepA was also overexpressed and administered into mice or rabbits to stimulate immune responses to FepA.\textsuperscript{177,178} The immunized mice tolerated oral challenge of *E. coli* O157:H7, *S. flexneri*, *K. pneumoniae*, and *S. enterica* serotype typhi better than the control group.\textsuperscript{177} Another study revealed that serum isolated from FepA-immunized rabbits displayed potent antibacterial activity against *E. coli* O157:H7, *S. flexneri*, *K. pneumoniae*, and *S. enterica* serotype typhi and the mice that were passively immunized with the rabbit serum tolerate bacterial challenge better than the control group.\textsuperscript{178}

Sequestering a ferric-siderophore complex is another possible strategy to target bacterial virulence and prevent the bacteria from colonizing and causing infection in the host. This notion is exemplified by the use of LCN2 by the mammalian innate immunity as discussed in Section 5.1.4. In 2009, Green and coworkers reported the development of a vaccine strategy based on vibriobactin (11, Figure 5.7), a siderophore produced by *Vibrio* spp.\textsuperscript{179} In this work, a vibriobactin analog was synthesized and conjugated to ovalbumin (OVA) and bovine serum albumin (BSA). When mice were immunized with the OVA-vibriobactin conjugate, IgG mAbs were isolated and characterized to be specific to vibriobactin moiety of both OVA-vibriobactin and BSA-vibriobactin conjugates; however, passive immunization with these isolated mAbs was not conducted to test their efficacy against *Vibrio* infection in an animal model.
5.4.3 Disrupting Siderophore-Mediated Iron Uptake Machinery

Siderophore-mediated iron acquisition machinery plays an important role in bacterial colonization in the host and many siderophores are shown to be virulence factors. Many bacterial strains that are deficient in siderophore biosynthesis turn to be avirulent in vivo. Furthermore, one of host-defense responses to bacterial infection is secretion of LCN2 to bind ferric siderophore complexes, and hence prevent the bacteria from colonization. As a result, targeting siderophore-dependent iron uptake systems is a promising strategy for developing new therapeutic approaches for bacterial infections. Studies on nonribosomal peptide synthetases (NRPS) that are involved in siderophore biosynthesis have provided useful insights into developing inhibitors targeting the enzymes.

The development of inhibitors that target the salicylate synthases Irp9 in *Yersinia peptis* and Mtbl in *M. tuberculosis* was investigated. These enzymes are involved in the first step towards the synthesis of yersiniabactin and mycobactin, respectively. They convert chorismate to salicylate in two steps (Figure 5.17a). Chorismate analogs (34, Figure 5.17b) were synthesized and tested for their inhibitory potency against Irp9\textsuperscript{181} and Mtbl.\textsuperscript{182} The reported $K_i$ values were in the 10-μM range. A high-throughput screening against Mtbl was also performed and revealed a series of noncompetitive reversible inhibitors, such as benzimidazole-2-thiones (35, Figure 5.17b), with the lowest 50%-inhibitory concentration (IC$_{50}$) of 7.6 μM.\textsuperscript{183} Nonetheless, investigation on antibacterial activity of these inhibitors under iron-deficient conditions is required.
Figure 5.17. (a) Reaction mechanism of salicylate synthase. (b) Inhibitors of salicylate synthase.

Activation of salicylate catalyzed by adenylation enzymes is another target for inhibiting siderophore biosynthesis. This process involves two steps: the carboxylate is condensed with ATP to form the corresponding adenylate and release pyrophosphate; the activating adenylate is then transferred to the peptidyl carrier protein and forms a thioester bond with the protein (Figure 5.18a). One example of this strategy was done via a high-throughput screening against the adenylation enzyme BasE, which is involved in the synthesis of acinetobactin in A. baumannii. The most potent inhibitor (36, Figure 5.18b) obtained from this method inhibits the enzyme by using the phenyl ring to block the channel, which is required for the PCP domain to reach the activated salicylate. A series of these inhibitors found from the screening was subsequently modified, resulting in an improved inhibitor (37, Figure 5.18b) with higher binding affinity to BasE ($K_d = 2$ nM). Nevertheless, these inhibitors displayed low in vitro antibacterial activity against A. baumannii probably due to the outer membrane acting as a permeability barrier for the inhibitors.
Figure 5.18. (a) Activation of salicylate catalyzed by adenylation enzymes. (b) Inhibitors of salicylate adenylation enzymes.

The biosynthesis of some siderophores involves a non-ribosomal peptide synthetase independent siderophore (NIS) synthetase.\textsuperscript{186} Examples of these siderophores include staphyloferrin B and petrobactin, which are produced by \textit{S. aureus} and \textit{B. anthracis}, respectively. A high-throughput screening using marine microbial-derived natural product extracts was performed against SbnE and AsbA, which are enzymes involved in the biosynthesis of staphyloferrin B and petrobactin, respectively.\textsuperscript{187} The screening yielded two potential inhibitors (Figure 5.19) with the lowest IC\textsubscript{50} values of 4.8 \(\mu\)M against SbnE and 180 \(\mu\)M against AsbA. An antimicrobial activity was also evaluated against \textit{S. aureus}, \textit{B. anthracis}, and \textit{E. coli} and the MIC values were in a range of 0.5-1 mM.\textsuperscript{187} Taken together, these proof-of-concept studies demonstrate that siderophore biosynthesis pathways are potential targets for developing new antibiotics although the current strategy requires further optimization.
For example, a Trojan Horse strategy using siderophore-inhibitor conjugates may be needed to overcome the permeability barrier by the outer membrane.

![Chemical structures of Baulamycins A and B](image)

**Figure 5.19.** Inhibitors of NIS enzymes SbnE and AsbA.\(^{187}\)

Another potential target in the siderophore-mediated iron transport system is the siderophore exporter. A recent study revealed that interfering with the export process resulted in self-poisoning of the producers.\(^{188}\) In this study, a knock-out mutant of two membrane proteins MmrS4 and MmrS5 in *M. tuberculosis*, which are responsible for exporting mycobactin and carboxymycobactin, exhibited impaired virulence in a mouse model. In addition, the mutant was unable to grow under iron-deficient conditions and addition of mycobactin and carboxymycobactin at low micromolar concentrations to the culture resulted in the growth inhibitory effect. Once the siderophore biosynthesis is inhibited, the growth of the mutant was recovered on a hemoglobin-supplemented plate. These results indicated that overaccumulation of siderophores was toxic to these bacteria probably by causing iron deprivation in the cytosol. Another example is that the knock-out mutants of membrane proteins EntS and IroC, which are responsible for exporting Ent and GlcEnt, displayed attenuated virulence in a chicken infection model.\(^{189}\) Intracellular accumulation of Ent and GlcEnt was observed in these mutant *E. coli* strains. Although no inhibitors for the siderophore receptors and exporters have been reported, many studies suggest that blocking either ferric-siderophore uptake or

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siderophore export results in impaired virulence in bacteria. Therefore, these membrane proteins are also interesting targets for new antibiotic development.

5.5 Goals and Organization of the Siderophore-Related Projects

This dissertation project focuses on two strategies that utilize siderophore-mediated iron acquisition to target Gram-negative bacteria. The emergence of antibacterial resistance and the paucity of new antibiotic development create a worldwide public health crisis.\textsuperscript{190,191} Narrow-spectrum antibiotics that target select pathogens are required to address this alarming issue. Siderophore-mediated iron uptake machinery plays an essential role in pathogenicity of bacteria, including \textit{E. coli} and \textit{S. Typhimurium}. In addition, different bacterial groups employ variable sets of siderophores. Therefore, the strategies that focus on siderophore and the dedicated uptake machinery are likely to provide specificity to select bacterial strains, as well as targeting pathogenicity and minimally perturbing commensal bacteria.

In Chapter 6, we present a strategy that specifically delivers antibiotics to bacterial pathogens, which utilizes the salmochelin (GlcEnt)-mediated iron uptake machinery. GlcEnt-β-lactam conjugates that harbor the antibiotics ampicillin (Amp) and amoxicillin (Amx), hereafter GlcEnt-Amp/Amx, were synthesized and the conjugates exhibit up to 1000-fold enhanced antimicrobial activity against uropathogenic \textit{E. coli} relative to the parent β-lactams. Moreover, GlcEnt-Amp/Amx based on a diglycosylated Ent platform selectively kill uropathogenic \textit{E. coli} that express the salmochelin receptor IroN in the presence of non-pathogenic \textit{E. coli} and other bacterial strains that include the commensal microbe \textit{Lactobacillus rhamnosus} GG. Moreover, GlcEnt-Amp/Amx evade
the host-defense protein LCN2, and exhibit low toxicity to mammalian cells. This work establishes that siderophore-antibiotic conjugates provide a strategy for targeting virulence, narrowing the activity spectrum of antibiotics in clinical use, and achieving selective delivery of antibacterial cargos to pathogenic bacteria on the basis of siderophore receptor expression.

In Chapter 7, we present an immunization approach that targets siderophores. This work was performed in collaboration with the Raffatellu Lab at UC Irvine. Conjugates of Ent and the immunogenic carrier protein cholera toxin subunit B (CTB) were synthesized and used to immunize mice. The immunized mice produce anti-siderophore antibodies in the gut mucosa. When infected with the enteric pathogen Salmonella, the immunized mice exhibit reduced intestinal colonization, reduced systemic dissemination of the pathogen, and increased survival. Moreover, analysis of the gut microbiota reveals that the reduction of Salmonella colonization is accompanied by the expansion of Lactobacillus spp., beneficial commensal organisms that thrive in a similar environment as Enterobacteriaceae. Taken together, our results demonstrate that antibodies against siderophores confer protection against infection with Salmonella and possibly other Enterobacteriaceae that deploy similar molecules to acquire iron in the host.

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Chapter 6

Targeting Virulence: Salmochelin Modification Tunes the Antibacterial Activity Spectrum of β-Lactams for Pathogen-Selective Killing of *Escherichia coli*

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6.1 Contributions

Dr. T. Zheng designed and developed the synthetic protocols for GlcEnt-Amp/Amx and conducted preliminary antimicrobial activity assays of these conjugates against *Escherichia coli* CFT073 and K12.

6.2 Introduction

Bacterial infections, the rise in antibacterial resistance in hospital and community settings, and the paucity of new antibiotics in the current drug pipeline create a worldwide public health crisis.\(^1,2\) New strategies to diagnose and treat bacterial infections as well as counteract the emergence and spread of antibiotic resistance in bacterial pathogens are urgently needed to reduce morbidity and mortality, as well as the economic burden, caused by these infections.\(^3,4\) The discovery of narrow-spectrum antibiotics that target select pathogens is one important and necessary facet of this large and complex problem.\(^2,5,6\) Pathogen-specific antibiotics that minimally perturb the normal microbial flora are expected to reduce the likelihood of colonization by pathogenic and drug-resistant microbes during or after antibiotic treatment, and prevent life-threatening secondary infections such as those caused by *Clostridium difficile*.\(^5,7\) Moreover, the availability of narrow-spectrum antibiotics, coupled with rapid diagnostics, is expected to reduce the use of broad-spectrum therapeutics and thereby slow down the evolution of drug resistance.\(^2,5,7\) Among current and emerging microbial threats, Gram-negative bacteria, including pathogenic *E. coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Salmonella* spp., pose a challenge for antibiotic drug discovery.\(^1,2,8\) These strains have an outer membrane that serves as a permeability
barrier and prevents the cellular entry of many antibiotics. In this work, we report a stealth antibiotic delivery strategy that overcomes the outer membrane permeability barrier of Gram-negative \textit{E. coli} and targets pathogenicity by hijacking the iron import machinery utilized by virulent strains during colonization in the mammalian host.

Iron is an essential nutrient for almost all bacterial pathogens. Because iron exhibits low solubility in aqueous solutions at physiological pH and enables Fenton chemistry, the levels of "free" iron in mammals (ca. $10^{-24} \text{ M in serum}$) are tightly regulated by homeostatic mechanisms, which include the expression of the iron transport and storage proteins transferrin and ferritin. Most bacterial pathogens require micromolar concentrations of iron to colonize and cause disease, and bacterial iron acquisition machineries contribute to virulence.

![Figure 6.1. Siderophores and outer membrane siderophore receptors relevant to this work. (a) Chemical structures of enterobactin 1, and its glucosylated derivatives 2-4. (b) A cartoon representation of the outer membrane receptors FepA and IroN considered in this work. (c) Chemical structures of Ent-Amp/Amx 5-6.](image_url)
One way that bacteria scavenge iron in the host environment is to biosynthesize and export siderophores, secondary metabolites that chelate Fe$^{3+}$ with high affinity.\textsuperscript{15} The ferric siderophores are recognized and transported into the cell by dedicated uptake machinery. In this work, we consider the catecholate sideophore enterobactin (Ent, \textbf{Figure 6.1a}), its glucosylated congeners 2-4 (GlcEnt, \textbf{Figure 6.1a}), and the outer membrane receptors for these iron chelators. Ent is biosynthesized by all \textit{E. coli} and the ferric complex is transported across the outer membrane by the TonB-ExbB-ExbD-dependent outer membrane receptor FepA (\textbf{Figure 6.1b}).\textsuperscript{12} In addition to Ent, many pathogenic \textit{E. coli} as well as \textit{Salmonella} spp. biosynthesize salmochelins, C-glucosylated derivatives of Ent (\textbf{Figure 6.1a}).\textsuperscript{16} The \textit{iroA} gene cluster (\textit{iroBCDEN})\textsuperscript{14,17,18} encodes enzymes that tailor the Ent scaffold to provide the salmochelins (IroBDE), and proteins for salmochelin transport (IroCN). Expression of genes encoded by the \textit{iroA} locus contributes to virulence by providing Gram-negative pathogens with additional iron acquisition machinery and enabling the pathogens to overcome the host innate immune response.\textsuperscript{19,20} In the battle against such invading pathogens, the mammalian host mounts a metal-withholding response and secretes lipocalin-2 (LCN2), a 22-kDa antimicrobial protein that captures ferric Ent.\textsuperscript{19,21,22} Gram-negative pathogens that utilize salmochelins for iron acquisition readily evade this innate immune mechanism because the salmochelins cannot be sequestered by LCN2.\textsuperscript{19}

Because bacteria utilize siderophores to acquire nutrient iron during infection, these molecules, as well as the corresponding biosynthetic and transport machineries, provide opportunities for antibiotic development.\textsuperscript{10,11,23-27} The notion of using siderophores or siderophore mimics to deliver antibacterial cargo into bacterial cells has
garnered attention over several decades. Our approach to siderophore-based targeting focuses on harnessing native siderophore platforms used by pathogens in the human host for cargo delivery, and we seek to modify these scaffolds in ways that minimally perturb iron binding and receptor recognition. We have designed and utilized a monofunctionalized Ent platform to assemble a variety of Ent-cargo conjugates, and we reported that the Ent uptake machinery (FepABCDG) provides a means to transport small-molecule cargo, including antibiotics in clinical use, into E. coli. For instance, the Ent-β-lactam conjugates 5-6 (Figure 6.1c) target and kill E. coli expressing FepA. Because all E. coli use Ent for iron acquisition, the Ent-β-lactam conjugates target and kill both non-pathogenic and pathogenic E. coli strains. Some E. coli are commensal microbes, comprising <1% of the total microbial community in the human gut, that biosynthesize vitamin K that is needed by the host. Thus, the ability to target pathogenic E. coli has utility for minimally perturbing the normal flora. Inspired by prior investigations of native siderophore transport, we hypothesized that salmochelin-antibiotic conjugates will be specifically recognized by IroN, the outer membrane receptor for the salmochelins, and afford a strategy for overcoming the outer membrane permeability barrier, achieving narrow-spectrum antibacterial activity against pathogenic E. coli, and evading capture by LCN2.

In this work, we report the design and chemoenzymatic preparation of siderophore-β-lactam conjugates based on salmochelin platforms, and demonstrate targeting of β-lactam antibiotics to pathogenic E. coli that harbor the iroA gene cluster and express IroN. Salmochelin-inspired GlcEnt-β-lactam conjugates based on the diglucosylated Ent (DGE, Fig. 1a) platform provide selective antibacterial activity
against pathogenic *E. coli* and up to 1000-fold enhanced potency relative to the parent β-lactam antibiotics. Moreover, the salmochelin-inspired conjugates remain antibacterial in the presence of LCN2. These investigations establish a chemoenzymatic route to functionalized salmochelins and provide a new approach for transforming a broad-spectrum antibiotic in clinical use into a narrow-spectrum therapeutic that targets microbial pathogens on the basis of siderophore receptor expression.

### 6.3 Experimental Section

#### 6.3.1 Synthetic Reagents

Anhydrous dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich and used as received. All other chemicals and solvents were purchased from Sigma-Aldrich or Alfa Aesar in the highest available purity and used as received. The syntheses of Ent 1,49 MGE 2,50 DGE 3,51 Ent-Amp 5,44 Ent-Amx 6,44 Ent-PEG3-N3 11,44 Amp-alkyne 14, and Amx-alkyne 1544 are reported elsewhere.

#### 6.3.2 Instrumentation

Analytical and semi-preparative high-performance liquid chromatography (HPLC) were performed using an Agilent 1200 series HPLC system outfitted with a Clipeus reverse-phase C18 column (5-μm pore size, 4.6 x 250 mm; Higgins Analytical, Inc.) at a flow rate of 1 mL/min and an Agilent Zorbax reverse-phase C18 column (5-μm pore size, 9.4 x 250 mm) at a flow rate of 4 mL/min, respectively. The multi-wavelength detector was set to read the absorbance at 220, 280, and 316 (catecholate absorption) nm. HPLC-grade acetonitrile (MeCN) and trifluoroacetic acid (TFA) were purchased from EMD and
Alfa Aesar, respectively. For HPLC analyses, solvent A was 0.1% TFA/H$_2$O and solvent B was 0.1% TFA/MeCN, unless stated otherwise. The HPLC solvents were prepared with HPLC-grade MeCN and TFA, and Milli-Q water (18.2 mΩ•cm), and filtered through a 0.2-μm filter before use. For analytical HPLC to evaluate conjugate purity, the entire portion of each HPLC-purified compound was dissolved in a mixture of 1:1 MeCN/H$_2$O and an aliquot was taken for HPLC analysis. The remaining solution was subsequently lyophilized.

High-resolution mass spectrometry was performed by using an Agilent LC-MS system comprised of an Agilent 1260 series LC system outfitted with an Agilent Poroshell 120 EC-C18 column (2.7-μm pore size) and an Agilent 6230 TOF system housing an Agilent Jetstream ESI source. For all LC-MS analyses, solvent A was 0.1% formic acid/H$_2$O and solvent B was 0.1% formic acid/MeCN (LC-MS grade, Sigma-Aldrich). The samples were analysed using a solvent gradient of 5–95% B over 10 min with a flow rate of 0.4 mL/min. The MS profiles were analysed and deconvoluted by using Agilent Technologies Quantitative Analysis 2009 software version B.03.02.

Optical absorption spectra were recorded on a Beckman Coulter DU800 spectrophotometer (1-cm quartz cuvettes, Starna). A BioTek Synergy HT plate reader was used to record absorbance at 600 nm (OD$_{600}$) for antimicrobial activity assays and absorbance at 550 nm for cytotoxicity assays.

6.3.3 Enzymatic Activity Assays for IroB and MceC

The enzymes MceC and IroB were overexpressed as N-terminal His$_6$-fusion proteins in *E. coli* BL21(DE3) and purified as reported.$^{50,51}$ To a 405-μL solution containing Ent-
PEG₃-N₃ 11 (100 μM), uridine diphosphoglucose (UDP-Glc, 3 mM), and MgCl₂ (5 mM) prepared in 75 mM Tris-HCl buffer, pH 8.0, MceC (10 μM, 45 μL) or IroB (10 μM, 45 μL) was added to afford a final enzyme concentration of 1 μM. The reaction was incubated at room temperature and an aliquot (100 μL) was quenched by adding 10 μL of 6% TFA (aq) after 0, 15, 30, 60 min. The quenched reaction aliquots were immediately vortexed, centrifuged (13,000 rpm x 10 min, 4 °C), and analysed by HPLC (0–100% B over 30 min, 1 mL/min). The results are shown in Figures 6.2, 6.3.

6.3.4 Synthesis of MGE/DGE-PEG₃-N₃ (12-13)
A 6.3-mL solution containing Ent-PEG₃-N₃ 11 (500 μM), uridine diphosphoglucose (UDP-Glc, 3 mM), and MgCl₂ (5 mM) was prepared in 75 mM Tris-HCl buffer, pH 8.0 and divided into seven 900-μL aliquots. MceC (50 μM, 100 μL) or IroB (50 μM, 100 μL) was added to each aliquot to afford a final enzyme concentration of 5 μM. The 1-mL reactions were incubated at room temperature and quenched by addition of 100 μL of 6% TFA (aq) after 15 min (MceC reaction) or 2 h (IroB reaction). The quenched reaction aliquots were immediately vortexed, combined, and lyophilized to dryness. The resulting powder was dissolved in 3 mL of 1:1 MeCN/water and centrifuged (13,000 rpm x 10 min, 4 °C). MGE-PEG₃-N₃ 12 and DGE-PEG₃-N₃ 13 were purified from the supernatants of MceC- and IroB-catalysed reactions, respectively, by using semi-preparative HPLC (20–45% B over 8.5 min, 4 mL/min). Both compounds were obtained as white powders (MGE-PEG₃-N₃ 12, 0.66 mg, 41% from MceC-catalyzed reaction; DGE-PEG₃-N₃ 13, 0.85 mg, 45% from IroB-catalysed reaction). HRMS (ESI): MGE-PEG₃-N₃ 12, [M+H]⁺ m/z calcd. 1076.3215, found 1076.3214; DGE-PEG₃-N₃ 13, [M+H]⁺ m/z calcd.
1238.3743, found 1238.3744. The analytical HPLC traces of the purified compounds are reported in Figures 6.9, 6.10.

