LC-MS/MS ANALYSIS OF THE PROTEIN PROFILES OF ESCHERICHIA COLI IN RESPONSE TO THE CHALLENGE OF ANTIBACTERIAL PEPTIDES

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Summary

Novel antibacterial drugs are in urgent need to overcome the continuous growth in the emergence of bacterial resistance to current antibiotics. Antibacterial peptides (ABPs), especially non-membrane-permeabilizing ABPs which kill bacteria by specific mechanisms other than direct membrane disruption, are excellent candidates for development as novel antibacterial drugs. Systematic and comprehensive understanding their mechanisms of action was thus urgently required.

In this study, liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique was utilized to analyze the protein profiles of Escherichia coli (E. coli) in response to the challenge of two representatives of non-membrane-permeabilizing ABPs, apidaecin IB and human neutrophil peptides 1 (HNP-1).

A number of proteins which take essential roles in cellular protein quality control were found to be significantly changed. Levels of 60 kDa charperonin (GroEL) and 10 kDa charperonin (GroES), which together form the only essential chaperon system in E. coli cytoplasm under all growth conditions, were decreased; in contrast, levels of ATP-dependent protease ClpX and FtsH, which located in cytoplasm and inner membrane respectively, were increased. The increase in the proteases was probably
involved in a compensatory response to the suppression effect. However, the overproduction of FtsH further intensified the degrading of UDP-3-O-acyl-N-acetylglucosamine deacetylase (LpxC), an enzyme catalyzing the first committed step in the biosynthesis of the lipid A moiety of lipopolysaccharide (LPS). As the same reaction precursor (R-3-hydroxymyristoyl-ACP) is used by LpxC for the biosynthesis of the lipid A moiety of LPS and by (3R)-hydroxymyristoyl-[acyl-carrier-protein] dehydratase (FabZ) for the synthesis of fatty acid, the reduction in LpxC led to further unbalanced synthesis of LPS and phospholipids and the loss of membrane lipid homeostasis.

However, in response to HNP-1 challenge, levels of a number of enzymes in glycolysis were decreased, including 6-phosphofructokinase isozyme 1, glyceraldehyde-3-phosphate dehydrogenase A, phosphoglycerate kinase, enolase, and pyruvate kinase; in contrast, levels of enzymes (dehydrogenase and aconitate hydratase 2) which regulate the conversion of pyruvate into isocitrate were increased. In concert with the decreasing in cellular ATP and the slowing down in the growth of *E. coli* culture, central metabolism was suggested to be involved in the *E. coli* response to HNP-1 challenge.

Our findings provide new insights into the antibacterial mechanism of action of apidaecin IB and HNP-1. The LC-MS/MS-based proteomic
analysis platform established here may be expanded into the mechanistic studies of all other non-membrane-permeabilizing ABPs and perhaps, by extension, all other candidates of novel antibacterial drugs. The identified altered proteins may well be novel targets for more effective antibacterial intervention.
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<td>catabolite repressor/activator</td>
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<td><em>E. coli</em></td>
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<td>ESI</td>
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<td>FabZ</td>
<td>(3R)-hydroxymyristoyl-[acyl-carrier-protein] dehydratase</td>
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<td>FT-ICR</td>
<td>Fourier transform-ion cyclotron resonance</td>
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<td>FtsH</td>
<td>cell division protease</td>
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<td>GapA</td>
<td>glyceraldehyde-3-phosphate dehydrogenase A</td>
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<td>GroEL</td>
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<td>GroES</td>
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<td>HNP-1</td>
<td>human neutrophil peptide 1</td>
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<td>ICATs</td>
<td>isotope-coded affinity tags</td>
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<td>IM</td>
<td>inner membrane</td>
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<td>iTRAQ</td>
<td>isobaric tags for relative and absolute quantification</td>
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<td>KDO</td>
<td>2-keto-3-deoxyheptonic acid</td>
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<td>LC-MS/MS</td>
<td>liquid chromatography-tandem mass spectrometry</td>
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<td>LPS</td>
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<td>LpxC</td>
<td>UDP-3-O-acyl-N-acetylglucosamine deacetylase</td>
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<td>MALDI</td>
<td>matrix-assisted laser desorption/ionization</td>
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<td>MH</td>
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<td>MIC</td>
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<td>m/z</td>
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<td>RF</td>
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<td>RpoA</td>
<td>DNA-directed RNA polymerase subunit alpha</td>
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<td>SCX</td>
<td>strong-cation exchange</td>
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<td>sodium dodecyl sulphate</td>
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<tr>
<td>SIM</td>
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<td>TOF</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>PfkA</td>
<td>6-phosphofructokinase isozyme 1</td>
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<td>Pgk</td>
<td>phosphoglycerate kinase</td>
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<td>PVDF</td>
<td>polyvinylidene fluoride</td>
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<td>PykF</td>
<td>pyruvate kinase I</td>
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List of Publications

(* for corresponding author and # for co-first author)


Chapter 1: Introduction

Few developments in the history of medicine have had such a profound effect on human life and society as the development of the power to control infections by bacteria. In the fight against infectious disease, antibacterial drugs play a remarkable role. The majority of antibacterial drugs were discovered in the 1940-1960s. Owing to the improved hygiene measures and the success story of treatment of bacterial pathogens by antibacterial drugs, in 1969 the Surgeon General of the United States stated that it was time “to close the book of infectious diseases” [1]. Unfortunately, since the heady optimism of the 1960s we have learned to our cost that bacterial pathogens still have the capacity to spring unpleasant surprises on the world. The problem of acquired bacterial resistance to antibiotics, recognized since the very beginning of antibacterial therapy, has become ever more threatening.

Antibacterial drugs act by impairing cellular functions and structures essential for bacterial growth and survival. However, bacteria are extremely adaptable. Their lifestyles require the swift phenotypic adaptation to a variety of changing environmental factors. Even genetic adaptation is comparably fast due to a concurrence of short generation times, haploid genomes, and various routes of transfer of genetic material. In most cases, only a few genes or a single gene is sufficient to render a bacterium resistant to a particular antibacterial drug. Such genes can encode efflux
pumps, antibacterial-drug degrading or modifying enzymes, or a mutated target resistant to the action of antibacterial drugs. As these resistance genes become essential for survival under drug selection pressure, carriers might be the only survivors. Once presenting within the gene pool of pathogenic bacteria, resistance traits may be disseminated within and between species [2]. Resistance to one antibacterial drug would not pose a major threat, as a variety of antibacterial drugs are available to us. However, frequently resistance traits accumulate and multidrug-resistant pathogens cause major problems especially in places with high antibacterial-drug use [3]. During the past decade, multidrug-resistant pathogens have also taken hold in the community so that a fair number of deaths are now caused by community-acquired multidrug-resistant strains [4]. To combat multidrug-resistant pathogens, novel antibacterial drugs are in urgent need.

Antibacterial peptides (ABPs) are a group of relatively short (less than 100 amino acid residues), positively charged and amphiphilic peptides produced by a wide range of organisms as part of their first line of defense [5-7]. They exhibit activities against both Gram-positive and Gram-negative bacteria [8-10]. Most of them even have activities against fungi [8, 11, 12], protozoa [8, 13, 14] and viruses [15-17]. The modes of action of ABPs can be generally classified as either membrane-permeabilizing or non-membrane-permeabilizing [18]. Membrane-permeabilizing peptides can disrupt the membrane by forming transmembrane pores, micellarizing or dissolving the membrane. In contrast, non-membrane-permeabilizing peptides can translocate across the membrane and accumulate in the cytoplasm, where they kill bacteria by targeting different essential cellular
processes.

Non-membrane-permeabilizing ABPs generally exhibit a broad range of activity, act by specific mechanisms other than direct membrane disrupting, do not easily induce antibacterial-drug resistance, are bactericidal as opposite to bacteriostatic, and require a short time to induce cell killing [19-21]. All these features make them excellent candidates for development as novel antibacterial drugs. Systematic and comprehensive understanding of their antibacterial mechanism of action is thus urgently required.