6.3.5 Synthesis of MGE-Amp (7)

Amp-alkyne 14 (50 µL of a 50-mM solution in DMSO, 2.5 µmol) and MGE-PEG₃-N₃ 12 (73 µL of an 11.3-mM solution in DMSO, 0.825 µmol) were combined and 100 µL of DMSO was added. An aliquot of aqueous CuSO₄ (50 µL of a 90-mM solution in water, 4.5 µmol) and tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, 100 µL of a 50-mM solution in DMSO, 5 µmol) were combined to give a blue-green solution, to which sodium ascorbate (NaAsc, 100 µL of a 180-mM solution in water, 18.0 µmol) was added. This solution became light yellow and was immediately added to the alkyne/azide solution. The reaction was gently mixed on a bench-top rotator for 2 h at room temperature and conjugate 7 was purified by semi-preparative HPLC (20% B for 5 min and 20–50% B over 11 min, 4 mL/min; 0.005% TFA was used in solvents A and B because of the acid-sensitive β-lactam moiety). Conjugate 7 was obtained as white powder (0.75 mg, 59%). HRMS (ESI): [M+H]+ m/z calcd., 1519.4730; found, 1519.4639. The analytical HPLC trace of the purified product is reported in Figure 6.5.

6.3.6 Synthesis of MGE-Amx (8)

As described for MGE-Amp with the exception that Amx-alkyne 15 was used instead of Amp-alkyne 14. Conjugate 8 was purified by semi-preparative HPLC (20% B for 5 min and 20–42% B over 11 min, 4 mL/min) and obtained as white powder (0.49 mg, 31%). HRMS (ESI): [M+H]+ m/z calcd., 1535.4679; found, 1535.4685. The analytical HPLC
trace of the purified product is reported in Figure 6.6.

6.3.7 Synthesis of DGE-Amp (9)
As described for MGE-Amp with the exception that DGE-PEG₃-N₃ 13 was used instead of MGE-PEG₃-N₃ 12. Conjugate 9 was purified by semi-preparative HPLC (0% B for 5 min and 0–50% B over 13 min, 4 mL/min) and obtained as white powder (0.67 mg, 48%). HRMS (ESI): [M+Na]^+ m/z calcd., 1703.5077; found, 1703.5069. The analytical HPLC trace of the purified product is reported in Figure 6.7.

6.3.8 Synthesis of DGE-Amx (10)
As described for MGE-Amp with the exception that Amx-alkyne 15 and DGE-PEG₃-N₃ 13 were used instead of Amp-alkyne 14 and MGE-PEG₃-N₃ 12. Conjugate 10 was purified by semi-preparative HPLC (0% B for 5 min and 0–50% B over 13 min, 4 mL/min) and obtained as white powder (0.36 mg, 26%). HRMS (ESI): [M+H]^+ m/z calcd., 1697.5207; found, 1697.5235. The analytical HPLC trace of the purified product is reported in Figure 6.8.

6.3.9 Storage and Handling of Siderophores and Siderophore-Antibiotic Conjugates
All (Glc)Ent 1-3 and siderophore-antibiotic conjugates 5-10 were stored as DMSO stock solutions at -20 °C. The stock solution concentrations for (Glc)Ent-Amp/Amx 5-10 ranged from 2 to 5 mM. These values were determined by diluting the DMSO stock solution in MeOH and using the reported extinction coefficient for enterobactin in MeOH (316 nm, 9,500 M⁻¹cm⁻¹). To minimize multiple freeze-thaw cycles, the resulting
solutions were divided into 50-μL aliquots and stored at -20 °C. The β-lactam moieties and enterobactin trilactone are susceptible to hydrolysis, and aliquots were routinely analyzed by HPLC to confirm the integrity of the samples.

6.3.10 General Microbiology Materials and Methods

Information pertaining to all bacterial strains used in this study is listed in Table 6.4. Freezer stocks of all *E. coli* strains (*E. coli* K-12, B, H9049, CFT073, and UTI89), *Staphylococcus aureus* ATCC 25923, and *Acinetobacter baumannii* ATCC 17961 were prepared from single colonies in 25% glycerol/Luria Broth (LB) medium. Freezer stocks of *Lactobacillus rhamnosus* GG ATCC 53103 were prepared from single colonies in 25% glycerol/de Man, Rogosa, and Sharpe (MRS) medium.

LB, MRS, 5x M9 minimal medium and agar were purchased from BD. Mueller Hinton Broth (MHB) was purchased from Fluka. Recombinant human LCN2 was purchased from R&D System (Minneapolis, MN). The iron chelator 2,2'-dipyridyl (DP) was purchased from Sigma-Aldrich. All growth medium and Milli-Q water used for bacterial cultures or for preparing solutions of the enterobactin-antibiotic conjugates were sterilized by using an autoclave. A DP stock solution (200 mM) was prepared in DMSO and used in the bacteria growth assays requiring iron-depleted conditions. Working dilutions of the siderophore and siderophore-antibiotic conjugate stock solutions were prepared in 10% DMSO/H₂O. For all assays, the final cultures contained 1% v/v DMSO. Sterile polypropylene culture tubes and sterile polystyrene 96-well plates used for culturing were purchased from VWR and Corning Incorporated, respectively. The optical density at 600 nm (OD₆₀₀) was recorded on a Beckman Coulter DU800
spectrophotometer or by using a BioTek Synergy HT plate reader.

6.3.11 Growth Studies of E. coli in the Presence of DP

Overnight cultures of E. coli were prepared by inoculating 5 mL of Luria Broth (LB) medium with bacterial freezer stocks. The cultures were incubated at 37 °C in a tabletop incubator with shaking at 150 rpm for 16-18 h. The overnight culture was diluted 1:100 into 5 mL of fresh LB medium containing DP (200 µM) and incubated at 37 °C with shaking at 150 rpm until OD$_{600}$ reached 0.6. The cultures were subsequently diluted to an OD$_{600}$ value of 0.001 using 50% MHB medium (11.5 g/L). A 90-µL aliquot of the diluted culture was combined with a 10-µL aliquot of a 10x solution of DP (0, 0.25, 0.5, 1, 2, 4, and 8 mM) in a 96-well plate, which was wrapped in Parafilm and incubated at 30 °C with shaking at 150 rpm. Bacterial growth was determined at t = 0, 2, 4, 6, 8, 10, and 20 h by measuring the OD$_{600}$ using a BioTek Synergy HT plate reader. Each well condition was prepared in duplicate and at least three independent replicates were conducted on different days and using two different DP stock solutions. The resulting mean OD$_{600}$ values are reported and the error bars represent the standard deviation.

6.3.12 Iron Content of the Antimicrobial Assay Medium

The iron content of the antimicrobial assay medium was determined by ICP-OES (University of Illinois Urbana-Champaign, UIUC) for two independently prepared batches. Both values are reported.
Table 6.1. Iron content of the antimicrobial assay medium.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Medium</th>
<th>Fe concentration (ppm)</th>
<th>Fe concentration ((\mu)M)</th>
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<tbody>
<tr>
<td>Luria Broth (LB)\textsuperscript{b}</td>
<td>0.339</td>
<td>6.05</td>
</tr>
<tr>
<td></td>
<td>0.342</td>
<td>6.11</td>
</tr>
<tr>
<td>50% Mueller Hinton Broth (MHB)\textsuperscript{b}</td>
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<td>4.73</td>
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<td></td>
<td>0.173</td>
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<tr>
<td>Modified M9 minimal medium\textsuperscript{c}</td>
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<tr>
<td></td>
<td>0.019</td>
<td>0.341</td>
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<tr>
<td>1:1 MRS/MHB medium\textsuperscript{c}</td>
<td>0.624</td>
<td>11.2</td>
</tr>
<tr>
<td></td>
<td>0.647</td>
<td>11.6</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The samples were stored in polypropylene tubes, and sent to UIUC for ICP-OES analysis. The samples were stored at room temperature and shipped at ambient temperature.\textsuperscript{b} Data previously reported.\textsuperscript{44} \textsuperscript{c} Growth media was prepared as described in the main text and autoclaved.

6.3.13 General Procedure for Antimicrobial Activity Assays

Overnight cultures of \textit{E. coli}, \textit{S. aureus}, and \textit{A. baumannii} were prepared by inoculating 5 mL of Luria Broth (LB) medium with bacterial freezer stocks. The cultures were incubated at 37 °C in a tabletop incubator with shaking at 150 rpm for 16-18 h. The overnight culture was diluted 1:100 into 5 mL of fresh LB medium containing DP (200 \(\mu\)M) and incubated at 37 °C with shaking at 150 rpm until \(\text{OD}_{600}\) reached 0.6. The cultures were subsequently diluted to an \(\text{OD}_{600}\) value of 0.001 using 50\% MHB medium (11.5 g/L) with or without DP (200 \(\mu\)M). A 90-\(\mu\)L aliquot of the diluted culture was combined with a 10-\(\mu\)L aliquot of a 10x solution of Amp/Amx or (Glc)Ent-Amp/Amx \textbf{5-10} in a 96-well plate, which was wrapped in Parafilm and incubated at 30 °C with shaking at 150 rpm for 19 h. Bacterial growth was determined by measuring the \(\text{OD}_{600}\) using a BioTek Synergy HT plate reader. Each well condition was prepared in duplicate and at least three independent replicates were conducted on different days and using different synthetic batches of each conjugate. The resulting mean \(\text{OD}_{600}\) values are reported and
For *L. rhamnosus* GG ATCC 53103, the bacterial culture was grown in MRS medium overnight. The resulting culture was diluted 1:50 into 5 mL of fresh MRS medium containing DP (200 μM) and incubated at 37 °C with shaking at 150 rpm until OD$_{600}$ reached 1.0. The culture was subsequently diluted to an OD$_{600}$ value of 0.004 in 1:1 MRS/MHB medium with or without DP (200 μM). The antibacterial activity assays were performed as described above for *E. coli*.

6.3.14 Time-Kill Kinetic Assays

A 5-mL overnight culture of *E. coli* CFT073 or UT189 grown in LB medium was diluted 1:100 into 5 mL of fresh LB medium with 200 μM DP and incubated at 37 °C with shaking at 150 rpm until OD$_{600}$ reached ≈0.3. The culture was centrifuged (3000 rpm x 10 min, room temperature) and the resulting pellet was resuspended in 50% MHB and centrifuged (3000 rpm x 10 min, room temperature). The resulting pellet was resuspended in 50% MHB with or without DP (200 μM) and the OD$_{600}$ was adjusted to 0.3. A 90-μL aliquot of the resulting culture was combined with a 10-μL aliquot of a 10x solution of Amp/Amx or (Glc)Ent-Amp/Amx 5-10 in a 96-well plate, which was wrapped in Parafilm and incubated at 37 °C with shaking at 150 rpm. The OD$_{600}$ values were recorded at t = 0, 1, 2, and 3 h. In a parallel experiment, a 10-μL aliquot of the culture was taken at t = 0, 1, 2, and 3 h and serially diluted by using sterile phosphate-buffered saline (PBS) and plated on LB agar to obtain colony forming units (CFU/mL). Each well condition was repeated at least three times on different days. The resulting mean OD$_{600}$ or CFU/mL is reported and the error bars are the standard deviation.
6.3.15 Antimicrobial Activity Assays in the Presence of Unmodified (Glc)Ent

These assays were performed following the general procedure described above except that varying concentrations of Ent, MGE, or DGE were mixed with Ent-Amp/Amx 5-6, MGE-Amp/Amx 7-8, or DGE-Amp/Amx 9-10. Ent was synthesized following a literature procedure,\textsuperscript{49} MGE 2 and DGE 3 were prepared from Ent using MceC and IroB as described for MGE-PEG\textsubscript{3}-N\textsubscript{3} 12 and DGE-PEG\textsubscript{3}-N\textsubscript{3} 13. Stock solutions of (Glc)Ent 1-3 were prepared in DMSO and stored at -20 °C.

6.3.16 Mixed-E. coli Assays

The pET29a plasmid (kanamycin resistance) was transformed into \textit{E. coli} K-12, and the pHSG398 plasmid (chloramphenicol resistance) was transformed into \textit{E. coli} CFT073 and UTI89, by electroporation. Overnight cultures of the bacterial strains were prepared by inoculating 5 mL of LB medium containing the appropriate antibiotic (kanamycin, 50 μg/mL; chloramphenicol, 34 μg/mL) with bacterial freezer stocks, and the cultures were incubated at 37 °C in a tabletop incubator shaker set at 150 rpm for 16-18 h. Each overnight culture was diluted 1:100 into 5 mL of fresh LB medium containing 200 μM DP, but no antibiotics, and incubated at 37 °C with shaking at 150 rpm until OD\textsubscript{600} reached 0.6. The cultures were diluted to an OD\textsubscript{600} value of 0.001 in 50% MHB separately or in a 1:1 mixture (10\textsuperscript{6} CFU/mL for each strain), with or without 200 μM DP. No antibiotic marker was included in these cultures. Aliquots of these cultures were serially diluted by using sterile PBS and plated on LB agar plates with or without the corresponding antibiotic to confirm the CFU of the starter cultures. A 90-μL aliquot of each culture was combined with a 10-μL aliquot of a 1-μM solution of Amp/Amx or (Glc)Ent-Amp/Amx 5-
10 in a 96-well plate. The plate was then wrapped in Parafilm and incubated at 30 °C with shaking at 150 rpm for 19 h. Bacterial growth was evaluated by measuring OD\textsubscript{600} as well as counting colonies formed on LB agar with or without kanamycin/chloramphenicol after serial dilution with sterile PBS. Each well condition was repeated at least three times independently on different days. The resulting mean OD\textsubscript{600} and CFU/mL values are reported and the error bars are the standard deviation.

6.3.17 Mixed-species Assays

These assays were performed following the mixed-\textit{E. coli} assay procedure except that \textit{E. coli} CFT073 and \textit{L. rhamnosus} GG ATCC 53103 were used. A 5-mL culture of \textit{E. coli} CFT073 or \textit{L. rhamnosus} GG was grown for 16-18 h in LB or MRS medium, respectively. The overnight culture was diluted 1:100 (\textit{E. coli}) or 1:50 (\textit{L. rhamnosus} GG) into 5 mL of fresh LB or MRS medium with 200 μM DP and incubated at 37 °C with shaking at 150 rpm until OD\textsubscript{600} reached 0.6 (\textit{E. coli}) or 1.0 (\textit{L. rhamnosus} GG). The cultures were diluted to an OD\textsubscript{600} value of 0.001 (\textit{E. coli}) or 0.004 (\textit{L. rhamnosus} GG) in 1:1 MRS/MHB containing 200 μM DP separately or in a 1:1 mixture (10\textsuperscript{6} CFU/mL for each strain). Aliquots of these cultures were serially diluted by using sterile PBS and plated on LB and MRS agar plates to confirm the CFU of the starter culture. A 90-μL aliquot of each culture was combined with a 10-μL aliquot of a 10-μM solution of Amp/Amx or (Glo)Ent-Amp/Amx 5-10 in a 96-well plate, which was wrapped in Parafilm and incubated at 30 °C with shaking at 150 rpm for 19 h. Bacterial growth was assayed by both measuring OD\textsubscript{600} and counting colonies formed on LB and MRS agar plates after serial dilution with sterile PBS. Each well condition was repeated at least three
times independently on different days. The resulting mean OD$_{600}$ and CFU/mL values are reported and the error bars are the standard deviation. Comment: *E. coli* CFT073 forms colonies more quickly than *L. rhamnosus* GG on LB agar plates, whereas *L. rhamnosus* GG colonies appear more quickly than those of *E. coli* CFT073 on MRS agar plates, and these behaviors allow for each strain to be monitored independently over a 24-h period.

The assays were also performed by co-culturing *E. coli* CFT073 or UTI89 with *S. aureus* ATCC 25923 or *A. baumannii* ATCC 17961. A 5-mL culture of each individual bacterial strain was grown for 16-18 h in LB. The overnight culture was diluted 1:100 into 5 mL of fresh LB with 200 μM DP and incubated at 37 °C with shaking at 150 rpm until OD$_{600}$ reached 0.6. The cultures were diluted to an OD$_{600}$ value of 0.001 in 50% MHB containing 200 μM DP separately or in a 1:1 mixture (10$^6$ CFU/mL for each strain). A 90-μL aliquot of each culture was combined with a 10-μL aliquot of a 10-μM solution of Amp/Amx or (Glc)Ent-Amp/Amx 5-10 in a 96-well plate, which was wrapped in Parafilm and incubated at 30 °C with shaking at 150 rpm for 19 h. Bacterial growth was assayed by both measuring OD$_{600}$ and counting colonies formed on HardyCHROM UTI plates after serial dilution with sterile PBS. Plating *E. coli* strains on these plates results in pink colonies, whereas *S. aureus* and *A. baumannii* provide white colonies. Each well condition was repeated at least three times independently on different days. The resulting mean OD$_{600}$ and CFU/mL values are reported and the error bars are the standard deviation.
6.3.18 Antimicrobial Activity Assays in the Presence of Lipocalin-2

Cultures of *E. coli* CFT073 were grown in modified M9 minimal medium \(^{53} (\text{Na}_2\text{HPO}_4 6.8 \text{ g/L}, \text{KH}_2\text{PO}_4 3 \text{ g/L}, \text{NaCl} 0.5 \text{ g/L}, \text{NH}_4\text{Cl} 1 \text{ g/L}, 0.4\% \text{ glucose}, 2 \text{ mM MgSO}_4, 0.1 \text{ mM CaCl}_2, 0.2\% \text{ casein amino acids}, \text{ and } 16.5 \mu \text{g/mL of thiamine})\) for 16-18 h. The overnight culture grew to saturation and was diluted 1:100 into 5 mL of fresh modified M9 minimal medium and incubated at 37 °C with shaking at 150 rpm until OD\(_{600}\) reached 0.6. The OD\(_{600}\) of the culture was adjusted to 0.001, and the culture was further diluted 1:100 with the M9 medium in two steps (1:10 x 1:10). The corresponding CFU was determined to be \(\approx10^4 \text{ CFU/mL}\) by plating on LB agar plates. Lipocalin-2 (LCN2, R&D Systems) was diluted into PBS, pH 7.4 to a concentration of 20 \(\mu\text{M}\) and frozen at -20 °C until use. Bovine serum albumin (BSA, Sigma-Aldrich) was prepared in PBS, pH 7.4 to achieve a concentration of 20 \(\mu\text{M}\). A 90-\(\mu\text{L}\) aliquot of the diluted culture was combined with a 5-\(\mu\text{L}\) aliquot of a 20x solution of (Glc)Ent-Amp/Amx 5-10 and a 5-\(\mu\text{L}\) aliquot of LCN2 or BSA in a 96-well plate, which was wrapped in Parafilm and incubated at 37 °C with shaking at 150 rpm for 24 h. Bacterial growth was determined by OD\(_{600}\). Each well condition was repeated at least three times independently on different days. The resulting mean OD\(_{600}\) is reported and the error bars are the standard deviation.

6.3.19 Cytotoxicity Assays

The human colon epithelial T84 cell line was purchased from ATCC and cultured in 1:1 DMEM/F12 medium with 10% fetal bovine serum, and 1% penicillin and streptomycin (v/v, ATCC). The cells were grown to approximately 95% confluency and treated with 3 mL of trypsin-EDTA (Corning). A 12-mL portion of fresh medium was added to the
detached cells, and the T84 cell suspension was centrifuged (600 rpm × 5 min, 37 °C). The supernatant was discarded and the cell pellet was resuspended in 6 mL of the fresh culture medium. The concentration of cells was quantified by using a manual hemocytometer (VWR International) and adjusted to 1 × 10^5 cells/mL. A 90-μL aliquot of T84 cells were then added to 96-well plates and incubated at 37 °C and 5% CO₂ for 24 h. Stock solutions (10x) of Amp/Amx or (Glc)Ent-Amp/Amx 5-10 were prepared in sterile-filtered 10% DMSO/H₂O and 10 μL of each solution was added to the appropriate well. The plate was incubated at 37 °C and 5% CO₂ for another 24 h. 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT, Alfa Aesar) was dissolved in sterile PBS and the concentration was adjusted to 5 mg/mL. The resulting yellow solution was filtered through a 0.2-μm filter and a 20-μL aliquot of the resulting MTT solution was added to each well. The plate was incubated at 37 °C and 5% CO₂ for 4 h and the supernatant was removed from each well. DMSO (100 μL) was added to each well and the absorbance at 550 nm was recorded by using a plate reader. Blank readings were recorded on wells that contained only the medium. The assay was repeated in triplicate on different days, and the mean and standard deviation are reported.

6.3.20 BLAST Search for iroN Sequence

The iroN sequence (2,178 bp) from E. coli CFT073 was used to search the sequences deposited in the nucleotide collection of GenBank (as of May 14, 2015) using the BLASTN 2.2.30+ program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The results (≤78% sequence identity) are summarized on the next page.
Table 6.2. Results from BLAST search using *iroN* of *E. coli* CFT073.

<table>
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<th>% Sequence Identity</th>
<th>Comments</th>
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<tr>
<td><em>E. coli</em> Nissle 1917</td>
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<td>Probiotic</td>
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<td><em>E. coli</em> CFT073</td>
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<td>Uropathogenic</td>
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<tr>
<td><em>E. coli</em> 83972</td>
<td>99</td>
<td>Uropathogenic, asymptomatic bacteriuria</td>
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<tr>
<td><em>E. coli</em> 536</td>
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<td>Uropathogenic</td>
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<td><em>E. coli</em> IHE3034</td>
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<td>Salmonellosis</td>
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6.4 Results and Discussion

6.4.1 Design and Syntheses of GlcEnt-β-Lactam Conjugates

We present a family of salmochelin-inspired GlcEnt-β-lactam conjugates 7-10 that exhibit ampicillin (Amp) or amoxicillin (Amx) attached to either monoglucosylated Ent (MGE, 2) or diglucosylated Ent (DGE, 3) by a stable polyethylene glycol (PEG₃) linker. The design of the GlcEnt-β-lactam conjugates 7-10 builds upon Ent-Amp/Amx 5-6
These conjugates are based on a monosubstituted Ent platform where one catechol moiety is modified at the C5 position for cargo attachment. We sought to install glucose moieties at the C5 position of one or both of the unfunctionalized catechol rings to afford MGE-Amp/Amx 7-8 and DGE-Amp/Amx 9-10, respectively. Although the total chemical syntheses of salmochelins have been reported, nine steps are required to achieve the requisite glucosylated 2,3-dihydroxybenzoic acid building block. We therefore established a chemoenzymatic approach that employs enzymes involved in salmochelin biosynthesis, which affords the desired glucosylated conjugates and requires only one additional step compared to the reported preparation of Ent-Amp/Amx.