Two representatives of non-membrane-permeabilizing ABPs are apidaecin IB and human neutrophil peptide 1 (HNP-1). Apidaecin IB is a 20 amino-acid, proline-rich ABP found in insects [22]. It is predominantly active against Gram-negative bacteria [23-25]. Previous studies showed that the antibacterial mechanism of apidaecin IB was based on its ability to bind the chaperone DnaK and inhibit the function of DnaK in assisting the folding of polypeptides [21]. However, it is possible that apidaecin IB kill bacteria by other mechanisms not yet explored. HNP-1 is a 30 amino-acid, β-sheet ABP found in human neutrophils [26, 27]. HNP-1 has a wide spectrum activity against bacteria (both Gram-positive and Gram-negative) [28], yeast [29, 30] and enveloped viruses [31]. It was originally proposed that permeabilization of the target cell membrane was the mechanism of action of this type of peptides [32]. However, there is an increasing body of evidence indicating that the targeting of essential cellular processes, such as inhibition of nucleic acid synthesis, plays more important roles in mediating cell death.
Proteomics is a new discipline that originated in the mid-1990s and has grown rapidly in a very short time. The best current definition of proteomics is “any large-scale protein-based systematic analysis of the proteome or defined sub-proteome from a cell, tissue, or entire organism” [33-35]. Quantitative protein profiling, which attempts to quantitatively compare changes in the level of proteins between two or more experimental conditions, is the most common type of proteomic study [36]. Antibacterial agents act by interfering with essential cellular functions or structures of bacteria. The bacterial proteome is dynamic in nature and quickly adjusts in response to an antibacterial-agent challenge to physiological homeostasis. Therefore, quantitatively comparing changes in the level of proteins between control and antibacterial-agent-treated conditions may aid mechanistic studies of antibacterial agents such as ABPs.

The overall objective of this Ph.D. research project is to investigate changes in the proteome of Escherichia coli (E. coli) in response to the challenge of apidaecin IB and HNP-1, two representatives of non-membrane-permeabilizing ABPs, using quantitative protein profiling technique and thus infer the possible antibacterial mechanisms involved. Experimentally, Isobaric tag for relative and absolute quantitation (iTRAQ)-coupled two dimensional (2D) liquid chromatography–tandem mass spectrometry (LC-MS/MS) was applied. Working on this sophisticated approach, we hope our results can shed new lights and thereby provide better understandings of the mechanism of action of these two ABPs. We also hope the platform developed in this study can be expanded into the mechanistic studies of all other non-membrane-permeabilizing ABPs and
perhaps, by extension, all other candidate novel antibacterial drugs; and the identified altered proteins can be used as the novel targets for more effective antibacterial intervention.

The work presented in this dissertation is organized as follows.

In chapter 2, a brief review of the available literature concerning ABPs and proteomics is presented. The purpose of this chapter is to elucidate the context and address the importance of the present work.

In chapters 3 and 4, alterations in the level of cytoplasmic and membrane proteins in *E. coli* in response to apidaecin IB challenge were analyzed by utilizing an iTARQ-coupled 2D LC-MS/MS technique. New targets of apidaecin IB were identified, and the underlying molecular mechanisms were explored.

In chapter 5, cytoplasmic protein profile of *E. coli* in response to HNP-1 challenge was investigated by utilizing the LC-MS/MS-based quantitative protein profiling platform established in chapter 3. Altered proteins, which may be novel targets of HNP-1, were identified.

In chapter 6, conclusions of the overall research achieved from this project are summarized, and limitations lying in the study are also discussed.

In chapter 7, the potential avenues for future work are presented.
Chapter 2: Literature Review

2.1 Overview of ABPs

ABPs are a group of relatively short (less than 100 amino acid residues), positively charged (net charge of +2 to +9) and amphiphilic peptides isolated from single-celled microorganisms, insects and other invertebrates, plants, amphibians, birds, fish, and mammals, including humans [5-7]. They form part of the ancient, nonspecific innate immune system, which is the principal defense system for the majority of living organisms. They process activities against both Gram-positive and Gram-negative bacteria [8-10]. Most of them even have activities against fungi [8, 11, 12], protozoa [8, 13, 14] and viruses [15-17]. In many cases, their primary role is in the killing of invading pathogenic organisms; however, it is increasingly recognized that they may also function as modulators of the innate immune response in higher organisms [37-40]. ABPs may be expressed constitutively in some cases or may be inducibly expressed in response to pathogenic challenge. In multicellular animals, they may be expressed systemically (for example, in insect hemolymph or vertebrate immune cells) and/or localized to specific cell or tissue types in the body most susceptible to infection, such as mucosal epithelia and the skin. To date, hundreds of such ABPs have been identified. Besides these naturally occurring ABPs,
numerous synthetic analogues have also been produced [41].

### 2.1.1 Structure

ABPs can be categorized into three subgroups on the basis of their amino-acid composition and structure: (i) α-helical peptides; (ii) cysteine stabilized β-sheet peptides; and (iii) those rich in specific amino acid residues, but variable in conformation. The first two subgroups are the most common in nature [42-44]. Representative structures from each of these subgroups are presented in Figure 2.1 [45].

![Figure 2.1](image)

**Figure 2.1** Structural classes of ABPs [45]. (A) α-helical, LL-37; (B) cysteine stabilized β-sheet, tachyplesin I; and (C) specific amino acid-rich, indolicidin (rich in tryptophan and proline). Disulfide bonds are in yellow.
Peptides from the first subgroup are short (less than 40 amino acids), lack cysteine residues, and sometimes have a hinge in the middle [46]. In aqueous solutions, many of these peptides are disordered; however, in the presence of trifluoroethanol, sodium dodecyl sulphate (SDS) micelles, phospholipid vesicles or lipid A, all or part of the molecule is converted into an α-helix [46]. LL-37, the only cathelicidin family of ABPs in human, is a representative of this group of peptides. In water, it exhibits a circular dichroism spectrum that is consistent with a disordered structure [47]. However, in 15 mM HCO₃⁻, SO₄²⁻ or CF₃CO₂⁻, the peptide forms a helical structure (Figure 2.1 A) [47].

The cysteine stabilized β-sheet peptides are characterized by the presence of at least one antiparallel β-sheet stabilized by disulfide bonds between cysteine residues. The tachyplesins isolated from the haemocytes of the Japanese horseshoe crab *Tachypleus tridentatus*, are representative of this class [9]. NMR studies of tachyplesin I showed the presence of an antiparallel β-sheet stabilized by two disulfide bridges formed between residues 3 and 16 and residues 7 and 12 (Figure 2.1 B) [48].

Peptides in the third subgroup are rich in certain amino acids. For example, apidaecins isolated from insects are rich in proline, and the indolicidin isolated from the cytoplasmic granules of bovine neutrophils is rich in tryptophan and proline (Figure 2.1 C) [10]. This subgroup of ABPs lack
cysteine residues and are linear, though some can form extended coils.

2.1.2 Physicochemical Features

2.1.2.1 Cationicity

ABPs generally display a net positive charge of +2 to +9 owing to an excess of basic amino acids (arginine, lysine and histidine) over acidic amino acids [49]. Cationicity is critical for the initial electrostatic attraction of ABPs to the surface of the bacterial cell, which is rich in negatively charged components including the acidic phospholipids (in the cytoplasmic membrane), the lipopolysaccharides (LPS, in the outer membrane of Gram-negative bacteria), and the teichoic or teichuronic acids (in the peptidoglycan of Gram-positive bacteria) [50].

2.1.2.2 Amphipathicity

Almost all ABPs form amphipathic structures upon interaction with target membranes [51]. Amphipathicity is an essential feature for ABPs-membrane interaction. It allows ABPs to partition into the membrane lipid bilayer [52].
2.1.3 Cell Selectivity