IroB and MceC are C-glucosyltransferases that catalyse C-glucosylation of Ent at the C5 positions of the catechol rings. MceC is encoded by the MccE492 gene cluster of *K. pneumoniae* RYC492, and has 75% amino acid sequence identity with IroB. IroB catalyzes up to three C-glucosylation events, affording MGE, DGE, and TGE as products (*Figure 6.1a*). MceC, in contrast, produces only MGE and DGE. On the basis of these observations, we hypothesized that both IroB and MceC would accept monofunctionalized Ent as a substrate, providing a preparative route to 7-10. Initial activity assays where either IroB or MceC was incubated with Ent-PEG3-N3 11, UDP-Glc, and Mg(II) revealed that both enzymes accept Ent-PEG3-N3 11 as a substrate and afford MGE-PEG3-N3 12 and DGE-PEG3-N3 13 as products (*Figures 6.2, 6.3*).
Figure 6.2. Analytical HPLC traces of 100 μL of 100 μM Ent-PEG₃-N₃ 11 incubated with 1 μM MceC in 100 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, and 3 mM UDP-Glc for the indicated time. The samples were quenched with 10 μL of 6% TFA. Method: 0–100% B over 30 min, 1 mL/min. (a) Absorbance monitored at 220 nm. (b) Absorbance monitored at 316 nm.

Accumulation of 12 was observed in the MceC-catalyzed reactions, whereas 13 accumulated in reactions catalyzed by IroB. When Ent-Amp/Amx 5-6 were employed as substrates, complex product mixtures were obtained. LC-MS analysis of the mixtures
revealed the desired products as well as multiple byproducts, including products of β-lactam decomposition. We therefore performed large-scale C-glucosylation reactions employing Ent-PEG$_3$-N$_3$ 11 as a substrate to afford milligram quantities of MGE-PEG$_3$-N$_3$ 12, and DGE-PEG$_3$-N$_3$ 13 (Figure 6.4). We subsequently employed copper-catalyzed azide/alkyne cycloaddition to install the β-lactam moieties (Figure 6.4).

**Figure 6.4.** Chemoenzymatic syntheses of GlcEnt-Amp/Amx 7-10. The synthetic route consists of MceC- or IroB-catalysed glucosylation of Ent-PEG$_3$-N$_3$ 11 followed by a copper-catalysed click reaction to achieve the GlcEnt-β-lactam conjugates 7-10. We abbreviate the siderophore family 1-4 (Figure 6.1a) as (Glc)Ent and the siderophore-β-lactam conjugates 5-10 as (Glc)Ent-Amp/Amx.

This route achieved the mono- and diglucosylated conjugates 7-10 in high purity and in yields of 26–59% from 7 following HPLC purification. The analytical HPLC traces of the pure compounds 7-10, 12, 13 are shown in Figures 6.5-6.10. As expected, the GlcEnt-β-lactam conjugates 7-10 bind iron. Each ferric complex exhibits a broad
absorption band (ca. 400-700 nm, MeOH) characteristic of ferric Ent and its derivatives (Figure 6.11).\textsuperscript{44,45,74}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{absorption_bands.png}
\caption{Analytical HPLC traces of purified MGE-Amp 7 (0\% B for 5 min followed by 0–100\% B over 30 min, 1 mL/min). (a) Absorbance monitored at 220 nm. (b) Absorbance monitored at 316 nm. The sample was dissolved in 1:1 MeCN/H\textsubscript{2}O.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{hplc_traces.png}
\caption{Analytical HPLC traces of purified MGE-Amx 8 (0\% B for 5 min followed by 0–100\% B over 30 min, 1 mL/min). (a) Absorbance monitored at 220 nm. (b) Absorbance monitored at 316 nm. The sample was dissolved in 1:1 MeCN/H\textsubscript{2}O.}
\end{figure}
Figure 6.7. Analytical HPLC traces of purified DGE-Amp 9 (0% B for 5 min followed by 0–100% B over 30 min, 1 mL/min). (a) Absorbance monitored at 220 nm. (b) Absorbance monitored at 316 nm. The sample was dissolved in 1:1 MeCN/H₂O.

Figure 6.8. Analytical HPLC traces of purified DGE-Amx 10 (0% B for 5 min followed by 0–100% B over 30 min, 1 mL/min). (a) Absorbance monitored at 220 nm. (b) Absorbance monitored at 316 nm. The sample was dissolved in 1:1 MeCN/H₂O.
Figure 6.9. Analytical HPLC traces of purified MGE-PEG$_3$-N$_3$ 12 (0% B for 5 min followed by 0–100% B over 30 min, 1 mL/min). (a) Absorbance monitored at 220 nm. (b) Absorbance monitored at 316 nm. The sample was dissolved in 1:1 MeCN/H$_2$O.

Figure 6.10. Analytical HPLC traces of purified DGE-PEG$_3$-N$_3$ 13 (0% B for 5 min followed by 0–100% B over 30 min, 1 mL/min). (a) Absorbance monitored at 220 nm. (b) Absorbance monitored at 316 nm. The sample was dissolved in 1:1 MeCN/H$_2$O.
Table 6.3. Characterization of compounds 7-10, 12, 13.

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\(^a\) HPLC gradient used for all compounds is 0% B for 5 min followed by 0–100% B over 30 min, 1 mL/min. \(^b\) All m/z values correspond to [M+H]\(^+\) unless specified otherwise. \(^c\) The m/z value corresponds to [M+Na]\(^+\).

Figure 6.11. Optical absorption spectra of 40 \(\mu\)M GlcEnt-Amp/Amx in the absence (gray) and presence (black) of 0.95 equivalents of Fe\(^{3+}\) (MeOH, room temperature). (a) MGE-Amp 7. (b) MGE-Amx 8. (c) DGE-Amp 9. (d) DGE-Amx 10.
6.4.2 DGE-ß-lactam Conjugates Target Pathogenic E. coli Expressing IroN

To evaluate whether GlcEnt-Amp/Amx 7-10 target pathogenic E. coli expressing IroN, we compared the antibacterial activities of the parent antibiotics Amp/Amx, Ent-Amp/Amx 5-6, and GlcEnt-Amp/Amx 7-10. We selected five E. coli strains on the basis of siderophore receptor expression (Table 6.4). E. coli CFT073 and UTI89 harbor the iroA gene cluster, biosynthesize and utilize salmochelins for iron acquisition in the host, and cause urinary tract infections. In contrast, E. coli H9049 is a clinical isolate that does not have the iroA cluster. E. coli K-12 and E. coli B are non-pathogenic laboratory strains that also lack the iroA cluster.

Table 6.4. Bacterial strains employed in this study.

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<td>FepA, IroN expression</td>
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<td>E. coli B</td>
<td>ATCC</td>
<td>Common lab strain, BL1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FepA expression</td>
</tr>
<tr>
<td>L. rhamnosus GG</td>
<td>ATCC</td>
<td>Clinical isolate, probiotic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Minimal metabolic iron requirement</td>
</tr>
<tr>
<td>S. aureus</td>
<td>ATCC</td>
<td>Clinical isolate, pathogenic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No FepA or IroN expression</td>
</tr>
<tr>
<td>A. baumannii</td>
<td>ATCC</td>
<td>Clinical isolate, pathogenic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No FepA or IroN expression</td>
</tr>
</tbody>
</table>
To ascertain the effect of iron limitation on antibacterial activity, we performed antibacterial activity assays in the absence or presence of the metal-ion chelator 2,2'-dipyridyl (DP, 200 μM). This concentration of DP inhibits *E. coli* growth (Figure 6.12). These assays revealed that DGE-Amp/Amx 9-10 target pathogenic *E. coli* that express IroN.

![Figure 6.12](image)

**Figure 6.12.** Growth curves of *E. coli* strains employed in this work in 50% MHB and in the presence of varying concentrations of DP (T = 30 °C) (mean ± standard deviation, n = 3).

Amp/Amx exhibit minimum inhibitory concentration (MIC) values of 10 μM against the five *E. coli* strains (±DP, Figures 6.13-6.17). Under conditions of iron limitation, Ent-Amp/Amx provide 100- to 1000-fold enhanced activity against all five strains (50% MHB, +DP). These results are in agreement with our prior studies of Ent-Amp/Amx killing of *E. coli*. Glucosylation affords strain-dependent antimicrobial activity that correlates with IroN expression (Figures 6.13, 6.14). Like Ent-Amp/Amx 5-6, GlcEnt-Amp/Amx 7-10
provide 100- and 1000-fold enhanced antimicrobial activity against *E. coli* UTI89 and *E. coli* CFT073, respectively (+DP). The susceptibility of *E. coli* CFT073 to GlcEnt-Amp/Amx remains enhanced in the absence of DP, as observed previously for Ent-Amp/Amx. The antibacterial activity of GlcEnt-Amp/Amx 7-10 against *E. coli* H9049, K-12, and B is attenuated relative to that of Ent-Amp/Amx 5-6 (+DP, Figures 6.15-6.17).

Figure 6.13. Antibacterial activity of (Glc)Ent-Amp/Amx 5-10 against *E. coli* CFT073 in 50% MHB medium in the absence and presence of 200 µM DP (t = 19 h, T = 30 °C) (mean ± standard deviation, n = 3).
Figure 6.14. Antibacterial activity of (Glc)Ent-Amp/Amx 5-10 against *E. coli* UTI89 in 50% MHB medium in the absence and presence of 200 μM DP (t = 19 h, T = 30 °C) (mean ± standard deviation, n = 3).
Figure 6.15. Antibacterial activity of (Glc)Ent-Amp/Amx 5-10 against E. coli H9049 in 50% MHB medium in the absence and presence of 200 μM DP (t = 19 h, T = 30 °C) (mean ± standard deviation, n = 3).
Figure 6.16. Antibacterial activity of (Glc)Ent-Amp/Amx 5-10 against *E. coli* K-12 in 50% MHB medium in the absence and presence of 200 μM DP (t = 19 h, T = 30 °C) (mean ± standard deviation, n = 3).
Figure 6.17. Antibacterial activity of (Glc)Ent-Amp/Amx 5-10 against E. coli B in 50% MHB medium in the absence and presence of 200 μM DP (t = 19 h, T = 30 °C) (mean ± standard deviation, n = 3).

Moreover, for these non-pathogenic strains, the MIC values of (Glc)Ent-Amp/Amx follow the trend Ent-Amp/Amx < MGE-Amp/Amx < DGE-Amp/Amx. The MGE modification provides enhanced potency relative to Amp/Amx because growth reduction (K-12) or complete growth inhibition (H9049 and B) occurs at 1 μM MGE-Amp/Amx (+DP). In contrast, the DGE-β-lactam conjugates exhibit negligible antibacterial activity
against the three strains that lack IroN (MIC > 10 μM). The growth medium contains ≈4 μM iron (Table 6.1) and we attribute the growth inhibition observed at 10 μM DGE-Amp/Amx to iron deprivation that results from DGE-Amp/Amx sequestering the iron in the growth medium (Table 6.1).

In the antibacterial activity assays described above, we treated the bacterial cultures with the apo conjugates and expected that the siderophore moieties chelate iron from the growth medium, allowing for recognition of the ferric-siderophore complexes by FepA and IroN. We previously reported that preloading of Ent-Amp/Amx with ferric ion prior to antibacterial activity assays against *E. coli* K-12 had negligible effect on the MIC value.44 Here we report that preloading of MGE-Amp/Amx and DGE-Amp/Amx also has a negligible effect on the growth inhibitory properties (Figure 6.18). This result is expected given that the concentration of iron in the growth medium far exceeds the MIC values obtained for the conjugates under conditions where FepA and IroN are expressed. Lastly, mixtures of unmodified Amp/Amx and (Glc)Ent 1-3 against *E. coli* CFT073 and UTI89 provide the same MIC values as Amp/Amx alone and confirm that the enhanced antibacterial activity of (Glc)Ent-Amp/Amx 5-10 requires the covalent attachment of β-lactams to the siderophore scaffolds (Figures 6.19, 6.20).
Figure 6.18. Antibacterial activity of (a,c) apo or (b,d) Fe$^{3+}$-preloaded (0.95 equiv of iron) (Glc)Ent-Amp/Amx 5-10 against E. coli CFT073 in 50% MHB medium in the presence of 200 μM DP (t = 19 h, T = 30 °C) (mean ± standard deviation, n = 3).
Figure 6.19. Antibacterial activity of Amp, and Amx in the presence of exogenous Ent 1, MGE 2, or DGE 3 against *E. coli* CFT073 in 50% MHB medium in the absence and presence of 200 μM DP (t = 19 h, T = 30 °C) (mean ± standard deviation, n = 3).
Figure 6.20. Antibacterial activity of Amp, and Amx in the presence of exogenous Ent 1, MGE 2, or DGE 3 against E. coli UT189 in 50% MHB medium in the absence and presence of 200 μM DP (t = 19 h, T = 30 °C) (mean ± standard deviation, n = 3).

6.4.3 Siderophore Modification Accelerates Killing of Pathogenic E. coli CFT073

E. coli CFT073 is rapidly killed by conjugates 5-10 (Figure 6.21). The OD₆₀₀ value of E. coli CFT073 culture (10⁸ CFU/mL) treated with 5 μM (Glc)Ent-β-lactam is reduced to almost the baseline value (≈0.04) after 1 h, which corresponds to a ≈2-fold log reduction.
in CFU/mL, whereas the change in OD<sub>600</sub> and CFU/mL for *E. coli* CFT073 treated with 50 μM Amp/Amx is negligible over this time period.

![Graphs showing time-kill kinetics](image)

**Figure 6.21.** Time-kill kinetics of (Glc)Ent-Amp/Amx 5-10 against *E. coli* CFT073 in 50% MHB medium in the absence and presence of 200 μM (T = 37 °C) (mean ± standard deviation, n = 3). (a,b) The bacteria (=10<sup>8</sup> CFU/mL) are treated with 50 μM Amp or 5 μM (Glc)Ent-Amp. (c,d) The bacteria are treated with 50 μM Amx or 5 μM (Glc)Ent-Amx.
Fig. 6.22. Time-kill kinetics of (Glc)Ent-Amp/Amx 5-10 against *E. coli* UTI89 in 50% MHB medium in the absence and presence of 200 μM (T = 37 °C) (mean ± standard deviation, n = 3). (a,b) The bacteria (∼10⁸ CFU/mL) are treated with 50 μM Amp or 50 μM (Glc)Ent-Amp. (c,d) The bacteria are treated with 50 μM Amx or 50 μM (Glc)Ent-Amx.

In contrast, siderophore modification has negligible effect on the time-kill kinetics observed for *E. coli* UTI89 (Figure 6.22); the (Glc)Ent-β-lactam conjugates provide similar profiles as observed for Amp/Amx. This result is reminiscent of our prior observations for *E. coli* K-12 where attachment of Ent to Amp/Amx provided only a modest increase in the time-kill kinetics compared to the parent antibiotics. The origin
of this strain-dependence is unclear and warrants further investigation. Nevertheless, these data show that glucosylation of Ent-Amp/Amx does not alter the time-kill kinetics of Ent-Amp/Amx for either \textit{E. coli} CFT073 or UTI89, and (Glc)Ent-Amp/Amx \textbf{5-10} kill CFT073 more rapidly than UTI89.

\textbf{6.4.4 Siderophore Competition Supports Recognition of (Glc)Ent-\(\beta\)-lactam Conjugates by IroN}

To investigate the interactions between (Glc)Ent-\(\beta\)-lactam conjugates \textbf{5-10} and the siderophore receptors FepA and IroN of \textit{E. coli} CFT073 and UTI89, we performed modified antimicrobial activity assays where varying concentrations (0-10 \(\mu\)M) of (Glc)Ent \textbf{1-3} were combined with 100 nM (Glc)Ent-Amp/Amx \textbf{5-10} (Figures \textbf{6.23}, \textbf{6.24}). These mixtures provide a means to probe competition between exogenous native siderophores and the conjugates for receptor recognition because siderophore uptake of the former molecules results in growth promotion whereas the latter afford growth inhibition. The competition assays establish that Ent and MGE attenuate the antibacterial activity of all (Glc)Ent-\(\beta\)-lactam conjugates \textbf{5-10} to varying degrees, whereas DGE only inhibits the activity of the glucosylated congeners \textbf{7-10}. Moreover, DGE fully attenuates DGE-Amp/Amx \textbf{9-10} but not MGE-Amp/Amx \textbf{7-8}. These conclusions are drawn from the following observations: (i) a 100-fold molar excess of Ent recovers the growth of \textit{E. coli} CFT073 treated with Ent/MGE-Amp/Amx \textbf{5-8} to levels comparable to that of the untreated control (Figures \textbf{6.23a}, \textbf{6.24a}).
Figure 6.23. Exogenous (Glc)Ent compete with (Glc)Ent-Amp conjugates for FepA and IroN recognition. (a)-(c) Growth of *E. coli* CFT073 in the presence of 100 nM (Glc)Ent-Amp 5/7/9 and mixtures of 100 nM (Glc)Ent-Amp 5/7/9 and 1, 5, 20, or 100 equiv of exogenous (a) Ent 1, (b) MGE 2, or (c) DGE 3 in the presence of 200 μM DP. (d)-(f) Growth of *E. coli* UT189 in the presence of 100 nM (Glc)Ent-Amp 5/7/9 and mixtures of 100 nM (Glc)Ent-Amp 5/7/9 and 1, 5, 20, or 100 equiv of exogenous (d) Ent 1, (e) MGE 2, or (f) DGE 3 in the presence of 200 μM DP. All assays were performed in 50% MHB medium (t = 19 h, T = 30 °C) (mean ± standard deviation, n = 3). An asterisk indicates OD$_{600}$ < 0.01.
Figure 6.24. (a)-(c) Growth of *E. coli* CFT073 in the presence of 100 nM (Glc)Ent-Amx 6/8/10 and mixtures of 100 nM (Glc)Ent-Amx 6/8/10 and 1, 5, 20, or 100 equiv of exogenous (a) Ent 1, (b) MGE 2, or (c) DGE 3 in the presence of 200 μM DP. (d)-(f) Growth of *E. coli* UTI89 in the presence of 100 nM (Glc)Ent-Amx 6/8/10 and mixtures of 100 nM (Glc)Ent-Amx 6/8/10 and 1, 5, 20, or 100 equiv of exogenous (d) Ent 1, (e) MGE 2, or (f) DGE 3 in the presence of 200 μM DP. All assays were performed in 50% MHB medium (t = 19 h, T = 30 °C) (mean ± standard deviation, n = 3). An asterisk indicates OD<sub>600</sub> < 0.01.

In contrast, a 100-fold excess of Ent provides only partial growth recovery of *E. coli* CFT073 treated with DGE-Amp/Amx 9-10. (ii) A 100-fold excess of MGE fully recovers the growth of *E. coli* CFT073 treated with (Glc)Ent-Amx 5-10 (Figures 6.23b, 6.24b). (iii) A 100-fold molar excess of DGE does not recover the growth of *E. coli* CFT073 treated with Ent-Am/Amx 5-6, whereas it provides partial and complete growth recovery of *E. coli* CFT073 treated with MGE-Amp/Amx 7-8 and DGE-Amp/Amx 9-10,
respectively (Figures 6.23c, 6.24c). In total, this work indicates that FepA recognises and delivers Ent/MGE-Amp/Amx 5-8 but not DGE-Amp/Amx 9-10, whereas IroN binds and transports all conjugates based on the three siderophore scaffolds. Competition assays employing E. coli UT189 afford overall trends that are similar to those observed for E. coli CFT073 except that lower concentrations of exogenous siderophores effectively block the antibacterial action of (Glc)Ent-Amp (Figures 6.23d-f, 6.24d-f).

6.4.5 GlcEnt-Amp/Amx Kill Pathogenic E. coli in the Presence of Other Microbes that Include Non-Pathogenic E. coli and Commensal Lactobacilli

To further probe the activity spectrum and investigate strain selectivity of GlcEnt-Amp/Amx, we performed mixed-species assays to determine whether these conjugates will selectively kill pathogenic E. coli that express IroN cultured in the presence of other organisms. These assays confirmed that GlcEnt-Amp/Amx 7-10 selectively kill pathogenic E. coli that express IroN in the presence of E. coli strains that do not express this receptor. Treatment of co-cultures of pathogenic E. coli (CFT073 or UT189, transformed with the chloramphenicol resistance plasmid pSG398) and non-pathogenic E. coli K-12 (transformed with the kanamycin resistance plasmid pET29a) with 100 nM Ent-Amp/Amx 5-6 results in complete killing of both strains (Figures 6.25a-d, 6.26a-d). In contrast, treatment of the co-cultures with 100 nM GlcEnt-Amp/Amx 7-10 affords killing of the uropathogenic E. coli concomitant with E. coli K-12 survival (Figures 6.25a-d, 6.26a-d). Taken together, these results demonstrate that GlcEnt-β-lactam conjugates 7-10 provide strain-specific targeting of the antibacterial cargo to virulent E. coli that express IroN.
Figure 6.25. MGE/DGE-Amp selectively kill uropathogenic *E. coli* in the presence of non-pathogenic *E. coli* K-12 and the probiotic *L. rhamnosus* GG. (a,b) Bacterial growth monitored by (a) OD<sub>600</sub> and (b) CFU/mL for cultures of *E. coli* K-12 only, CFT073 only, and 1:1 K-12/CFT073 mixtures treated with 100 nM Amp or 100 nM (Glc)Ent-Amp 5/7/9 in the presence of 200 μM DP. (c,d) Bacterial growth monitored by (c) OD<sub>600</sub> and (d) CFU/mL for cultures of *E. coli* K-12 only, UT189 only, and 1:1 K-12/UT189 mixtures treated with 100 nM Amp or 100 nM (Glc)Ent-Amp 5/7/9 in the presence of 200 μM DP. (e,f) Bacterial growth monitored by (e) OD<sub>600</sub> and (f) CFU/mL for cultures of *L. rhamnosus* GG only, *E. coli* CFT073 only, and 1:1 *L. rhamnosus* GG/*E. coli* CFT073 mixtures treated with 1 μM Amp or 1 μM (Glc)Ent-Amp 5/7/9 in the presence of 200 μM DP. All mixed-*E. coli* antimicrobial assays were performed in 50% MHB medium and all mixed-species antimicrobial assays were conducted in 1:1 MRS/MHB medium (t = 19 h, T = 30 °C) (mean ± standard deviation, n = 3). An asterisk indicates OD<sub>600</sub> < 0.01 or no colony formation.
Figure 6.26. (a,b) Bacterial growth monitored by (a) OD$_{600}$ and (b) CFU/mL for cultures of *E. coli* K-12 only, CFT073 only, and 1:1 K-12/CFT073 mixtures treated with 100 nM Amx or 100 nM (Glc)Ent-Amx in the presence of 200 μM DP. (c,d) Bacterial growth monitored by (c) OD$_{600}$ and (d) CFU/mL for cultures of *E. coli* K-12 only, UT189 only, and 1:1 K-12/UT189 mixtures treated with 100 nM Amx or 100 nM (Glc)Ent-Amx in the presence of 200 μM DP. (e,f) Bacterial growth monitored by (e) OD$_{600}$ and (f) CFU/mL for cultures of *L. rhamnosus* GG ATCC 53103 only, *E. coli* CFT073 only, and 1:1 *L. rhamnosus* GG/*E. coli* CFT073 mixtures treated with 1 μM Amx or 1 μM (Glc)Ent-Amx in the presence of 200 μM DP. All mixed-*E. coli* antimicrobial assays were performed in 50% MHB medium and all mixed-species antimicrobial assays were conducted in 1:1 MRS/MHB medium (t = 19 h, T = 30 °C) (mean ± standard deviation, n = 3). An asterisk indicates OD$_{600}$ < 0.01 or no colony formation.

(Glc)Ent-Amp/Amx 5-10 also target pathogenic *E. coli* in the presence of commensal microbes. Lactobacilli are Gram-positive commensal bacteria of the human gastrointestinal tract, and are also found in the urinary and genital tracts. Some Lactobacilli reduce recurrent urinary tract infections in women. Lactobacilli have little-to-no minimal metabolic iron requirement, and do not employ enterobactin or salmochelins for iron acquisition. *Lactobacillus rhamnosus* GG (ATCC 53103), a human commensal that is considered to be a probiotic, is susceptible to β-lactam antibiotics, and we obtained a MIC value of 10 μM for Amp/Amx against this strain.
MRS/MHB medium, ±DP) (Figure 6.27). In contrast, 10 μM (Glc)Ent-Amp/Amx 5-10 have negligible effect on L. rhamnosus GG growth (Figure 6.27). Treatment of E. coli CFT073 and L. rhamnosus GG co-cultures with (Glc)Ent-Amp/Amx 5-10 affords selective killing of E. coli CFT073 (Figures 6.25e,f, 6.26e,f).

**Figure 6.27.** Antibacterial activity of (Glc)Ent-Amp/Amx 5-10 against L. rhamnosus GG (ATCC 53103) in 1:1 MRS/MHB medium in the absence and presence of 200 μM DP (t = 19 h, T = 30 °C) (mean ± standard deviation, n = 3).
We previously reported that modification of Amp/Amx with Ent attenuated the activity of the β-lactam against *Staphylococcus aureus* ATCC 25923. In the current work, we obtained a similar result with GlcEnt-Amp/Amx, and found that the salmochelin modification lowers the antibacterial activity of Amp/Amx against *S. aureus* by 10-fold (Figure 6.28).

**Figure 6.28.** Antibacterial activity of (Glc)Ent-Amp/Amx 5-10 against *S. aureus* ATCC 25923 in 50% MHB medium in the absence and presence of 200 μM DP (t = 19 h, T = 30 °C) (mean ± standard deviation, n = 3).
Moreover, treatment of *E. coli* CFT073 and *S. aureus* co-cultures with DGE-Amp/Amx 9,10 affords selective killing of *E. coli* CFT073 (Figures 6.29a,b, 6.30a,b). Selective killing of *E. coli* CFT073 co-cultured with *Acinetobacter baumannii* ATCC 17961 also occurred (Figures 6.29a,b, 6.30a,b, 6.31). Substitution of *E. coli* CFT073 with UT189 in these assays afforded similar selectivity trends (Figures 6.32, 6.33). In total, the mixed-species assays provide support for DGE-based targeting of the antibacterial cargo to IroN-expressing strains.

**Figure 6.29.** (a,b) Bacterial growth monitored by (a) OD, and (b) CFU/mL for cultures of *S. aureus* ATCC 25923 only, *E. coli* CFT073 only, and 1:1 *S. aureus / E. coli* CFT073 mixtures treated with 1 μM Amp or 1 μM (Glc)Ent-Amp 5/7/9 in the presence of 200 μM DP. (c,d) Bacterial growth monitored by (c) OD, and (d) CFU/mL for cultures of *A. baumannii* ATCC 17961 only, *E. coli* CFT073 only, and 1:1 *A. baumannii / E. coli* CFT073 mixtures treated with 1 μM Amp or 1 μM (Glc)Ent-Amp 5/7/9 in the presence of 200 μM DP. These assays were performed in 50% MHB medium (t = 19 h, T = 30 °C) (mean ± standard deviation, n = 3). An asterisk indicates OD < 0.01 or no colony formation.
Figure 6.30. (a,b) Bacterial growth monitored by (a) \( \text{OD}_{600} \) and (b) CFU/mL for cultures of \textit{S. aureus} ATCC 25923 only, \textit{E. coli} CFT073 only, and 1:1 \textit{S. aureus} / \textit{E. coli} CFT073 mixtures treated with 1 \( \mu \text{M} \) Amx or 1 \( \mu \text{M} \) (Glc)Ent-Amx 6/8/10 in the presence of 200 \( \mu \text{M} \) DP. (c,d) Bacterial growth monitored by (c) \( \text{OD}_{600} \) and (d) CFU/mL for cultures of \textit{A. baumannii} ATCC 17961 only, \textit{E. coli} CFT073 only, and 1:1 \textit{A. baumannii} / \textit{E. coli} CFT073 mixtures treated with 1 \( \mu \text{M} \) Amx or 1 \( \mu \text{M} \) (Glc)Ent-Amx 6/8/10 in the presence of 200 \( \mu \text{M} \) DP. These assays were performed in 50\% MHB medium (t = 19 h, T = 30 °C) (mean ± standard deviation, n = 3). An asterisk indicates \( \text{OD}_{600} < 0.01 \) or no colony formation.
Figure 6.31. Antibacterial activity of (Glc)Ent-Amp/Amx 5-10 against A. baumannii ATCC 17961 in 50% MHB medium in the absence and presence of 200 μM DP (t = 19 h, T = 30 °C) (mean ± standard deviation, n = 3).

Note: This A. baumannii strain is insensitive to Amp/Amx over the concentration range tested. The activity observed for the conjugates in the presence of DP likely arises from iron deprivation caused by the conjugates sequestering Fe³⁺ in the growth medium. The origin of the enhanced activity of MGE-Amx against A. baumannii (+DP) is as-yet undetermined.
Figure 6.32. (a,b) Bacterial growth monitored by (a) OD₆₀₀ and (b) CFU/mL for cultures of S. aureus ATCC 25923 only, E. coli UT189 only, and 1:1 S. aureus / E. coli UT189 mixtures treated with 1 μM Amp or 1 μM (Glc)Ent-Amp 5/7/9 in the presence of 200 μM DP. (c,d) Bacterial growth monitored by (c) OD₆₀₀ and (d) CFU/mL for cultures of A. baumannii ATCC 17961 only, E. coli UT189 only, and 1:1 A. baumannii / E. coli UT189 mixtures treated with 1 μM Amp or 1 μM (Glc)Ent-Amp 5/7/9 in the presence of 200 μM DP. These assays were performed in 50% MHB medium (t = 19 h, T = 30 °C) (mean ± standard deviation, n = 3). An asterisk indicates OD₆₀₀ < 0.01 or no colony formation.
Figure 6.33. (a,b) Bacterial growth monitored by (a) OD_{600} and (b) CFU/mL for cultures of S. aureus ATCC 25923 only, E. coli UT189 only, and 1:1 S. aureus / E. coli UT189 mixtures treated with 1 µM Amx or 1 µM (Glc)Ent-Amx 6/8/10 in the presence of 200 µM DP. (c,d) Bacterial growth monitored by (c) OD_{600} and (d) CFU/mL for cultures of A. baumannii ATCC 17961 only, E. coli UT189 only, and 1:1 A. baumannii / E. coli UT189 mixtures treated with 1 µM Amx or 1 µM (Glc)Ent-Amx 6/8/10 in the presence of 200 µM DP. These assays were performed in 50% MHB medium (t = 19 h, T = 30 °C) (mean ± standard deviation, n = 3). An asterisk indicates OD_{600} < 0.01 or no colony formation.

6.4.6 GlcEnt-Amp/Amx Kill E. coli in the Presence of Lipocalin-2

To ascertain whether GlcEnt-Amp/Amx 7-10 overcome LCN2 sequestration, in analogy to LCN2 evasion by the salmochelins,\textsuperscript{14,18} we conducted antibacterial assays with E. coli CFT073 in the absence or presence of LCN2 or bovine serum albumin (BSA, control). These assays were conducted in modified M9 medium,\textsuperscript{53} and 100 nM (Glc)Ent-Amp/Amx 5-10 provide complete growth inhibition of E. coli CFT073 in this medium.
A 10-fold excess of LCN2 attenuates the antibacterial activity of Ent-Amp/Amx 5-6, in agreement with prior work. In contrast, LCN2 has negligible effect on the antimicrobial activity of GlcEnt-Amp/Amx 7-10 against E. coli CFT073 (Figures 6.34, 6.35).

**Figure 6.34.** Antibacterial activity of (Glc)Ent-Amp/Amx against E. coli CFT073 in the presence of LCN2 or bovine serum albumin (BSA). E. coli CFT073 was treated with (a) 100 nM (Glc)Ent-Amp 5/7/9 or (b) 100 nM (Glc)Ent-Amx 6/8/10 in the absence (control) and presence of 1 μM LCN2 or 1 μM BSA. The assays were conducted in modified M9 medium (t = 24 h, T = 37 °C) (mean ± standard deviation, n = 3). An asterisk indicates OD₆₀₀ < 0.01.

**Figure 6.35.** Antibacterial activity of (Glc)Ent-Amp/Amx 5-10 against E. coli CFT073 in the presence of LCN2 or BSA. E. coli CFT073 was treated with (a) 100 nM ferric-preloaded (Glc)Ent-Amp 5/7/9 or (b) 100 nM ferric-preloaded (Glc)Ent-Amx 6/8/10 in the absence and presence of 1 μM LCN2 or 1 μM BSA. For Fe³⁺ preloading, 0.95 equiv of Fe³⁺ was added to each conjugate. The assays were conducted in modified M9 medium (t = 24 h, T = 37 °C) (mean ± standard deviation, n = 3). An asterisk indicates OD₆₀₀ < 0.01.
6.4.7 GlcEnt-Amp/Amx Exhibit Low Cytotoxicity to Human T84 Cells

The cytotoxicity of apo and iron-bound GlcEnt-Amp/Amx 7-10 (≤10 μM) against human T84 colon epithelial cells was evaluated by the MTT assay. In all cases, the cell viability was ≥80% of that of the untreated control, indicating that the conjugates exhibit negligible cytotoxicity to T84 cells over a 24-h period (Figure 6.36).

![Figure 6.36](image)

**Figure 6.36.** Cytotoxicity studies of apo and Fe^{3+}-preloaded (Glc)Ent-Amp/Amx 5-10 against human colonic epithelial cells (T84 cells) in 1:1 DMEM/F-12 with 10% FBS, and 1% penicillin and streptomycin (t = 24 h, T = 37 °C, 5% CO₂). Percentage of cell viability is quantified by MTT assay after a 24-h treatment of the conjugates (mean ± standard deviation, n = 3).

6.5 Summary and Outlook

In this work, inspired by the siderophore recognition strategies utilized by *E. coli* for iron acquisition in the host, we report a siderophore-based approach for antibiotic delivery that targets strains that express IroN, a siderophore receptor that contributes to virulence. First, we establish that the tailoring enzymes IroB and MceC can C-glucosylate monofunctionalized Ent and therefore be employed in chemoenzymatic synthesis to afford functionalized salmochelins. Next, we demonstrate that GlcEnt-β-
lactam conjugates are recognised by siderophore transport machinery, target IroN, provide ≥100-fold enhanced antibacterial activity against uropathogenic *E. coli* relative to the parent β-lactams, afford killing of virulent *E. coli* in the presence of non-pathogenic *E. coli* and other commensal strains, and overcome the enterobactin-sequestering host-defense protein LCN2. Our results establish that conjugation of a broad-spectrum antibiotic to a siderophore tunes the activity profile of the parent antibiotic. With the appropriate choice of siderophore, the antibacterial activity spectrum can be modulated to afford species- and strain-specific targeting. In broad terms, targeting pathogens is important for pharmaceutical development, which will ultimately afford treatment options that minimally perturb the commensal microbiota.\(^{84,85}\)

IroN was first discovered in *Salmonella*\(^{18}\) and subsequently identified in other Enterobacteriaceae. Our current work focuses on antibiotic delivery to uropathogenic *E. coli* that harbour the *iroA* gene cluster, and we expect that this strategy will be applicable to other pathogens that employ salmochelins for iron acquisition. As of May 14, 2015, 121 completely sequenced *E. coli* genomes are available, which include 46 human pathogens. A BLAST search using *iroN* from *E. coli* CFT073 afforded hits with ≥99% sequence identity for three uropathogenic *E. coli* (UT189, 536, and 83792), adherent invasive *E. coli* UM146, the meningitis isolate *E. coli* IHE3034, and a carbapenemase-producing isolate *E. coli* ECONIH1 (Table 6.2). The probiotic *E. coli* Nissle 1917 and the laboratory reference strain for antimicrobial testing *E. coli* ATCC 25922 were the only other *E. coli* revealed as hits. Studies of the distribution of siderophore biosynthetic machinery in *E. coli* isolated from feces of healthy mammals indicate that ≈20% of the commensal isolates produce salmochelins.\(^{86}\) This observation
suggests that one potential limitation of GlcEnt-based antibiotic delivery is that a fraction of commensal *E. coli* harbour the *iroA* cluster are susceptible and, conversely, that some pathogenic *E. coli* do not. Regarding the former possibility, the healthy gut is considered to be a reservoir for *E. coli* that cause infections of the urinary tract,\(^{77,87-89}\) and the ability to target such pathobionts using siderophores may be advantageous in certain cases. In addition to *Salmonella* and *E. coli*, BLAST revealed that the genomes of the human pathogens *Shigella dysenteriae* 1617 and Sd197, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Enterobacter aerogenes* encode *iroN* (Table 6.2). Thus, it will be informative to determine whether DGE also provides targeted antibiotic delivery to these problematic strains.

Our current investigations also provide fundamental insights into siderophore recognition and transport. Prior studies of siderophore uptake in *Salmonella* revealed that both FepA and IroN recognize and transport Ent.\(^{90}\) Our competition assays employing uropathogenic *E. coli* are in agreement with this observation, and indicate that both receptors deliver Ent-Amp/Amx 5-6 into *E. coli*. Moreover, our competition data suggest that MGE 2 and MGE-Amp/Amx 7-8 are recognised and transported by FepA as well as IroN of *E. coli*. In contrast, DGE 3 only competes with GlcEnt-Amp/Amx 7-10 and most effectively blocks the activity of DGE-Amp/Amx 9-10. These observations support exclusive transport of DGE-Amp/Amx 9-10 through IroN. Indeed, prior studies demonstrated that IroN is required for transporting salmochelin extracts isolated from several *S. enterica* strains,\(^{16}\) and *in vitro* activity assays reveal that IroB accumulates DGE 3.\(^ {51}\)
We previously reported that *E. coli* CFT073 exhibits greater sensitivity to Ent-Amp/Amx 5-6 than *E. coli* UTI89,44 and we observe the same trend with GlcEnt-Amp/Amx 7-10. The physiological origins of this observation remain unclear. One possible explanation may be differences in the siderophore biosynthetic and uptake machineries employed by these two uropathogens. *E. coli* CFT073 expresses a third catecholate siderophore receptor, IhA,91 whereas *E. coli* UTI89 biosynthesizes yersiniabactin, a siderophore mainly used by *Yersinia* spp.92 Alternatively, as-yet unidentified factors may account for these trends, and further studies are warranted to understand these observations.

In closing, this investigation establishes that siderophores and the siderophore uptake machinery employed by virulent bacteria provide a powerful approach for targeting pathogenesis in the context of antibacterial drug discovery. Narrow-spectrum and species-specific antibiotics are needed for treating infections where the causative agent is known and, when coupled with rapid diagnostics, will ultimately reduce the onset of secondary infections and evolution of antibiotic resistance.2,5,7 The current study focuses on targeting broad-spectrum β-lactam antibiotics to pathogenic *E. coli* on the basis of iron acquisition machinery that is employed by these pathogens during colonisation in the host. We establish that native salmochelins can be used as scaffolds for "Trojan horse" antibiotic delivery to hijack the iron acquisition machinery that contributes to pathogenicity. It will be important to ascertain whether this salmochelin-inspired strategy is applicable to other Gram-negatives, such as *Salmonella* and *K. pneumoniae*, which cause human disease and utilise salmochelins for iron acquisition. Leveraging this strategy to target other antibacterial cargos and thereby modulate
activity and mitigate off-target effects is another important avenue of future chemical and biological investigation.

6.6 Acknowledgements
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6.7 References
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Chapter 7

A Siderophore-Based Immunization Strategy to Inhibit

Growth of Enteric Pathogens

The work presented in this Chapter is submitted for publication.
7.1 Contributions

Ms. M. Sassone-Corsi, and Dr. Araceli Perez-Lopez conducted the animal studies under the supervision of Professor M. Raffatellu at UC Irvine. Dr. M. D. George and Matthew Rolston at the UC Davis School of Medicine Host-Microbe Systems Biology Core performed mouse microbiota analysis and processed samples for Illumina MiSeq analysis. Professor R. A. Edwards at UC Irvine analyzed histopathology. Dr. T. Zheng synthesized Ent-PEG₃-biotin.

7.2 Introduction

Iron is an essential nutrient for almost all organisms, including microbial pathogens. Because the vertebrate host tightly controls the concentration of free iron (e.g., ≈10⁻²⁴ M in serum), many microbes biosynthesize and export secondary metabolites named siderophores to scavenge iron from the host. These small molecules chelate ferric iron (Fe³⁺) with high affinity (Kₐ ≈ 10⁻²⁵ M at neutral pH). Once a siderophore coordinates iron in the extracellular space, the iron-bound siderophore is recognized and transported into a microbial cell by a dedicated membrane receptor. Following cellular uptake, the iron is released from the siderophore and utilized, which enables microbial growth and replication. Siderophores are regarded as major virulence factors during infection with bacterial and fungal pathogens that include Salmonella enterica, uropathogenic Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Mycobacterium tuberculosis, Staphylococcus aureus, and Aspergillus fumigatus.

In response to infection and during inflammation, the host limits iron availability in a process known as “nutritional immunity.” In this process, epithelial cells and
neutrophils secrete the antimicrobial protein lipocalin-2 (LCN2). This host-defense protein inhibits siderophore-mediated iron uptake by capturing ferric enterobactin (Figure 7.1) in the extracellular milieu. Enterobactin (Ent) is a siderophore employed by commensal and pathogenic Enterobacteriaceae. LCN2 thereby inhibits the growth of Enterobacteriaceae that rely on Ent-mediated iron acquisition, which prevents lethal infection by these organisms. Nevertheless, various Gram-negative pathogens evade LCN2 by producing and utilizing "stealth siderophores" that cannot be captured by this host-defense protein. For example, Salmonella spp. and strains of pathogenic E. coli overcome LCN2 by biosynthesizing a family of C-glycosylated Ent derivatives named salmochelins (DGE, Figure 7.1). These stealth siderophores allow the pathogen to thrive in the inflamed gut and outcompete the microbiota. Indeed, Salmonella mutants that lack the DGE receptor IroN are susceptible to the LCN2-mediated host response; these mutants exhibit reduced colonization in the inflamed intestine and cannot outcompete the microbiota.

1. R = R' = R" = H, Enterobactin (Ent)
2. R = R' = Glc, R" = H, Salmochelin S4 (DGE)
3. CTB-Ent

Figure 7.1. Chemical structures of enterobactin (Ent, 1), salmochelin S4 (DGE, 2), and a cartoon depicting CTB-Ent 3 conjugate. Ent and DGE are cyclic trimers of N-2,3-dihydroxybenzoyl-L-serine.
Inspired by seminal studies exemplifying the importance of siderophore-mediated iron acquisition during infection with *Salmonella* as well as other pathogens,\textsuperscript{15,16} we hypothesized that boosting host nutritional immunity by blocking siderophore-based iron acquisition would reduce microbial replication and therefore improve the outcome of infection. To address this hypothesis, we designed and synthesized conjugates of a native siderophore employed by *Salmonella* and an immunogenic carrier protein, and immunized mice with the compounds to induce an antibody response against siderophores. We demonstrated that the immunized mice produced anti-siderophore antibodies in the gut mucosa and these mice exhibited reduced intestinal colonization, reduced systemic dissemination of the pathogen, and increased survival against the challenge of enteric pathogen *Salmonella*.