Cell selectivity describes the ability of ABPs to preferentially interact with the bacterial cells. This feature enables ABPs to kill bacteria without being significantly toxic to mammalian cells. The cationic property of ABPs mainly contributes to this cell selectivity. The bacterial cell membrane is rich in acidic phospholipids, such as phosphatidylglycerol and cardiolipin. Therefore, the outer leaflet of the bilayer of the bacterial membrane is negatively charged. Moreover, LPS in the outer membrane of Gram-negative bacteria and the teichoic or teichuronic acids present in the peptidoglycan of Gram-positive bacteria impart additional negative charges to the surface of these cells. By contrast, in the case of mammalian cells, acidic phospholipids are usually located in the inner leaflet of the membrane; the outer leaflet is mainly composed of zwitterionic phosphatidylcholine, and sphingomyelin. As a result, the surface of bacterial cells is more negatively charged than that of mammalian cells, and thus more attractive to cationic ABPs (Figure 2.2). Other factors also contribute to cell selectivity such as higher transmembrane potential of bacterial cells and the existence of membrane-stabilizing cholesterol in mammalian cells [53]. However, ABPs with strong membrane-disrupting activities can also damage mammalian cell membranes, and thus are toxic to these cells.
Figure 2.2 Molecular basis of cell selectivity of ABPs [53]. ABPs form amphipathic structures with a positively charged face (blue) and a hydrophobic face (brown).

2.1.4 Mode of Action

Regardless their specific antibacterial mechanisms, the activities of ABPs are almost universally dependent on the interaction with the bacterial cell membrane [54]. The first step of this interaction is the initial attraction between the peptides and the target cell, which is thought to achieve by electrostatic bonding between cationic peptides and negatively charged components present on the bacterial cell surface, such as phosphate groups within the LPS of Gram-negative bacteria, or the teichoic acids on the surface of Gram-positive bacteria [43, 55]. Once close to the bacterial surface, ABPs are inserted into the outer membrane in a process driven by...
hydrophobic interactions in the case of Gram-negative bacteria. Alternatively, ABPs traverse capsular polysaccharides, teichoic acids and lipoteichoic acids in the case of Gram-positive bacteria before they can interact with the cytoplasmic membrane [54]. Once ABPs have gained access to the cytoplasmic membrane, they can associate with the lipid bilayer through electrostatic and hydrophobic interactions. The events that occur at the cytoplasmic membrane surface are the subject of extensive debate, and several models (called “aggregate”, “toroidal-pore”, “barrel-stave” and “carpet” models) have been proposed. Each of these models describes different forms of intermediates which can lead to one of three kinds of events: formation of a transient channel, micellarization or dissolution of the membrane, or translocation across the membrane. Consequently, peptides can permeabilize the membrane, and/or translocate across the membrane and into the cytoplasm without causing significant membrane disruption. For this reason, the mode of action of ABPs can be broadly classified as either membrane permeabilizing or non-membrane permeabilizing.

2.1.4.1 Membrane-permeabilizing ABPs

Four different models have been proposed to explain how, following initial attachment, ABPs insert into the bacterial membrane to form transmembrane pores, which result in membrane permeabilization. The
amphipathic feature of ABPs mainly contributes to this process, as hydrophobic regions are necessary to interact directly with the lipid components of the membrane, while the hydrophilic regions either interact with the head groups of the phospholipid or face the lumen of the pore. Once arriving at the cytoplasmic membrane, membrane-permeabilizing ABPs first interact with the negatively-charged group of lipids at the membrane surface, adopting an orientation parallel to the membrane [18, 51]. It is at this point that the “aggregate”, the “toroidal-pore”, the “barrel-stave”, and the “carpet” models separate. In the “aggregate” model, ABPs reorient to span the membrane as micelle-like complexes of peptides and lipids (the peptides adopt no particular orientation) (Figure 2.3 A). Lacking of a formal channel structure makes the peptide have the capacity to translocate across the lipid bilayer as the aggregates collapse. This model can explain both membrane permeabilization, whereby informal channels with a variety of sizes and lifetimes form, and translocation across the bilayer [18]. In the “toroidal-pore” model, peptides insert perpendicularly into the membrane and induce the lipid monolayer to bend continuously along the pore so that the water core is lined by both the inserted peptides and lipid head groups (Figure 2.3 B) [56-59]. In the “barrel stave” model, peptides reorient and insert perpendicularly into the membrane and align (like the staves in a barrel) in a manner in which the hydrophobic surface interacts with the lipid core of the membrane while the
hydrophilic surface points inward to form transmembrane pores; this is a true protein-pore model (Figure 2.3 C) [60-63]. In the mechanism known as the “carpet” model, peptides align parallel to the phospholipids bilayer (like a carpet), remaining in contact with the phospholipids head groups (Figure 2.3 D) [64-66]. Once a threshold concentration of peptides is reached, this orientation results in a detergent-like effect which causes formation of micelles and membrane pores [64-66]. The α-helical subgroup of ABPs generally exhibits their antibacterial action through this membrane-permeabilizing mode.