7.3 Experimental Section

7.3.1 General Materials and Methods for Chemical Synthesis and the Preparation of CTB-Ent

Dimethylformamide (DMF) and dichloromethane (CH$_2$Cl$_2$) were dried by using a VAC solvent purification system (Vacuum Atmospheres). Anhydrous dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich and used as received. Enterobactin acid 6,\textsuperscript{17} Ent-azide 11,\textsuperscript{18} and biotin-alkyne 12\textsuperscript{19} were synthesized by following literature procedures. All other solvents, reagents, and chemicals were purchased from commercial suppliers and used as received unless noted otherwise. EMD TLC silica gel 60 F254 plates were used for analytical thin-layer chromatography. EMD PLC silica gel 60 F254 plates of 1-mm thickness were used for preparative TLC. Column
chromatography was carried out using Zeoprep 60HYD silica gel (40-63 μm) from Zeochem using a positive pressure of nitrogen. For procedures requiring mixtures of solvents, the reported ratios are by volume.

Analytical and semi-preparative high-performance liquid chromatography (HPLC) were performed on an Agilent 1200 instrument equipped with a thermostatted autosampler set at 4 °C and thermostatted column compartment generally set at 20 °C, and a multi-wavelength detector set at 220, 280, and 316 nm (500-nm reference wavelength with 100-nm bandwidth). A Clipeus C18 column (5-μm pore, 4.6 x 250 mm, Higgins Analytical, Inc.) set at a flow rate of 1 mL/min was employed for all analytical HPLC experiments. A ZORBAX C18 column (5-μm pore, 9.4 x 250 mm, Agilent Technologies, Inc.) set at a flow rate of 4 mL/min was employed for all semi-preparative-scale HPLC purification. A Luna 100 Å C18 LC column (10-μm particle size, 21.2 x 250 mm, Phenomenex) set at a flow rate of 10 mL/min was utilized for all preparative-scale HPLC purification. HPLC-grade acetonitrile (MeCN) and HPLC-grade trifluoroacetic acid (TFA) were routinely purchased from EMD and Alfa Aesar, respectively. For all HPLC runs, solvent A was 0.1% TFA/H₂O and solvent B was 0.1% TFA/MeCN.

All 1-D ¹H and ¹³C NMR spectra were recorded at ambient probe temperature using a Varian 500 MHz spectrometer. Chemical shifts are quoted as parts per million (ppm) relative to the internal signal of the solvent (CDCl₃/DMSO-d₆/CD₃OD), coupling constants (J) are quoted in Hertz (Hz) and are accurate to ± 0.2 Hz.

High-resolution mass spectrometry was performed by using an Agilent LC-MS system comprised of an Agilent 1260 series LC system outfitted with an Agilent
Poroshell 120 EC-C18 column (2.7-μm pore size) and an Agilent 6230 TOF system housing an Agilent Jetstream ESI source. For all LC-MS analyses, solvent A was 0.1% formic acid/H₂O and solvent B was 0.1% formic acid/MeCN. The samples were analyzed by using a gradient of 5–95% B over 5 min with a flow rate of 0.4 mL/min. The MS profiles were analyzed and deconvoluted by using Agilent Technologies Quantitative Analysis 2009 software version B.03.02.

The extinction coefficient (10,095 M⁻¹cm⁻¹ at 280 nm) of CTB was calculated by using ExPASy ProtParam. Concentrations of CTB solutions were determined by using a calibrated Bio-Tek Take3 micro volume plate and the calculated extinction coefficient.

To determine the extinction coefficients of [FeEnt]³⁻ in PBS pH 7.2, a stock solution of Ent (20 mM) in MeOH was prepared and its concentration was confirmed by using the reported extinction coefficient of Ent (9,500 M⁻¹cm⁻¹ at 316 nm in MeOH²⁰). Next, 5 μL of the 20-mM Ent stock solution was added into 95 μL of PBS pH 7.2 to achieve a 1-mM solution of Ent. One equivalent of FeCl₃ (4 μL, 25 mM in Chelex-treated Milli-Q water and 0.3 M HCl) was then added. The solution turned dark red and was incubated at room temperature for 15 min. The solution was diluted with PBS pH 7.2 (10-90 μM) and the optical absorption spectra of the resulting solutions were recorded. These experiments were done in triplicate with two different synthetic batches of Ent. The corresponding extinction coefficients of [FeEnt]³⁻ in PBS pH 7.2 were determined to be 8,260 and 5,480 M⁻¹cm⁻¹ at 280 and 498 nm, respectively.

Optical absorption spectra were recorded on a Beckman Coulter DU800 spectrophotometer (1-cm quartz cuvettes, Starna). Solution and buffer pH values were verified by using a Mettler Toledo S20 SevenEasy pH meter.
7.3.2 Synthesis of BnEnt-PEG₃-CO₂H (7)

1-(3,4-Bis(benzyloxy)-5-(((3S,7S,11S)-7,11-bis(2,3-bis(benzyloxy)benzamido)-2, 6,10-trioxo-1,5,9-trioxacyclocododecan-3-yl)carbamoyl)phenyl)-1-oxo-5,8,11-trioxa- 2-azatetradecan-14-oic acid (7). To a 10-mL round-bottom flask, tert-butyl 12-amino- 4,7,10-trioxadodecanoate 4 (32.2 mg, 116 μmol), triethylsilane (27.7 μL, 290 μmol), and 1 mL of CH₂Cl₂ were added. Trifluoroacetic acid (TFA, 350 μL) was added to this mixture and the solution was stirred at room temperature for 3 h. The solution was concentrated under reduced pressure to obtain PEG₃•TFA 5 as yellow oil, which was used in the next step without further purification.

Ent acid 6 (96.3 mg, 76.8 μmol), 1-[bis(dimethylamino)methylene]-1H-1,2,3- triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU, 29.2 mg, 76.8 μmol), and 1-hydroxy-7-azabenzotriazole (HOAt, 10.5 mg, 76.8 μmol) were dissolved in 1 mL of anhydrous DMSO and the mixture was purged with N₂ three times. Diisopropylethylamine (DIPEA, 83.0 μL, 464 μmol) was slowly added and the reaction was stirred at room temperature for 5 min. To the resulting bright yellow solution, a 500-μL pre-mixed solution of PEG₃•TFA 5 and DIPEA (40.4 μL, 232 μmol) in DMSO was added dropwise and the reaction was stirred at room temperature. After 1 h, the reaction was partitioned in water (20 mL) and CH₂Cl₂ (20 mL). The organic layer was washed with 1 M HCl (3 x 10 mL) and brine (1 x 10 mL), and dried over anhydrous Na₂SO₄. The solvents were removed by rotary evaporation. The crude product was purified by preparative TLC (EtOAc, dried and then 7% MeOH/ CH₂Cl₂) to yield 7 as a white solid (67.7 mg, 61%). TLC Rₜ = 0.5 (10% MeOH/ CH₂Cl₂). ¹H NMR (500 MHz, CDCl₃) δ 2.59 (t, J = 6.0 Hz, 2H), 3.58-3.72 (m, 12H), 3.77 (t, J = 6.5 Hz, 2H), 3.97-4.07
(m, 3H), 4.08-4.17 (m, 3H), 4.85-4.94 (m, 3H), 5.01-5.22 (m, 12H), 7.06-7.46 (m, 31H), 7.65 (dd, J = 6.8, 2 Hz, 2H), 7.79 (br, 1H), 7.90 (d, J = 1.6 Hz, 1H), 8.16 (d, J = 1.6 Hz, 1H), 8.49 (d, J = 6.8 Hz, 2H), 8.66 (d, J = 6.8 Hz, 1H).  

$^{13}$C NMR ($125$ MHz, CDCl$_3$) δ  
30.0, 40.0, 51.4, 63.8, 66.7, 69.7, 69.9, 70.2, 71.2, 76.3, 117.5, 121.0, 123.0, 124.3, 126.2, 127.6, 127.7, 128.0, 128.2, 128.4, 128.5, 128.6, 128.8, 128.9, 129.0, 135.4, 135.9, 136.0, 136.2, 146.8, 146.9, 149.0, 151.6, 165.0, 168.9, 169.1. 

HRMS (ESI): [M+H]$^+$ m/z calcd, 1479.5213; found, 1479.5500.

7.3.3 Synthesis of Ent-PEG$_3$-CO$_2$H (8) 

1-(3-((((3S,7S,11S)-7,11-Bis(2,3-dihydroxybenzamido)-2,6,10-trioxo-1,5,9-trioxa
cyclododecan-3-yl)carbamoyl)-4,5-dihydroxyphenyl)-1-oxo-5,8,11-trioxa-2-aza
tetradecan-14-oic acid (8). In a 10-mL round-bottom flask, compound 7 (49.6 mg, 34.1 
μmol) was dissolved in 4 mL of 1:1 EtOH/EtOAc. The flask was purged with N$_2$ three 
times, and 50 mg of Pd/C (10% wt, Sigma-Aldrich) was added. A balloon of H$_2$ was 
atached and the reaction was stirred under an atmosphere H$_2$ (1 atm) at room 
temperature for 3 h. The flask was purged with N$_2$ and the reaction was transferred to a 
centrifuge tube. The Pd/C was removed by centrifuging at 13,000 rpm for 10 min. The 
supernatant was transferred to a round-bottom flask and concentrated by rotary 
evaporation. The resulting crude product was dissolved in 2:2:1 H$_2$O/MeCN/DMSO, 
 purified by semi-preparative HPLC (25–34% B over 15 min, 4 mL/min), and lyophilized 
to yield 8 as white powder (24.6 mg, 78%). The product eluted at 17.5 min (0–100% B 
over 30 min, 1 mL/min, Figure 7.3).  

$^1$H NMR (500 MHz, CD$_3$OD) δ 2.49 (t, J = 6.2 Hz, 2H), 3.50-3.56 (m, 4H), 3.57-3.64 (m, 8H), 3.66 (t, J = 6.3 Hz, 2H), 4.58-4.72 (m, 6H),
5.04 (t, J = 4.9 Hz, 3H), 6.70 (t, J = 8.0 Hz, 2H), 6.94 (ddd, J = 7.9, 2.4, 1.6 Hz, 2H), 7.23 (ddd, J = 8.2, 5.3, 1.6 Hz, 2H), 7.44 (d, J = 2.2 Hz, 1H), 7.86 (d, J = 2.2 Hz, 1H).

$^{13}$C NMR (125 MHz, CD$_3$OD) δ 35.7, 41.0, 53.6, 65.8, 67.8, 70.5, 71.2, 71.3, 71.4, 71.5, 116.6, 116.7, 118.6, 119.5, 119.7, 120.1, 126.5, 143.1, 147.2, 149.4, 152.2, 169.3, 170.0, 175.4. HRMS (ESI): [M+Na]$^+$ m/z calcd, 939.2396; found, 939.2395.

### 7.3.4 Synthesis of CTB-Ent (3)

The conjugation procedure was adapted from literature procedures for the attachment of haptens to carrier proteins. Cholera toxin subunit B (1 mg, CTB, Sigma-Aldrich) was dissolved in 1 mL of Milli-Q water. The solution was divided into two 500-µL aliquots and each aliquot was transferred to an Amicon ultra-0.5 centrifugal filter unit with an ultracel-10 membrane (3 kDa MWCO, EMD Millipore) and buffer exchanged into phosphate buffered saline (PBS) pH 7.2 (5 x 500 µL, 13,000 rpm x 15 min, 4 °C), and then buffer exchanged into 100 mM sodium phosphate (NaP) pH 8.0 (3 x 500 µL, 13,000 rpm x 15 min, 4 °C). Following buffer exchange, the concentration of CTB was quantified by the calculated extinction coefficient (Section 7.2.1), and the concentration of CTB was adjusted to 2 mg/mL by addition of 100 mM NaP pH 8.0.

To activate the carboxylate group of Ent-PEG$_3$-CO$_2$H 8, stock solutions of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, 100 µg/µL) and N-hydroxysuccinimide (NHS, 60 µg/µL) were prepared in anhydrous DMSO. A 12.5-µL aliquot of the EDC solution was added to the powder of Ent-PEG$_3$-CO$_2$H 8 (2 mg, 0.2 µmol) followed by the addition of a 12.5-µL aliquot of the NHS solution. The progress of the reaction was monitored by analytical HPLC (0–100% B over 30 min, 1 mL/min) and
the new peak at 18.9 min was identified as the product Ent-PEG$_3$-NHS 9 by LC-MS. The reaction was complete after 2 h. Iron(III) ethylacetoacetate (Fe(acac)$_3$, 10 μL, 212 mM in DMSO, 0.95 equiv) was then added to Ent-PEG$_3$-NHS 9 to afford the iron-bound form. The colorless solution turned dark purple within 5 min after addition of Fe(acac)$_3$. Without any further purification, Fe-Ent-PEG$_3$ 10 was added to a solution of CTB (500 μL, 1 mg in NaP pH 8.0). The reaction was incubated for 3 h at room temperature. At this time, 500 μL of PBS pH 7.2 was added to the reaction and the resulting solution was subjected to eight rounds of spin filtration (13,000 rpm x 15 min, 4 °C) with PBS pH 7.2 by using an Amicon ultra-0.5 centrifugal filter unit with an ultracel-10 membrane (3 kDa MWCO, EMD Millipore) to remove coupling reagents and any unreacted 9. Following buffer exchange, the concentration of CTB-Ent 3 was determined by employing a Bradford protein assay and the concentration was adjusted to 2 mg/mL by adding PBS pH 7.2. CTB-Ent 3 was stored in PBS pH 7.2 as a dark red solution at 4 °C up to one month prior to immunization. The ratio of Ent to CTB was determined to be ≈4 based on the absorbance at 280 (CTB and Fe-Ent) and 495 nm (Fe-Ent only).

7.3.5 Synthesis of Ent-PEG$_3$-biotin (13)

DMSO solutions of Ent-PEG$_3$-azide 11 (26 mM, 125 μL) and biotin-alkyne 12 (13 mM, 250 μL), a DMF solution of benzoic acid (450 mM, 500 μL), and an aqueous solution of CuSO$_4$ (10 mg/mL, 45 mM, 500 μL) were combined, and 400 μL of DMSO was added to yield a clear yellow-brown solution. An aqueous solution of sodium ascorbate (NaAsc, 90 mM, 500 μL) was subsequently added. The reaction become light yellow and was stirred at room temperature for 15 min, at which time another 500 μL of aqueous NaAsc
was added. After stirring for an additional 15 min, the reaction was flash frozen in liquid N$_2$ and lyophilized to give a yellow oil. The oil was dissolved in a 1:1 ratio of H$_2$O/MeCN, purified by semi-preparative HPLC (20% B for 5 min followed by 20–50% B over 11 min, 4 mL/min), and lyophilized to yield 13 as white powder (1.8 mg, 46%). The product eluted at 17.4 min (0–100% B over 30 min, 1 mL/min, Figure 7.7). HRMS (ESI): [M+Na]$^+$ m/z calcd., 1217.3704; found, 1217.3711.

7.3.6 Gel Electrophoresis
Solutions of the conjugate (1 mg/mL) were prepared in SDS-PAGE sample buffer (10 mM Tris, 1 mM EDTA, 2.5% SDS, and 5% mercaptoethanol, pH 8.0) and heated to 100 °C for 3 min. Gels (16% tricine$^{22}$) were loaded with 5 μL of sample and run in under denaturing conditions for 90 min at 120 V using a Bio-Rad electrophoresis apparatus and stained with Coomassie blue.

7.3.7 Mice
Six-to-eight weeks old C57BL/6 females were purchased from Taconic. When indicated, six-to-eight weeks old males or females C57BL/6 or C57BL/6 NRAMP1 mice bred in our vivarium were also used for the experiments. Mouse colonies were maintained in pathogen-free barrier facilities for the duration of the immunization studies. The Institutional Animal Care and Use Committee at University of California Irvine approved all of the mouse experiments.
7.3.8 Mucosal Immunization with CTB/CTB-Ent Conjugates

C57BL/6 mice were immunized by intranasal injection with either 100 µg of CTB-Ent or CTB (Sigma). CTB-Ent (2 mg/mL) was prepared in PBS pH 7.2 as described above. CTB (2 mg/mL) was dissolved in ultrapure water. Mice were briefly anesthetized with isoflurane and injected intranasally with 25–50 µL of CTB-Ent/CTB. Mice were also boosted 14 days later with the same amount. To monitor the health of the animals during the immunization period, mice were weighed weekly. When indicated, mice were immunized by intraperitoneal injection with same timing and doses.

7.3.9 Preparation of Fecal Samples for ELISA and Dot blot

Fresh fecal samples were collected weekly before and after immunization. Fecal pellets were weighted and 50-60 mg of fecal pellets were resuspendend in 400 µL of sterile PBS containing a protease cocktail inhibitor (Roche) and incubated at room temperature shaking for 30 min. Samples were centrifuged at 7,200 x g for 20 minutes and the supernatants were collected individually and stored at -20 °C until further use.

7.3.10 Enzyme-Linked Immunosorbent Assays (ELISA)

To measure anti-Ent and anti-DGE IgA antibodies by ELISA, 96-well plates (NUNC MAXIsorp) were incubated overnight at 4 °C or 2 h at 37 °C with 1 µg/mL of Ent-PEG₃-biotin 13 (Figure 7.10) or DGE diluted in sterile PBS pH 7.4. The plates were then blocked for 1h at 37 °C with 5% non-fat dry milk. Serial 2-fold dilutions of fecal extracts from CTB-immunized mice and mice immunized with CTB-Ent were added to the wells, starting from 1:10 dilution. To evaluate the presence of specific IgA, we added a goat
anti-mouse IgA conjugated with horseradish peroxidase (1:5,000; Southern Biotechnology Associates, Inc., Birlingham, Al, USA) and incubated for 1 h at 37 °C. This step was followed by detection with horseradish peroxidase substrate. Briefly, o-phenylenediamine dihydro-chloride 3 mg tablets (Sigma) were dissolved in 1x phosphate-citrate buffer and 60 μL of hydrogen peroxide 3 wt% (Sigma) was added. The reaction was quenched with 2 N hydrogen sulfate.

7.3.11 Enzyme-Linked ImmunoSpot (ELISPOT)

A multi-screen ELISPOT PVDF (Millipore) membrane plate was washed with 35% ethanol and then coated with 1 μg/mL of the antigen (Ent/DGE) diluted in sterile PBS. The plate was then blocked by the addition of RPMI +10% FBS (Gibco, Life Technology) and incubated for 1 h at room temperature shaking. Cells isolated from the intestinal mucosa (Peyer’s patches, mesenteric lymph nodes) and, as a control, a non-mucosal site (spleen) from CTB-immunized (mock) and CTB-Ent immunized mice were resuspended in RPMI +10% FBS and seeded at a density of 2.5x10^5 cells/well or lower in duplicates. Then, the plate was incubated at 37 °C 5% CO₂ for at least 18 h. The cells were washed out and specific antibodies were detected after incubation with 0.5 g/mL goat anti-mouse IgA secondary antibody for 2 h at room temperature shaking (Southern Biotechnology Associates, Inc., Birlingham, Al, USA), and addition of 3-amino-9-ethylcarbazole (AEC), then visualized and counted with the CTL Immunospot software (Cellular Technology Limited).
7.3.12 Bacterial Strains and Growth Conditions

IR715 is a fully virulent, nalidixic acid-resistant derivative of *Salmonella enterica* serovar Typhimurium wild-type isolate ATCC 14028. The *S. Typhimurium* mutant strain, AJB52, lacking the salmochelin receptor IroN was also used in this study. The strains were grown aerobically in Luria-Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) or on LB agar plates at 37 °C. When needed, antibiotics were added to the media at the following concentration: 0.03 mg/mL chloramphenicol (Cm), 0.1 mg/mL carbenicillin (Carb), 0.05 mg/mL kanamycin (Kan). For animal infections, all strains were grown in LB media aerobically at 37 °C overnight.

7.3.13 Salmonella Acute Model of Gastrointestinal Infection

Between 36-51 days post-immunization, immunized mice were inoculated intragastrically with streptomycin (100 μL of a 200 mg/mL solution in sterile water) 1 day prior to infection. The following day, the mice were inoculated intragastrically with $10^9$ CFU of *S. Typhimurium* (resuspended in 0.1 mL LB broth) or with 1:1 ratio of $5 \times 10^8$ CFU each of *S. Typhimurium* wild-type and the *iroN* mutant for competitive colonization experiments. Fecal samples were collected daily after bacterial administration and were harvested in sterile PBS. The bacterial load was determined by plating serial 10-fold dilutions on selective agar plates. Between 4-6 days post infection, mice were euthanized. Each mouse caecum was collected and one portion was fixed in formalin for histopathology, whereas a second portion was flash frozen in liquid nitrogen and stored at -80 °C for isolation of mRNA and protein. Bacteria were enumerated in the colon content, terminal ileum, Peyer’s patches, mesenteric lymph nodes, and spleen by
plating serial dilutions on LB agar plates containing the appropriate antibiotics. When noted, the competitive indices were calculated by dividing the output ratio (S. Typhimurium wild-type CFU/ S. Typhimurium iroN CFU) by the input ratio (S. Typhimurium wild-type CFU/ S. Typhimurium iroN CFU).

7.3.14 Quantitative Real-time PCR

For analysis of gene expression by quantitative real-time PCR, total RNA was extracted from cecal tissues with TRI Reagent (Molecular Research Center, Cincinnati, OH). Reverse transcription reagents were employed to generate cDNA from all RNA samples. Real-time PCR was performed using SYBR Green (Roche, Indianapolis, IN) and the Roche Lightcycler 480 system (Roche, Indianapolis, IN). The data were analyzed using the comparative ΔΔ-Ct method. Target gene transcription of each sample was normalized to the respective levels of mRNA β-actin.

Table 7.1. Primers for quantitative Real-Time PCR.

<table>
<thead>
<tr>
<th>Species</th>
<th>Target</th>
<th>Primer Pairs</th>
</tr>
</thead>
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<tr>
<td><em>Mus musculus</em></td>
<td>LCN2</td>
<td>5'-ACATTTGTTCCAAGCTCCAGGGC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-CATGGCGAAACTTTGAGTCC-3'</td>
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<tr>
<td><em>Mus musculus</em></td>
<td>Il17a</td>
<td>5'-GCTCCAGAAGGCTCTCAGA-3'</td>
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<td></td>
<td></td>
<td>5'-AGCTTCCCCTCGATTGA-3'</td>
</tr>
<tr>
<td><em>Mus musculus</em></td>
<td>Ly6g</td>
<td>5'-TGCCTTGCTCTGAGTACA-3'</td>
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<td></td>
<td></td>
<td>5'-CAGAGTGTGGGCAGATGG-3'</td>
</tr>
<tr>
<td><em>Mus musculus</em></td>
<td>Il22</td>
<td>5'-GGCCAGCCTTGCAGATAACA-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-GCTGATGTGCAGAGGACTGA-3'</td>
</tr>
<tr>
<td><em>Mus musculus</em></td>
<td>Cxcl-1</td>
<td>5'-TGCACTAAGCCAGCTGGACCTGAC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-TGCACCCAAACCAGGTCAT-3'</td>
</tr>
<tr>
<td><em>Mus musculus</em></td>
<td>Nos2</td>
<td>5'-TGGGCTTCTTTCACCTCCAGGG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-CCTCTTTGAGTCTCTTCTGAGG-3'</td>
</tr>
<tr>
<td><em>Mus musculus</em></td>
<td>β-actin</td>
<td>5'-GGCTGTATTCCCTCCATCG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-CCAGTTGGTAACAATGCCC-3'</td>
</tr>
</tbody>
</table>
7.3.15 Histopathology Analysis

Tissue samples were fixed in formalin, processed according to standard procedures for paraffin embedding, sectioned at 5 mm, and stained with hematoxylin and eosin. The pathology score of cecal samples was determined by blinded examinations of cecal sections from a board-certified pathologist using previously published methods. Each section was evaluated for the presence of neutrophils, mononuclear infiltrate, submucosal edema, surface erosions, inflammatory exudates, and cryptitis. Inflammatory changes were scored from 0 to 4 according to the following scale: 0 = none; 1 = low; 2 = moderate; 3 = high; 4 = extreme. The inflammation score was calculated by adding up all the scores obtained for each parameter and interpreted as follows: 0–2 = within normal limit; 3–5 = mild; 6–8 = moderate; 8+ = severe.

7.3.16 Microbiota analysis

DNA from the colon content was extracted with the QIAamp DNA stool kit (QIAGEN). Bacterial DNA was amplified by a two-step PCR enrichment of the 16S rDNA (V4 region) encoding sequences from each sample with primers 515F and 806R modified by addition of barcodes for multiplexing. Libraries were sequenced using an Illumina MiSeq system. Uncalled bases, incorrect primer sequence, and runs of ≥12 identical nucleotides sequences were removed. Following quality filtering, the sequences were demultiplexed and trimmed before performing sequence alignments, identification of operational taxonomic units (OTU), clustering, and phylogenetic analysis using QIIME open-source software.
7.3.17 Statistical analysis

Statistical analysis was performed by using Prism 6. In most cases, we applied the non-parametric Mann-Whitney-Wilcoxon test. Analysis of the microbiota was performed with a 2-way ANOVA.

7.4 Results and Discussion

7.4.1 Design and Synthesis of CTB-Ent Conjugate

We selected cholera toxin subunit B (CTB) as the immunogenic carrier protein because it induces a strong mucosal immune response. We reasoned that immunizing mice with a CTB-siderophore conjugate should result in the production of anti-siderophore antibodies in the gut mucosa, which is the primary site of Salmonella infection. Salmonella biosynthesize Ent and DGE, and the vast majority of enteric pathogens have receptors for these siderophores. Because Ent 1 and DGE 2 share several structural features, including the catecholate moieties and trilactone ring (Figure 7.1), we designed and prepared a CTB-Ent conjugate that harbors the native Ent scaffold, hypothesizing that immunization with CTB-Ent would elicit an antibody response against both Ent and DGE. These antibodies would capture Ent, which would be advantageous if LCN2 levels are low (for instance, in immunocompromised patients) and also capture DGE. We assembled CTB-Ent 3 from CTB and an Ent derivative monofunctionalized with a polyethylene glycol (PEG3) linker (Figures 7.1, 7.2) using standard coupling methods.
Figure 7.2. Synthesis of CTB-Ent conjugate 3.
Figure 7.2 illustrates the synthesis of CTB-Ent. The synthesis of Bn6EntCO₂H 6 was previously reported, and we coupled H₂N-PEG₃-CO₂H 5 to 6 to obtain compound 7 (Figure 7.2). We prepared 5 from the commercially available t-butyl ester analog 4 and removed the t-butyl group by treatment with trifluoroacetic acid (TFA) in dichloromethane. It was necessary to remove the t-butyl group before coupling to Bn6EntCO₂H 6 because Ent contains a trilactone ring with three ester linkages, which readily decomposes in the presence of TFA. The ¹H and ¹³C NMR spectra of Bn₆Ent-PEG₃-CO₂H 7 are shown in Appendix C (Figures C.1, C.2). The benzyl protecting groups of 7 were removed by hydrogenation to afford Ent-PEG₃-CO₂H 8 (Figure 7.2). This key intermediate was obtained in high purity and in 48% yield over two steps. The analytical HPLC traces of compound 8 are shown in Figure 7.3. The ¹H and ¹³C NMR spectra of Ent-PEG₃-CO₂H 8 are shown in Appendix C (Figures C.3, C.4). We routinely store compound 8 as lyophilized powder at -20 °C. Storage of 8 and other Ent derivatives as a lyophilized powder minimizes the decomposition of the Ent moiety.

![Figure 7.3. Analytical HPLC traces at (a) 220 nm and (b) 316 nm (Method: 0–100% B over 30 min, 1 mL/min) of Ent-PEG₃-CO₂H 8.](image-url)
We used standard EDC/NHS coupling to attach Ent-PEG$_3$-CO$_2$H 8 to the surface-exposed lysine residues of CTB. These reactions are typically performed in aqueous buffer at pH 8.0.$^{25}$ However, the trilactone ring of Ent is also sensitive to base-catalyzed hydrolysis. Thus, the potential for hydrolytic decomposition of Ent or any Ent derivative must be taken into account when handling the compound in basic aqueous solution. We found that the ferric-bound Ent has enhanced stability to hydrolysis under these conditions (Figure 7.4). As a result, we decided to perform the EDC/NHS coupling of Ent-PEG$_3$-NHS 9 to CTB using the ferric-bound form. In addition, we expected that the Ent moieties of CTB-Ent 3 would chelate Fe$^{3+}$ once injected into mice due to its high affinity for Fe$^{3+}$.
Figure 7.4. Fe$^{3+}$ enhances the stability of Ent in buffered solutions. Analytical HPLC traces (316 nm, 0% B for 5 min followed by 0–100% B over 30 min, 1 mL/min) of Ent incubated under the described conditions. Ent eluted at 24.5 min.
Compound 8 was activated with N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) to achieve Ent-PEG3-NHS 9, and 9 was used without further purification. We prepared iron-bound Ent-PEG3-NHS 10 by addition of ≈1 equivalent of Fe(acac)₃ to 9 in DMSO. An aliquot of this solution was added to CTB in 100 mM NaP at pH 8.0. These reaction conditions contain a 25-fold excess of the siderophore relative to CTB. After incubation at room temperature for 3 h and eight rounds of spin-filtration, CTB-Ent 3 was obtained as dark purple solution. The purple color arises from the ferric-bound Ent species. A Bradford protein assay indicated a yield of 67% of CTB relative to the initial amount of CTB used in the coupling reaction.

CTB-Ent 3 was characterized by SDS-PAGE (16% tricine gel) and optical absorption spectroscopy (Figure 7.5). The tricine gel revealed the CTB-Ent sample (lane 2) contains several bands, and the lowest band runs higher than unmodified CTB (lane 1) or unmodified CTB incubated with EDC/NHS (lane 4). This comparison suggests that some Ent moieties are attached to CTB in the CTB-Ent reaction product. The samples from conjugation reactions (lane 2) showed no band for unmodified CTB and prominent new bands at ca.12 kDa (broad) and 25 kDa. We attribute the former to CTB-Ent conjugate 3. The identity of the 25-kDa band is unclear and may be a result of cross-linking of CTB-Ent subunits in the presence of EDC/NHS because we also observed a similar band when CTB was incubated only with EDC/NHS (lane 4). One subunit of CTB contains nine lysine residues, eight of which are surface exposed (PDB ID = 1XTC²⁶).
Figure 7.5. Characterization of CTB-Ent. (a) SDS-PAGE (16% tricine gel) of samples from conjugation reactions. Unmodified CTB is 11.6 kDa, Ent-PEG₃-CO₂H is approximately 900 Da. All samples were run on the same gel and the gel was cut to facilitate comparison of lane 4 with lanes 1-3. (b) Optical absorption spectra of CTB (30 μM, blue) and iron-bound CTB-Ent 3 (20 μM, red). The samples were prepared in PBS pH 7.2.

The optical absorption spectrum of CTB-Ent 3 revealed an absorption feature at 316 nm, which results from the Ent catechol moieties, and a broad absorption band centered at 498 nm, which corresponds to the charge transfer band of [FeEnt]⁻. The optical absorption spectrum of CTB-Ent 3 provides a means to quantify the Ent/CTB ratio by using the absorbance at 280 (CTB and [FeEnt]⁻) and 498 nm ([FeEnt]⁻ only) and calculated extinction coefficient. This ratio was found to be ≈4, indicating that four lysine residues of each CTB are covalently modified with the Ent-PEG₃ moiety. To date, we have prepared nine independent batches of CTB-Ent 3 (0.5–4 mg scale) and the calculated Ent/CTB ratio varies from 3.5 to 5 (n = 9).

This procedure results in the Ent haptens covalently attached to surface-exposed lysine residues of CTB (Figure 7.1). We selected a PEG₃ linker because it is stable and...
affords both flexibility and enhanced solubility in aqueous solution. In prior work, we modified the native Ent scaffold with a PEG₃ linker and established that these molecules coordinate Fe³⁺ and are recognized and transported by the Ent receptor FepA. From these prior studies, we reasoned that antibodies that bind the siderophore moiety of CTB-Ent would also bind to unmodified Ent. In addition, we synthesized and used Ent-PEG₃-biotin (13, Figures 7.6, 7.7) to coat the wells employed in ELISA to determine the specificity of the isolated antibodies against Ent (Section 7.3.10).

![Figure 7.6. Synthesis of Ent-PEG₃-biotin conjugate 13.](image)

![Figure 7.7. Analytical HPLC traces at (a) 220 nm and (b) 316 nm (Method: 0–100% B over 30 min, 1 mL/min) of Ent-PEG₃-biotin 13.](image)
7.4.2 **Intranasal Immunization with CTB-Ent Induces the Development of Anti-Ent and Anti-DGE IgA Antibodies in the Gut Mucosa**

We immunized mice at day 0 and boosted at day 14 (Figure 7.8a) by intranasal administration of either unmodified CTB (100 μg/mouse, mock-immunization), or CTB-Ent (100 μg/mouse). To determine whether mice immunized with CTB-Ent developed a specific antibody response against Ent, blood and fecal samples were collected weekly for detection of anti-Ent antibodies by an in-house ELISA and by dot-blot. As expected, the amount of total fecal IgA was similar between the two groups of mice (Figure 7.9a). However, only mice immunized with CTB-Ent developed specific fecal IgA antibodies that recognized Ent (Figure 7.8c, 7.9b). The production of anti-Ent IgA significantly increased at day 21 post-immunization (Figure 7.8c,d), in conjunction with the detection of specific anti-CTB IgA found in both groups of mice (Figure 7.9c). An ELISPOT assay confirmed that B cells isolated from the Peyer’s patches of mice immunized with CTB-Ent were the source of anti-Ent IgA (Figure 7.8d). In contrast, IgA that recognized unmodified CTB were secreted by B cells isolated from the Peyer’s patches in both groups of mice (Figure 7.9d).
Figure 7.8. Intranasal immunization with CTB-Ent induces the development of anti-enterobactin and anti-salmochelin IgA antibodies in the gut mucosa. (a) Description and timeline of the immunization protocol. Timing of intranasal immunization (day 0 and day 14) and dosage of antigen (100 μg/mL) are indicated. Feces were collected weekly. ELISPOT assay was carried out at day 21 post-immunization. Mice were infected with *Salmonella enterica* serovar Typhimurium *per os* between day 35 and day 51 post-immunization. (b) Anti-Ent IgA were quantified by using an in-house ELISA in fecal samples from mice immunized with either CTB (n = 5) or CTB-Ent (n = 7) during the indicated time course. (c) 2-Fold dilution for detection of fecal anti-Ent IgA by ELISA in mice immunized with either CTB (n = 15-20) or CTB-Ent (n = 15-20) at day 21 post-immunization. (d) Representative ELISPOT images of supernatant from B cells producing anti-Ent IgA isolated from Peyer's patches of mice immunized with either CTB or CTB-Ent at day 21 post-immunization. Average number of spots detected in CTB or CTB-Ent immunized mice (n = 7). (e) 2-Fold dilution for detection of fecal anti-DGE IgA by ELISA in mice immunized with either CTB (n = 15-20) or CTB-Ent (n = 15-20) at day 21 post-immunization. (f) Representative ELISPOT images of supernatant from B cells producing anti-DGE IgA isolated from Peyer's patches of mice immunized with either CTB or CTB-Ent at day 21 post-immunization. Average number of spots detected in CTB or CTB-Ent immunized mice (n = 5). In (b)-(f), bars represent the mean ± standard error. **** P < 0.0001, *** P < 0.001, ** P < 0.01.
Figure 7.9. Detection and characterization of antibodies in response to CTB/CTB-Ent immunization. (a) Representative DOTBLOT image for detection of anti-siderophore antibodies. Ent (1 μg) or DGE (1 μg) was spotted on a PVDF membrane and CTB-Ent fecal extract (day 21 post-immunization) was used to detect specific IgA anti-Ent and anti-DGE. (b) Total fecal IgA detected at day 0, day 14 and day 21 post-immunization in CTB (n = 6) and CTB-Ent (n = 6). (c) Anti-CTB fecal IgA detected in CTB (n = 6) and CTB-Ent (n = 6) by in-house ELISA. (d) Representative ELISPOT images of CTB-IgA from Peyer’s patches of mice immunized with either CTB or CTB-Ent at day 21 post-immunization. (e) Representative ELISPOT images of Ent-IgG and CTB-IgG from spleen of mice immunized with either CTB or CTB-Ent at day 21 post-immunization. In (d) and (e), bar graph represents the number of spots detected in the CTB or CTB-Ent immunized mouse. In (a) and (c), bars represent the mean ± standard error.
We next investigated whether the mucosal IgA elicited by CTB-Ent immunization would also recognize DGE. Consistent with our expectation based on the structural attributes shared by Ent and DGE (Figure 7.1), specific fecal IgA from mice immunized with CTB-Ent also recognized DGE (Figures 7.8e,f, 7.9b). This immunization appeared to specifically trigger mucosal immunity to Ent and DGE. We found no evidence for the production of anti-Ent and anti-DGE IgG in the spleen or blood of mice immunized with CTB-Ent, even though we detected IgG recognizing unmodified CTB in these tissues (Figure 7.9e). Additionally, the response to the CTB-Ent antigen was independent of the route of immunization. Mice immunized intraperitoneally with CTB-Ent also developed only anti-Ent/DGE IgA in the Peyer's patches (Figure 7.10a,b). We thus conclude that our immunization strategy specifically affords the generation of mucosal IgA that recognize Ent and DGE. These results are in agreement with the known effects of CTB in boosting mucosal immunity.27
Figure 7.10. Intraperitoneal immunization with CTB-Ent induces the development anti-siderophores IgA antibodies. (a) Representative ELISPOT images of Ent-IgA of DGE-IgA from Peyer's patches of mice immunized intraperitoneal injection with either CTB or CTB-Ent at day 21 post-immunization and boosted with 100 µg/mouse of CTB or CTB-Ent. (b) Average number of spots detected with ELISPOT assay in CTB and CTB-Ent immunized mice. Bars represent the mean ± standard error. (c) Bacterial load in the colon of C57BL/6 immunized by intraperitoneal injection with CTB or CTB-Ent and infected with Salmonella. CTB (n = 4) and CTB-Ent immunized mice (n = 5) at day 4 post-infection. (d) Bacterial load in the spleen of C57BL/6 immunized by intraperitoneal injection with CTB or CTB-Ent and infected with Salmonella. CTB immunized mice (n = 4) and CTB-Ent immunized mice (n = 5) at day 4 post-infection. In (c) and (d), each circle represents an individual mouse. Bars represent the geometric mean. * P < 0.05, n.s. = not significant.

7.4.3 Immunization with CTB-Ent Reduces Salmonella Gut Colonization and Systemic Dissemination.

Once we established the antibody response, we investigated whether the production of anti-Ent/DGE IgA would limit gut colonization and systemic dissemination of Salmonella. We employed the mouse model of Salmonella colitis and infected mice that are highly...
susceptible to *Salmonella* (C57BL/6 mice). Groups of 10-20 C57BL/6 mice purchased from Taconic, and immunized with either CTB (mock-immunized) or CTB-Ent, were treated with a single oral dose of streptomycin, followed by infection with $1 \times 10^9$ CFU/mouse of *Salmonella enterica* serovar Typhimurium. At both day 1 and day 2 post-infection, we observed similar levels of *Salmonella* colonization in the colon content between the groups (Figure 7.11a and data not shown). In contrast, we found a significant reduction of *Salmonella* in the colon content of mice immunized with CTB-Ent at day 3, and especially at day 4, post-infection (Figure 7.11a). *Salmonella* colonization was lower in mice with the highest anti-Ent antibody titer, with some mice showing as few as 100-1,000 *Salmonella* CFU/mg of colon content (Figure 7.11b). *Salmonella* colonization of the terminal ileum and Peyer's patches (Figure 7.11c,d), but not of the mesenteric lymph nodes (Figure 7.12d), was also reduced in mice immunized with CTB-Ent. Moreover, mice immunized with CTB-Ent exhibited reduced weight loss (Figure 7.11e) and reduced *Salmonella* dissemination to the spleen (Figure 7.11f) compared to mock-immunized mice, which indicates a slower progression of disease resulting from CTB-Ent immunization. In contrast to mock-immunized mice, which had to be sacrificed at day 4 because of the high weight loss and bacterial burden, we could monitor mice immunized with CTB-Ent to day 6 post-infection (Figure 7.12a). Moreover, when mice were immunized intraperitoneally with CTB-Ent, we observed similar reductions of *Salmonella* gut colonization and dissemination to the spleen (Figure 7.10c,d).
Figure 7.11. Immunization of mice with CTB-Ent reduces *Salmonella* gut colonization and systemic dissemination. (a) *Salmonella* colonization in the colon content of mice immunized intranasally with either CTB (n = 35) or CTB-Ent (n = 27), then infected with $10^8$ CFU of *Salmonella* by oral gavage. (b) Scatter plot showing an inverse correlation between *Salmonella* CFU in the colon content at day 4 post-infection and anti-Ent IgA detected by ELISA assay in individual mice that were immunized with CTB-Ent prior to infection (n = 21). c,d, *Salmonella* CFU/mg in the terminal ileum (c) and Peyer’s patches (d) of mice immunized with CTB (n = 33) or CTB-Ent (n = 22) at day 4 post-infection. (e) Weight loss of mice immunized with either CTB or CTB-Ent at day 4 post-infection (n = 15 per group). (f) *Salmonella* CFU/mg in the spleen of mice immunized with either CTB (n = 33) or CTB-Ent (n = 22) at day 4 post-infection. In (a)-(f), each circle represents an individual mouse. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, n.s. = not significant.
Figure 7.12. Immunization with CTB-Ent reduces *Salmonella* gut colonization. (a) Bacterial load in colon content of immunized mice infected with *Salmonella*. CTB immunized mice (n = 35) and CTB-Ent (n = 27) immunized mice at day 4, 5 and 6 post-infection. Grey dots (n = 5) represent CTB-Ent immunized mice that survived after day 4 post-infection. (b) Blinded histopathology scores of mice immunized with CTB or CTB-Ent at day 6 post-infection. (c) Competitive index (CI) of *S. Typhimurium* wild-type over *S. Typhimurium iroN* mutant in CTB or CTB-Ent immunized mice at day 3 and day 4 post-infection. Bars represent the geometric mean. (d) *Salmonella* CFU/mg in mesenteric lymph nodes (MLN) of CTB (n = 33) and CTB-Ent (n = 22) immunized mice at day 4 post-infection. Bars represent the mean ± standard error. (e) Bacterial load in the colon of C57BL/6 bred in our vivarium immunized with CTB or CTB-Ent at day 4 post-*Salmonella* infection. (f) *Salmonella* colon content in C57BL/6 NRAMP1 mice immunized with CTB (n = 6) or CTB-Ent (n = 10) at day 8 post-*Salmonella* infection. In (a), (d), (e), and (f), each circle represents an individual mouse. **** P < 0.0001, * P < 0.05, n.s. = not significant.
To ensure that the success of our immunization strategy was independent of housing conditions and differences in the gut microbiota, we immunized and infected mice from two colonies bred in our vivarium. The first colony consisted of conventional C57BL/6 mice, and the second colony consisted of C57BL/6 mice that have been engineered to express a functional Natural Resistant Macrophage Protein 1 (NRAMP1) protein which, in the Raffatellu Lab, survive up to 8-9 days post-infection. Analogous to the mice purchased from Taconic, the C57BL/6 mice from the first colony produced anti-Ent IgA in the Peyer's patches (data not shown) and exhibited reduced Salmonella colonization in the colon at day 4 post-infection (Figure 7.12e). Moreover, in the CF7BL/6 NRAMP1+ mice, we observed a significant reduction in Salmonella burden in the colon at day 8 post-infection as a result of CTB-Ent immunization (Figure 7.12f). Taken together, these results indicate that our immunization strategy was successful in different mouse lines and highly reproducible.

A Salmonella mutant in the DGE receptor IroN is defective in colonization of the inflamed gut where LCN2 is highly induced. Thus, we evaluated whether we could reduce the competitive advantage of wild-type Salmonella over the iroN mutant in mice immunized with CTB-Ent. We reasoned that anti-Ent/DGE IgA produced in response to CTB-Ent immunization sequester DGE in the gut lumen, thereby inhibiting growth of wild-type Salmonella. Consistent with our hypothesis, the competitive advantage of wild-type Salmonella over the iroN mutant was significantly reduced in mice immunized with CTB-Ent (Figure 7.12c). These results further support the model that sequestration of DGE by specific IgA contributes to the reduction of Salmonella colonization in mice immunized with CTB-Ent.
7.4.4 Immunization with CTB-Ent Does Not Lower Intestinal Inflammation upon Salmonella Infection

Because *Salmonella* thrives in the inflamed gut and benefits from inflammatory responses from the host during infection, we questioned whether the lower *Salmonella* levels in the colon content resulted from reduced inflammation in mice immunized with CTB-Ent. Histopathology analysis ruled out this possibility because similar levels of intestinal inflammation were observed in mock-immunized and CTB-Ent immunized mice upon *Salmonella* infection (Figure 7.12b, 7.13a,b).

**Figure 7.13.** Mice immunized with CTB-Ent show similar pathology and inflammation as mock-immunized mice. (a) Blinded histopathology scores of mice immunized with CTB or CTB-Ent at day 4 or day 6 post-infection. (b) H&E stained sections from representative animals for each group. (c) Relative expression of *Lcn2*, *Il17a*, and *Il22* in cecal samples of mice immunized with CTB (n = 12) or CTB-Ent (n = 12) then mock-infected (n = 7) or infected with *Salmonella* for 4 days. (d) Relative expression of *Ly6g*, *Cxcl-1* and *Nos2* in cecal samples of mice immunized with CTB (n = 12) or CTB-Ent (n = 12) at day 4 post-infection. In (c) and (d), bars represent the mean ± standard error.
Moreover, several inflammatory markers, including genes encoding LCN2, interleukin (IL)-17A, IL-22, CXCL-1, LY6G, and iNOS, were similarly upregulated in both groups of mice after infection (Figure 7.13c,d). Taken together, these results demonstrate that lower levels of Salmonella gut colonization occurred in mice immunized with CTB-Ent despite similar high levels of intestinal inflammation.

One consequence of intestinal inflammation is a profound alteration of the gut microbiota, termed "dysbiosis". The highly oxidative environment in the inflamed gut reduces the growth of obligate anaerobes, which constitute approximately 99% of microbes in the normal, non-inflamed gut. In contrast, inflammation promotes the bloom of Enterobacteriaceae, including Salmonella. These facultative anaerobes can respire novel electron acceptors (e.g., nitrate, tetrathionate) that become available only in the inflamed gut, thus outcompeting the resident microbiota.\textsuperscript{33,34} We therefore questioned the impact of our immunization strategy on the microbiota in the inflamed gut, especially in light of the lower levels of Salmonella observed in CTB-Ent immunized mice (Figure 7.11a). In the absence of infection and thus of intestinal inflammation, composition of the gut microbiota was not affected by immunization with CTB or CTB-Ent (Figure 7.14a, Table 7.2). Consistent with earlier studies in naive mice,\textsuperscript{31} we found that Salmonella infection induced a significant decrease in bacterial diversity at day 4 post-infection in both mice immunized with CTB or CTB-Ent (Figure 7.14a). Salmonella constituted an average of 50% of the gut microbes in mock-immunized mice, and this value dropped to an average of 15% in mice immunized with CTB-Ent (Figures 7.14b,c, 7.15). Moreover, other Enterobacteriaceae (e.g. Proteus spp.) did not expand in the inflamed gut of mice immunized with CTB-Ent. Because all Enterobacteriaceae employ
Figure 7.14. Analysis of the gut microbiota in mice immunized with CTB or CTB-Ent before and after infection with *Salmonella*. (a) Gut bacterial diversity, measured by the Shannon diversity index, in mice immunized with CTB or CTB-Ent, before infection and 4 days after infection with *Salmonella* (n = 8 per group). Bars represent the mean ± standard error. (b) Analysis of the relative abundance of *Salmonella* and *Lactobacillus* in individual mice (n = 8 per group), measured by Illumina MiSeq in mice immunized with CTB or CTB-Ent, before infection and 4 days after infection with *Salmonella*. Each circle represents an individual mouse. (c) Relative abundance of order/family/genus 16S rDNA sequence assignments (Illumina MiSeq) in fecal samples from mice immunized with CTB or CTB-Ent, collected before infection (mock, n = 8) and at day 4 post-infection (infected, n = 8). Bar represent the geometric mean. **P < 0.01, n.s. = not significant.
Table 7.2. Analysis of the gut microbiota of CTB/CTB-Ent-immunized mice before and after *Salmonella* infection.

### Mean and Standard Deviation

<table>
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<tr>
<th>Bacterial Taxa</th>
<th>CTB mock</th>
<th>CTB-Ent mock</th>
<th>CTB d4 p.i.</th>
<th>CTB-Ent d4 p.i.</th>
<th>CTB mock vs CTB-Ent mock</th>
<th>CTB mock vs CTB d4 p.i.</th>
<th>CTB-Ent mock vs CTB d4 p.i.</th>
<th>CTB-Ent mock vs CTB-Ent d4 p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteobacteria. Enterobacteriaceae</td>
<td>0.075+/-.0139</td>
<td>0.01428571+/-.0038</td>
<td>49.6375+/-.21.125</td>
<td>17.0125+/-.19.458</td>
<td>NS</td>
<td>****</td>
<td>****</td>
<td>****</td>
</tr>
<tr>
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<td>2.057143+/-.2.084</td>
<td>0+/-.0</td>
<td>0.0125+/-.0.036</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Proteobacteria. Enterobacteriaceae</td>
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<td>0+/-.0</td>
<td>5.2875+/-.6.222</td>
<td>0.7+/-.1.333</td>
<td>NS</td>
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<tr>
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<td>0.02857143+/-.0.049</td>
<td>0.4125+/-.0.429</td>
<td>0.9625+/-.0.717</td>
<td>NS</td>
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Tukey’s multiple comparisons test
Ent for iron acquisition, we reason that the production of anti-Ent IgA limits the expansion of this family by sequestering this common siderophore. Although most other bacterial genera were equally affected by *Salmonella* infection in both groups of mice (Figure 7.14c, Table 7.2), we detected a significant expansion of *Lactobacillus* spp. in the inflamed gut of mice immunized with CTB-Ent (50%), but not in mock-immunized mice (15%) (Figures 7.14b,c, 7.15). One likely explanation is that *Lactobacillus* spp. also replicate in a similar environment as Enterobacteriaceae and benefits from inflammation, although by unknown mechanisms.\(^{31}\) Importantly, *Lactobacillus* spp. are considered beneficial microbes and a few species are even administered as therapeutics agents.

Figure 7.15. Single mouse microbiota analysis of CTB-Ent/CTB immunized mice before and after *Salmonella* infection. Single mice for analysis of the microbiota (Illumina MiSeq). Relative abundance of order/family/genus 16S rDNA sequence assignments from CTB and CTB-Ent immunized mice stool samples, collected before (mock, \(n = 8\)) and at day 4 post-infection (infected, \(n = 8\)).
7.5 Summary and Outlook

For many years, siderophores as well as the biosynthetic and transport machineries for these virulence factors have garnered significant interest for the development of new antibiotics. Reported efforts as discussed in Chapter 5 (Section 5.4) include the design and application of siderophore-antibiotic conjugates for targeted drug delivery, the identification of small-molecule inhibitors of siderophore biosynthesis, and the inhibition of siderophore uptake by immunization against siderophore receptors or siderophores. A prior study described the development of antibodies against vibrobactin; however, there was no indication as to whether this vaccination provided protection to the host during infection with *Vibrio cholerae*.\(^{35}\) Our work establishes that immunization against bacterial siderophores results in the production of anti-siderophore antibodies and affords reduced colonization and disease severity during infection with an enteric pathogen. This siderophore-capture mechanism mediated by the adaptive immune system boosts metal-withholding by the innate immune system and provides narrow-spectrum growth inhibitory activity. We expect that this strategy will be broadly applicable for preventing or ameliorating disease during infection caused by pathogens that utilize other siderophores to acquire iron and thrive in the mammalian host. Because synthesis and uptake of new siderophores would require acquisition of new gene clusters by horizontal gene transfer, we predict that resistance against our antibody-based strategy would not readily develop. Broadly, our work indicates that new preventive and therapeutic strategies for microbial infections should target siderophore-mediated iron acquisition, a virulence trait shared by almost all bacterial and fungal pathogens.
7.6 Summary of the Siderophore-Related Projects

Due to the emergence of bacterial resistance to antibiotics, an alternative therapeutic strategy is required to treat bacterial infections. Furthermore, broad-spectrum antibiotics typically wipe out both pathogens and commensal bacteria. More recent studies reveal the benefits of the gut microbiota to the host; for example, the gut microbiota confer protection against pathogenic invaders. The altered population of the gut microbiota caused by broad-spectrum antibiotics often lead to secondary infection (e.g. by *C. difficile*). Therefore, having narrow-spectrum antibacterial activity is one of the most essential properties for this new therapy.

To date, one of the most explored strategies to combat bacterial infection is to target bacterial virulence. This approach provides specificity to pathogens and minimally perturbs the commensal bacteria. Siderophores are considered to be virulence factors among bacterial pathogens. Many siderophore-based approaches have been studied for decades. Examples as discussed in Chapter 5 are the targeted drug delivery using siderophore-antibiotic conjugates (also known as "Trojan Horse" strategy),\textsuperscript{36} the inhibition of siderophore-mediated iron acquisition by using antibodies against siderophore or siderophore receptors,\textsuperscript{35,37} and the identification of small-molecule inhibitors of siderophore biosynthesis and transport.\textsuperscript{38}

My siderophore-based dissertation project involves two distinct strategies that focus on Ent and its glycosylated derivatives to target enteric bacteria, such as *E. coli* and *Salmonella* spp. The first approach is the Trojan Horse strategy, in which we utilize siderophores and the specific recognition between the siderophores and the uptake receptors to deliver toxic compounds into bacteria. Many siderophore-antimicrobial
conjugates have been synthesized and tested for the antibacterial activity as discussed in Chapter 5 (Section 5.4.1); however, there are mixed results of enhanced antibacterial activity compared to the parent drug. The less successful studies are probably due to the improper design of the siderophore-antimicrobial conjugates.

Before I started to work on this approach, Dr. Zheng in the Nolan Lab had synthesized the first monofunctionalized Ent, which allows the attachment of cargos, including antibiotics, to the Ent scaffold. Later, she demonstrated that the Ent-β-lactam conjugates exhibited enhanced antibacterial activity up to 1,000 fold against several E. coli strains when compared to the parent antibiotic. The next goal was to use salmochelin (DGE), which is a glycosylated analog of Ent, to target enteric pathogens, including uropathogenic E. coli and S. Typhimurium. I then continued working from Dr. Zheng’s preliminary data and as discussed in Chapter 6, we demonstrate that glycosylated Ent-β-lactam conjugates display enhanced antibacterial activity up to 1,000 fold only against uropathogenic E. coli strains, but not when against commensal E. coli strains. In addition, these conjugates selectively kill uropathogenic E. coli in the co-cultures of these organisms with commensal E. coli or L. rhamnosus, which is a probiotic.

Taken together, we successfully show that the broad-spectrum antibacterial activity of β-lactams can be tuned into species-specific and pathogen-specific antibacterial activity by using DGE and the receptor IroN in the targeted delivery. The next step in this project is to evaluate the efficacy of the Ent-β-lactam conjugates and the glycosylated derivatives in a murine Salmonella infection model. Another avenue that is worth pursuing is to employ other toxic molecules. Potential candidates include
the drugs that exhibit high toxicity to host cells and the antibiotics that have not encountered bacterial resistance. The siderophore-mediated delivery of these compounds may direct the drugs to bacteria, and thereby reduce the toxicity to the host.

The other siderophore-based approach is based on immunization (Chapter 8). The central idea of this strategy is to produce and employ siderophore-specific antibodies to sequester siderophores to reduce bacterial iron uptake, and thereby prevent bacterial colonization in the host. This approach is fairly new to the field when compared to the Trojan Horse strategy. There are only a few studies on using antibodies against siderophore or siderophore receptors. The goal of this project is to raise antibodies against Ent and DGE and use them as passive immunization to fight against S. Typhimurium infection. We first synthesized conjugates of a carrier protein CTB and a siderophore Ent by attaching Ent to the lysine residues of CTB via amide-bond formation. The conjugation of small-molecule Ent to a larger protein enhances the immunogenicity of Ent. Subsequently, in collaboration with the Raffatellu Lab at UC Irvine, we immunized mice with CTB-Ent and characterized the produced antibodies. The immunized mice produce anti-Ent and anti-DGE antibodies in the gut mucosa and these mice exhibit reduced intestinal colonization, reduced systemic dissemination of S. Typhimurium, and increased resistance against the challenge of this pathogen. Although there have been studies on immunization against siderophores and the uptake receptors, our work is the first report that provides promising in vivo results from immunization against siderophores. The next step in this project is to isolate monoclonal antibodies and use them as passive immunization in a mouse model. In addition, we expect that this siderophore-based immunization will be broadly applicable for other
pathogens that use different siderophores to acquire iron. In summary, both of our siderophore-based studies support that siderophore-mediated iron acquisition system in bacteria is a potential target for developing new treatment for bacterial infection.

7.7 Acknowledgments

We thank Professor M. Raffatellu, Ms. M. Sassone-Corsi, and Dr. Araceli Perez-Lopez at UC Irvine for the animal studies and the corresponding data. We thank Professor R. A. Edwards at UC Irvine for histopathology analysis. We thank Dr. M. D. George and Matthew Rolston at the UC Davis School of Medicine Host-Microbe Systems Biology Core for mouse microbiota analysis and processing samples for Illumina MiSeq analysis. We thank Dr. T. Zheng for the synthesis of Ent-PEG3-biotin. This work was supported by a pilot project from the Pacific Southwest Regional Center of Excellence for Biodefense and Emerging Infectious Disease (supported by Public Health Service Grants U54AI065359), and by Public Health Service Grants AI101784 and AI114625 to MR and EMN. Work in MR lab is also supported by Public Health Service Grants AI083663, AI105374, and DK058057. Work in EMN lab is also supported by the Kinship Foundation (Searle Scholar award to EMN) and the MIT Department of Chemistry. MR holds an Investigator in the Pathogenesis of Infectious Disease Award from the Burroughs Wellcome Fund. APL was funded by a UC MEXUS-CONACYT award. PC is a recipient of a Royal Thai Government Fellowship. NMR instrumentation in the MIT Department of Chemistry Instrumentation Facility is supported by NSF grants CHE-9808061 and DBI-9729592.
7.8 References


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Appendix A

Methionine Oxidation Disrupts

Self-Assembly of Human Defensin 6
A.1 Introduction

In Chapter 2, we discussed the preparation of native HD6 and its variants and the effect of hydrophobicity on the self-assembly of HD6. During the development of the preparation protocol for HD6, we accidentally obtained a new HD6 species as a byproduct. The characterization of this new species, which will be discussed in Section A.3, indicated that this species corresponded to HD6, of which Met23 was oxidized to methionine sulfoxide, hereafter described as Met(O)23-HD6. Our study on HD6 and its variants as discussed in Chapter 2 indicates that the hydrophobic residues of HD6 (Figure A.1), especially F2 and F29, are important for its ability to oligomerize in solution. Met23 is hydrophobic and it is located in the hydrophobic pocket as shown in Figure A.1. As a result, we questioned its effect on the self-assembly of HD6. Moreover, a methionine residue exhibits several biological functions, including acting as an antioxidant against oxidative stress during infection. It can be oxidized to methionine sulfoxide and such an oxidative post-translational modification can lead to some consequences, such as disruption or enhancement of protein-protein interactions. Therefore, we desire to characterize the oxidation of Met23 and investigate its relevance in vivo. In this Appendix, we report a robust protocol for preparing Met(O)23-HD6 from HD6. Our preliminary biophysical studies reveal that oxidation of Met23 causes defects in the morphology of HD6 fibrils, suggesting that Met23 is also essential for the self-assembly of HD6.
Figure A.1. Left: previously reported extended crystal structure of HD6 (PDB: 1ZMQ).\textsuperscript{5} Hydrophobic residues are shown in orange. Inset: a HD6 monomer unit illustrates the three-stranded \( \beta \)-sheet fold and Met23 residue. Right: close-up view of the hydrophobic pocket located among HD6 monomers. Individual HD6 monomers are labeled A-D.

A.2 Experimental Section

A.2.1 General Materials and Methods

All solvents, reagents, and chemicals were purchased from commercial suppliers and used as received unless noted otherwise. The mature 32-residue form of HD6 was recombinantly overexpressed and purified as described in Chapter 2. All functional and biophysical studies were performed with oxidatively folded peptides. All buffers, aqueous solutions, and peptide stock solutions were prepared in Milli-Q water (18.2 m\( \Omega \cdot \text{cm} \)) after it was passed through a 0.22-\( \mu \text{m} \) filter. All details of the instrumentation are described in Chapter 2 (Section 2.2.2). Extinction coefficients (280 nm) for native HD6 and Met(O)23-HD6 were calculated by using ExPASy ProtParam and both values are 4845 M\(^{-1}\) cm\(^{-1}\).
A.2.2 Time-Point Analysis of Acid-Catalyzed Methionine Oxidation of HD6

Oxidation of Met23 in HD6 was set up following a literature protocol for oxidizing a methionine residue with modifications. A solution of HD6 (1 mg/mL, 700 μL) was prepared in 6 M GuHCl, 1 M HCl, and 5% DMSO at room temperature. An 80-μL aliquot was taken after 10 min, 2, 3, 4, 5, 8, and 16 h and basified with 30 μL of 3 M Tris pH 8.0. The resulting samples were vortexed, centrifuged (13000 rpm x 10 min, 4 °C), and analyzed by HPLC (10–60% B over 30 min at 1 mL/min).

A.2.3 Synthesis of Met(O)23-HD6

HD6 (3.8 mg, 1.0 μmol) was dissolved in a mixture of 6 M GuHCl, 1 M HCl, and 5% DMSO (3.8 mL). The reaction was incubated at room temperature. After 3 h, 3 M Tris pH 8.0 (1.4 mL) was added to quench the reaction. The crude mixture was purified by semi-preparative HPLC to yield 2.5 mg of Met(O)23-HD6 (66% yield). The product eluted at 15.5 min and the analytical HPLC trace of Met(O)23-HD6 is shown in Figure A.4. The calculated m/z is 3,721.5 and the observed m/z is 3,721.5.

A.2.4 Negative-Staining Transmission Electron Microscopy

The detailed protocol for preparing samples for TEM is in Chapter 2 (Section 2.2.10). TEM images were obtained with at least two independently prepared peptides and representative images are presented.
A.3 Results and Discussion

The first evidence of the existence of Met(O)23-HD6 was found during the purification of native HD6 by semi-preparative HPLC. A fraction collector was employed, and the collected fractions were left in the fraction collector overnight. When we analyzed the fractions corresponding to HD6 (16.9 min) by analytical HPLC, we observed the HD6 peak as well as a new peak, which eluted at 15.5 min. The LC-MS analysis of the new peak revealed its mass to be 3,721.50 Da, which corresponds to the mass of HD6 (3,705.5 Da) with an additional oxygen atom. The crystal structure of HD6 (Figure A.1) shows that Met23 is located on the loop and exposed to the surface. It is known that low pH can promote methionine oxidation.⁶ Taken together, we hypothesized that the presence of 0.1% TFA in the HPLC eluent catalyzed the oxidation of Met23 in HD6 (Figure A.2).

![Figure A.2. Analytical HPLC trace of purified HD6 after being left in the fraction collector overnight. Absorbance at 220 nm was monitored with a reference wavelength of 500 nm. Method: 10–60% B over 30 min at 1 mL/min.](image)

We have established a preparation protocol for converting native HD6 to Met(O)23-HD6 to enable further characterization of this HD6 species and the effect of
methionine oxidation on the self-assembly of HD6. We set up the reaction in the presence of 1 M HCl and 5% DMSO to oxidize the methionine residue. GuHCl (6 M) was added to disrupt the self-assembly of HD6, which may cause Met23 to be less solvent-exposed. We observed that a new peak corresponding to Met(O)23-HD6 formed after 10 min and native HD6 was fully converted to Met(O)23-HD6 after 4 h (Figure A.3). There was also another peak showing up after an overnight incubation of HD6 under acidic conditions; however, its identity is not determined. We then scaled up this reaction to prepare Met(O)23-HD6 in milligram quantities (Figure A.4).

![Figure A.3](image)

**Figure A.3.** Analytical HPLC trace of oxidation of Met23 in HD6 at different time points. Absorbance at 220 nm was monitored with a reference wavelength of 500 nm. Method: 10–60% B over 30 min at 1 mL/min.
Figure A.4. Analytical HPLC traces of purified Met(O)23-HD6. Absorbance at 220 nm was monitored with a reference wavelength of 500 nm. Method: 10–60% B over 30 min at 1 mL/min.

Our study with F2A and F29A variants described in Chapter 2 revealed that the hydrophobic residues of HD6 are important for its self-assembly. Therefore, we questioned whether the oxidation of Met23, which also reduces the hydrophobicity of this residue, would affect the self-assembly of HD6. We employed negative-staining transmission electron microscopy (TEM) and analytical ultracentrifugation (AUC) to address this question. Preliminary TEM (Figure A.5) reveals that the Met(O)23-HD6 fibrils display distinct morphology from the HD6 fibrils. Met(O)23-HD6 forms relatively short fibrils compared to HD6.

Figure A.5. Transmission micrographs of 20 μM HD6 (left) and Met(O)23-HD6 (right) in 10 mM sodium phosphate pH 7.4. Scale bar = 100 nm.
Our preliminary AUC study of Met(O)23-HD6 in 10 mM sodium phosphate, pH 7.4 were not successful, but indicated that Met(O)23-HD6 has different oligomerization properties from HD6. When HD6 and Met(O)23-HD6 (100 μM in 10 mM sodium phosphate, pH 7.4) were centrifuged at 3,000 rpm, HD6 immediately sedimented as described in Chapter 2 (Section 2.3.2), whereas Met(O)23-HD6 remained evenly distributed over 6 h, indicating that the rotor speed was not enough to cause Met(O)23-HD6 to sediment. When the rotor speed was increased to 20,000 rpm, Met(O)23-HD6 immediately sedimented. Taken together, our preliminary data suggest that Met(O)23-HD6 forms higher-order oligomers, but these oligomers are smaller than those of HD6. AUC experiments at speed between 3,000 and 20,000 rpm may be employed to obtain the S value of Met(O)23-HD6 in future work. In addition, biological assays, including bacterial agglutination and bacterial invasion assays, are needed to further elucidate the functional properties of Met(O)23-HD6.

A.4 Summary and Outlook

This Appendix details the preparation of Met(O)23-HD6 and preliminary biophysical studies on its self-assembly property. Negative-staining TEM and AUC reveal that Met(O)23-HD6 forms higher-order oligomers; however, the Met(O)23-HD6 fibrils display defective morphology when compared to those of HD6. Additional experiments, including bacterial agglutination and bacterial invasion assays, are needed to confirm these observations. We question whether methionine oxidation modulates the functional properties of HD6, including the self-assembly. Oxidation of Met23 may be another way of temporally controlling the self-assembly of HD6 because the methionine oxidation is
reversible by the methionine reductases. In addition, a methionine residue has several biological functions, including acting as an antioxidant, a redox sensor, and a signal molecule and some defensins display immunomodulatory properties. Therefore, HD6 may serve these biological roles by utilizing the Met23 residue and its oxidation. Further investigations are needed to address these questions and determine whether the significance of Met(O)23-HD6 in the biological systems.

A.5 Acknowledgements

We thank Ms. D. Pheasant and Dr. A. Wommack for assistance with the AUC experiments, and Dr. E. J. Brignole for helpful advice on TEM sample preparation and data collection.

A.6 References


Appendix B

An N-terminal Extension of Human Defensin 6
Attenuates Its Self-Assembly and Biological Function
B.1 Introduction

Our previous study on proHD6\(^1\) as discussed in Chapter 3 showed that its 49-residue pro region is utilized to temporally and spatially control the self-assembly of HD6 and allow HD6 to be stored in the granules of intestinal Paneth Cells as an inactive propeptide. In addition, the crystal structure of HD6\(^2\) indicates that HD6 monomers must properly align to form a hydrophobic pocket to drive the self-assembly of HD6. The N-terminal pro region interferes with the alignment of the HD6 monomers and thereby blocks the self-assembly and functional properties observed for HD6. We speculated that a short peptide sequence at the N- or C-terminus of HD6 is likely sufficient to disrupt the formation of the hydrophobic pocket among HD6 monomers, and hence to suppress the self-assembly of HD6. To test our hypothesis, we prepared truncated proHD6 (hereafter described as proHD6\(^{62-100}\)) that contains a 7-residue N-terminal extension (RALGSTR-HD6) by utilizing chymotrypsin to cleave His\(_6\)proHD6 that we obtained from overexpression in *Escherichia coli*. We then employed negative-staining transmission electron microscopy (TEM), bacterial agglutination assays, and *Listeria monocytogenes* invasion assays to evaluate the effect of the short N-terminal extension on the self-assembly of HD6 and its host-defense function.

B.2 Experimental Section

B.2.1 Materials and General Methods

All solvents, reagents, and chemicals were purchased from commercial suppliers and used as received unless noted otherwise. The His\(_6\)proHD6 and the mature 32-residue form of HD6 were recombinantly overexpressed and purified as described in Chapter 2.
These procedures involve oxidative folding, which allows for isolation of the native CysI—CysVI, CysII—CysIV, CysIII—CysV regioisomer following purification. All functional and biophysical studies were performed with oxidatively folded peptides. Milli-Q water (18.2 MΩ•cm) was passed through a 0.22-μm filter before it was used to prepare all buffers, aqueous solutions, and peptide stock solutions. All details of the instrumentation are listed in Chapter 2 (Section 2.2.2). Both extinction coefficients of native HD6 and proHD6$^{62-100}$ were calculated by using ExPASy ProtParam and both values are 4845 M$^{-1}$cm$^{-1}$, respectively.

**B.2.2 Preparation and Purification of Truncated proHD6**

A 400-μL aliquot of a 1-mg/mL stock solution of chymotrypsin (Worthington) in Milli-Q water was added to a 40-mL solution of His$_6$-proHD6 (1 mg/mL in 100 mM Tris-HCl pH 8.2 containing 20 mM CaCl$_2$) to afford a final chymotrypsin concentration of 0.01 mg/mL. The reaction was incubated at room temperature for 1 h and subsequently quenched by addition of 6% TFA/H$_2$O (10% v/v). The quenched reaction was immediately vortexed, flash frozen in liquid N$_2$, and lyophilized to dryness. The resulting powder was resuspended in 25 mL of 6 M GuHCl for 15 min and passed through a 0.22-μm filter. RALGSTR-HD6 was purified by semi-preparative HPLC using a solvent gradient of 25–33% B over 18 min at 4 mL/min. The fractions containing proHD6$^{62-100}$ were lyophilized to dryness to provide a white fluffy powder. The yield was 2.5 mg/L culture. The product eluted at 16.3 min and the analytical HPLC trace of purified proHD6$^{62-100}$ is shown in Figure B.3. The calculated m/z is 4,450.06 Da and the observed m/z is 4,450.73 Da.
B.2.3 Negative-Staining Transmission Electron Microscopy

The detailed protocol for preparing samples for TEM is in Chapter 2 (Section 2.2.10). TEM images were obtained with at least two independently prepared peptides and representative images are presented.

B.2.4 Bacterial Agglutination Assays

Stock solutions (50 μL) of HD6 and proHD6\textsuperscript{62-100} (0, 50, 100, and 200 μM; 10x concentrations) were prepared in sterile Milli-Q water. If trypsin was required, a 5-μL aliquot of a 1-mg/mL stock solution of TPCK-treated trypsin (Worthington) prepared in sterile Milli-Q water was added to solution of proHD6\textsuperscript{62-100}. The assays were then conducted as described in Chapter 2 (Section 2.2.14). All assays were conducted with at least two independently prepared and purified samples of each peptide and in three independent trials. The resulting averages with standard deviations are reported.

B.2.5 Listeria Invasion Assays

Concentrated (20x, 10 mL) solutions of HD6 and proHD6\textsuperscript{62-100} were prepared in sterile Milli-Q water (0, 50, 100, and 200 mM). For conditions that required trypsin, a 5-μL aliquot of a 1-mg/mL stock solution of TPCK-treated trypsin (Worthington) prepared in sterile Milli-Q water was added to solution of proHD6\textsuperscript{62-100}. A 190-mL aliquot of the diluted bacterial culture was added to each 10x peptide solution and the resulting mixtures were incubated at room temperature for 30 min. The invasion assays were then performed as described in Chapter 2 (Section 2.2.16). All invasion assays were conducted with at least two independently prepared and purified samples of each.
peptide and in three independent trials and the resulting averages with standard deviations are reported.

B.2.6 Antimicrobial Activity Assays

The assays were performed as described in Chapter 2 (Section 2.2.13). Briefly, a 90-μL aliquot of the diluted bacterial culture was added to each well and then to each well was added 10 μL of a 10x concentrated aqueous peptide solution (500 μM) or sterile Milli-Q water as a no-peptide control. The plate was incubated for 1 h (37 °C, 150 rpm). The serial dilution for CFU counting was conducted as previously reported. These assays were performed with at least two independently prepared and purified samples of each peptide and in three independent trials. The resulting averages and standard deviations are reported.

B.3 Results and Discussion

B.3.1 Design and Preparation of proHD6

We prepared proHD6 (Figure B.1) to evaluate the effect of a short N-terminal extension of HD6 on its self-assembly and host-defense function. The crystal structure of HD6 shows that four HD6 monomers align to form a hydrophobic pocket and we hypothesized that disrupting the formation of the hydrophobic pocket by extending the N-terminus of HD6 would suppress its self-assembly and biological function. In prior studies of HD6, we overexpressed His6-proHD6 and used trypsin cleavage to afford native HD6. In this work, we employed chymotrypsin to cleave His6-proHD6 and obtain...
proHD6^{62-100}, which contains additional seven residues at the N-terminus of HD6 (Figure B.1).

![Diagram of amino acid sequences](image)

**Figure B.1.** Amino acid sequences of native proHD6 (proHD6^{20-100}), proHD6^{62-100}, and HD6. (a) Trypsin cleaves Arg7 to afford mature HD6 peptide. (b) Chymotrypsin cleaves Leu61 to afford proHD6^{62-100}.

Analytical HPLC revealed that after an hour of incubation, chymotrypsin cleavage of His_{6}proHD6 consumed all the starting peptide and yielded proHD6^{62-100} along with other truncated propeptides, which have not been characterized. We therefore prepared proHD6^{62-100} on a larger scale and analytical HPLC (Figure B.3) indicated that proHD6^{62-100} was obtained in high purity and the yield of 2.5 mg/L of culture. All experiments were performed with the oxidized species and in the absence of reducing agents.
Figure B.2. Analytical HPLC trace of His$_6$proHD6 after the treatment with chymotrypsin for 1 h. Absorbance at 220 nm was monitored with a reference wavelength of 500 nm. Method: 10–60% B over 30 min at 1 mL/min.

Figure B.3. Analytical HPLC trace of purified proHD6$^{62-100}$. Absorbance at 220 nm was monitored with a reference wavelength of 500 nm. Method: 10–60% B over 30 min at 1 mL/min.

B.3.2 TEM Reveals that proHD6$^{62-100}$ Exhibits Defective Self-Assembly

Transmission electron microscopy (TEM) provided the first evidence for defective self-assembly of proHD6$^{62-100}$ (Figure B.4). Notably, the 7-residue N-terminal extension exhibited a pronounced effect on fibril formation and morphology. Instead of having elongated structure as observed for HD6, proHD6$^{62-100}$ only forms short fibrils, which
aggregate together (Figure B.4). These observations indicate that in addition to hydrophobic residues, a close alignment among monomers is essential for self-assembly of HD6 and that the N-terminal extension causes defects in fibril formation.

Figure B.4. Transmission electron micrographs of 20 μM native HD6 (left) and proHD6^{52-100} (middle and right) in 10 mM sodium phosphate pH 7.4.

B.3.3 The Truncated Pro Region Suppresses Biological Function of HD6

The prior studies of HD6 host-defense function demonstrated that HD6 entangles several bacterial strains and prevents them from invading into host cells.\(^3\,^4\) Moreover, we discussed in Chapter 2 that disrupting HD6 self-assembly causes a reduced propensity to entrap and agglutinate bacteria and to prevent their invasion.\(^3\) Thus, we first employed a bacterial agglutination assay to evaluate the ability of proHD6^{52-100} to cause bacterial sedimentation (Figure B.5). When we introduced proHD6^{52-100} into a bacterial culture (10^8 CFU/mL) housed in a sterile plastic cuvette, we observed that proHD6^{52-100} only at high concentration (20 μM) caused bacterial clumping. These clumps sedimented slowly over the period of 6 h. These results indicated that the 7-residue N-terminal extension attenuated the ability of HD6 to agglutinate bacteria. In agreement with trypsin cleavage of native proHD6 discussed in Chapter 3, trypsin cleaved the N-terminal extension to unleash mature HD6 and yield similar sedimentation profiles to those of HD6 (Figure B.5).
Figure B.5. Agglutination assays of *L. monocytogenes* ATCC 19115 and *E. coli* ATCC 25922 incubated with 0-20μM proHD6<sub>62-100</sub> in the absence of trypsin (left column) or in the presence of trypsin (right column). The plots represent mean ± SDM, n = 2.

In addition, we performed a series of *L. monocytogenes* invasion assays to investigate the effect of the truncated pro region on this biological function of HD6. Consistent with our previous study, mature HD6 (≥2.5 μM) blocks *L. monocytogenes* invasion into human intestinal epithelial cells (Figure B.6). In contrast, proHD6<sub>62-100</sub> did not inhibit *Listeria* invasion over this concentration range (Figure B.6). In agreement with trypsin cleavage of native proHD6 discussed in Chapter 3, when proHD6<sub>62-100</sub> (≥2.5 μM) was treated with trypsin, the percentage of *L. monocytogenes* invasion decreased
from \( \approx 10\% \) to \(< 2\% \), similar to the decrease observed for equivalent concentrations of mature HD6 (Figure B.6). Based on the bacterial agglutination and *Listeria* invasion assays, we conclude that the N-terminal extension can impair the self-assembly of HD6 and consequently its ability to agglutinate bacteria and block *Listeria* invasion.

![Graph showing bacterial agglutination and Listeria invasion](image)

**Figure B.6.** Invasion of human T84 colon epithelial cells by *L. monocytogenes* ATCC 19115 pre-treated with proHD6 in the absence and presence of 0.4 \( \mu \text{M} \) trypsin or HD6 (control). The bacteria (2 \( \times 10^6 \) CFU/mL) were incubated with the indicated peptides for 30 min prior to the infection of the T84 cells (mean ± SDM, \( n = 3 \)).

### B.3.4 Truncation of the Pro Region Confers Antibacterial Activity

We questioned whether proHD6\(^{62-100}\) exhibits antibacterial activity as a result of disrupted oligomerization. In addition, recent work by Mathew and Nagaraj demonstrated that introducing arginine residues into the HD6 sequence turned on bactericidal activity.\(^5\) In this work, proHD6\(^{62-100}\) contains two additional arginine residues at the N-terminus of HD6; therefore, we reasoned that proHD6\(^{62-100}\) may exhibit antibacterial activity. We compared the antibacterial activity of proHD6\(^{62-100}\) and native
HD6 against four bacterial species. *Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* ATCC 19115 were chosen as representative Gram-positive organisms whereas *Escherichia coli* ATCC 25922 and *Salmonella Typhimurium* ATCC 14028 were selected as representative Gram-negative microbes. In these assays, HD5, which has broad-spectrum antibacterial activity, was employed as a positive control and HD6, which exerts negligible antibacterial activity, was used as a negative control. The results presented in Figure B.7 clearly show that proHD6<sup>62-100</sup> displays significant antibacterial activity against *E. coli*, whereas proHD6<sup>62-100</sup> at the same concentration exhibits mild antibacterial activity against the other three bacterial strains. The truncated propeptide causes a one-fold log reduction in the CFU/mL values. We reasoned that introducing two arginine residues at the N-terminus of HD6 confers antibacterial activity. These results are in general agreement with the prior studies on arginine residues and HD6.⁵

![Graph showing antibacterial activity assays](image)

**Figure B.7.** Antibacterial activity assays against *L. monocytogenes* ATCC 19115, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *S. Typhimurium* ATCC 14028 (mean ± SDM, n = 3). The peptide concentrations were 20 μM.
B.4 Summary and Outlook

In this Appendix, we prepared proHD6\textsuperscript{62-100}, which contains a 7-residue extension from the N-terminus of HD6 and we then employed TEM, bacterial agglutination assays, and *Listeria* invasion assays to evaluate the effect of the short N-terminal extension on the self-assembly of HD6 and its host-defense function. Our results indicate that the additional seven residues at the N-terminus of HD6 is sufficient to suppress the self-assembly of HD6 and consequently its biological function, including bacterial agglutination and *Listeria* invasion blockage. Nevertheless, we found that proHD6\textsuperscript{62-100} exhibited antibacterial activity against *E. coli*. Building upon these observations and our work on proHD6 discussed in Chapter 3,\textsuperscript{1} we question why proHD6 is expressed with a 49-residue pro region, which is fairly long compared to the 32-residue mature HD6 when a short peptide sequence is sufficient to suppress the self-assembly of HD6. All defensins contain long pro regions and many studies show that the pro regions interact with the mature defensins and facilitate the oxidative folding of the peptides.\textsuperscript{6-8} Therefore, beyond temporally and spatially controlling the self-assembly of HD6, a pro region may have additional function, such as intramolecular interaction with HD6 during storage. Further studies are required to address functional roles of the pro region in proHD6.

B.5 References


Appendix C

NMR Spectra of Reported Compounds
Chapter 7

Figure C.1. $^1$H NMR spectrum of Bn$_6$Ent-PEG$_3$-CO$_2$H 7 in CDCl$_3$. 
Figure C.2. $^{13}$C NMR spectrum of Bn$_6$Ent-PEG$_3$-CO$_2$H 7 in CDCl$_3$. 
Figure C.3. $^1$H NMR spectrum of Ent-PEG$_3$-CO$_2$H 8 in CD$_3$OD.
Figure C.4. $^{13}$C NMR spectrum of Ent-PEG$_3$-CO$_2$H 8 in CD$_3$OD.
Biographical Note

The author was born and raised in Bangkok, Thailand. During high school, he participated in the 37th International Chemistry Olympiad in Taipei, Taiwan and received a silver medal. In addition, he received a Royal Thai Government Scholarship to pursue his higher education outside Thailand. He earned his master degree in chemistry at St. John's College, Oxford University, United Kingdom. During his final year there, he conducted a research developing thiophene-based dyes for probing neuronal activity via second harmonic generation (SHG) under the supervision of Professor Harry L. Anderson, and his work resulted in a Part-II Thesis Runner-Up Prize in Organic Chemistry. In 2011, he graduated from Oxford University with First Class Honors and then moved to the United States to attend a graduate program in the Department of Chemistry, MIT. During his graduate study, he has worked under the guidance of Professor Elizabeth M. Nolan. His graduate research focuses on understanding host-pathogen interactions. He works to elucidate the mechanism of action of a host-defense peptide called human defensin 6. He also aims to develop alternative strategies beyond typical antibiotics to target Gram-negative bacteria. These strategies are based on small-molecule iron chelators that bacteria use to sequester iron from the host named siderophores in particular enterobactin and its glycosylated derivatives.
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EDUCATION
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• Synthesis and evaluation of antimicrobial activity of enterobactin-
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• Synthesis of enterobactin-immunogenic protein conjugate for
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• Studies of maturation and self-assembly of human α-defensin 6
• Investigations of anti-biofilm activity of human α-defensin 6
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Advisor: Professor Harry L. Anderson
• Synthesis and evaluation of biophysical properties of thiophene-
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generation
Undergraduate Research Assistant
Advisor: Professor Harry L. Anderson
- Synthesis of the hexadentate template and porphyrins, which are used in the syntheses of various nanorings

Undergraduate Research Assistant
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- Synthesis of DGJNAC-[2-acetamido-1,2-dideoxy-D-galactonojirimycin] from D-glucuronolactone, the first sub-micromolar inhibitor of α-N-acetylgalactosaminidases

TEACHING EXPERIENCE
02/2012-05/2012 Massachusetts Institute of Technology, Cambridge, MA, USA
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LABORATORY TECHNIQUES
Multistep organic synthesis, structure determination (NMR, LC/MS, and IR), HPLC usage for purifying small molecules and peptides and HPLC basic maintenance, analytical ultracentrifugation (AUC), transmission electron microscopy (TEM), scanning electron microscopy (SEM), mammalian cell culture technique, antimicrobial activity assays, experienced with several microorganisms (BL1: Lactobacillus rhamnosus, Escherichia coli, and Candida albicans, and BL2: Listeria monocytogenes, Staphylococcus aureus, uropathogenic Escherichia coli, Salmonella enterica serovar Typhimurium, and Acinetobacter baumannii), DNA cloning, RNA extractions, RT-qPCR, bacterial transformation via electroporation and heat-shock method, protein overexpression in Escherichia coli and purification.
HONORS AND AWARDS

07/2015 Honorable Mention Poster Award at the 2015 Cell Biology of Metals Gordon Research Conference
08/2013 Morse Travel Grant to present at the 2014 Chemistry and Biology of Peptides Gordon Research Conference (Chemistry Department, MIT, USA)
08/2012 Department of Chemistry Award for Outstanding Teaching (MIT, USA)
06/2011 Part-II Thesis Runner-Up Prize in Organic Chemistry (University of Oxford, UK)
02/2011 Clarendon Scholarship to study in a Ph.D. program at University of Oxford (Declined, University of Oxford, UK)
01/2010 Selected as a student case study for 2010 Nuffield Science Bursary as a result of excellent outcome of the summer project conducted at the University of Oxford (Nuffield Foundation, UK)
06/2009 Department of Chemistry Award for achieving the second highest mark on the Part IA Examination (University of Oxford, UK)
06/2009-08/2009 Nuffield Science Bursary for summer undergraduate research project (Nuffield Foundation, UK)
10/2008-06/2011 Astrazeneca Chemistry Grant for achieving the third highest mark on the University of Oxford Chemistry Preliminary Examination (Astrazeneca, UK)
10/2008-06/2011 Casberd Scholarship for the outstanding performance on examinations (St. John's College, University of Oxford, UK)
08/2006-present Royal Thai Government Scholarship
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PUBLICATIONS


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ORAL PRESENTATIONS

“Understanding Self-Assembly of Human Defensin 6 and Its Role in Innate Immunity,” to be presented in the MIT Biological Chemistry Seminar Series, MIT, Cambridge, MA, USA (March 28, 2016).

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SERVICE ACTIVITIES
10/2014-present Massachusetts Institute of Technology Cambridge, MA, USA
Member, the Chemistry Department Environment Health and Safety (EHS) Committee
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EHS representative of the Nolan Lab
- Assisting Principal Investigator in complying with regulations and MIT practices under MIT’s EHS Management System
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Vice President of the Oxford Thai Society
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Sport Officer of the Oxford Thai Society
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