2.1.4.2 Non-membrane-permeabilizing ABPs

However, a growing number of ABPs have been shown to translocate across the membrane and accumulate in the cytoplasm, where they target various essential cellular processes to mediate cell death. Mechanisms of action which have been demonstrated include inhibiting actual nuclear acid synthesis (Figure 2.3 E), protein synthesis and folding (Figure 2.3 F and D) enzyme activity (Figure 2.3 H) and cell wall synthesis (Figure 2.3 I). The frog ABP buforin II translocates across the bacterial membrane without causing permeabilization and binds to both DNA and RNA within the cytoplasm of *E. coli* [67]. Similarly, peptides such as derivatives of pleurocidin, a fish-derived ABP, and dermaseptin, isolated from frog skin, cause inhibition of DNA and RNA synthesis at their MICs without
destabilizing the membrane of \textit{E. coli} cells [68]. Inhibition of nucleic acid synthesis has also been demonstrated for ABPs from different structural classes, such as the \(\beta\)-sheet human defensin, HNP-1 [32], and the extended-structure bovine peptide indolicidin [69]. Additionally, some of these peptides have been shown to interfere with protein synthesis. Pleurocidin and dermaseptin can block tritiated leucine uptake in \textit{E. coli}, and PR-39- and indolicidin-treated cells also exhibit reduced rates of protein synthesis [68-71]. Inhibition of cellular enzymatic activity by proline-rich insect ABPs has also been observed. Pyrrhocidin enters the target cell and binds to DnaK, a heat shock protein that is involved in chaperone-assisted protein folding. Specifically, the peptide inhibits the ATPase activity of DnaK, preventing protein folding, which results in the accumulation of misfolded proteins and cell death [72, 73]. ABPs can also target the formation of structural components, such as the cell wall. The bacterially produced lantibiotic mersacidin interferes with transglycosylation of lipid II, a necessary step in the synthesis of peptidoglycan [74]. Nisin, another lantibiotic, can also bind to lipid II, thus inhibiting cell wall synthesis in addition to its pore-forming activity [75]. Interestingly, this is the same biosynthetic process that is targeted by the antibiotic vancomycin; however, mersacidin and nisin are thought to act by interacting with distinct molecular moieties within lipid II, explaining why these peptides are still active against vancomycin-resistant bacteria [75,
Figure 2.3 Mechanisms of action of ABPs [18]. The bacterial membrane is yellow lipid bilayer with the peptides shown as cylinders, where the hydrophilic regions are red and the hydrophobic regions are blue. Cell wall-associated peptidoglycan molecules are depicted as purple cylinders. (A) the "aggregate" model; (B) the "toroidal pore" model; (C) the "barrel-stave" model; (D) the "carpet" model; (E) nuclear acid synthesis inhibition; (F) and (G) protein synthesis and folding inhibition; (H) enzyme activity inhibition; and (I) cell wall synthesis inhibition.
2.1.5 Candidates for Development as Novel Antibacterial Drugs

Non-membrane-permeabilizing ABPs generally exhibit a broad range of activity, act by specific mechanisms other than direct membrane disruption, do not easily induce antibacterial-drug resistance, are bacteriocidal as opposite to bacteriostatic, and require a short time to induce killing [19-21]. All these features make non-membrane-permeabilizing ABPs excellent candidates for development as novel antibacterial drugs. Systematic and comprehensive understanding of the underlying mechanisms of action of such ABPs is thus urgently required.

2.2 Apidaecin IB and HNP-1

Apidaecin IB and HNP-1 are two representatives of non-membrane-permeabilizing ABPs. Apidaecin IB is a 20 amino-acid, proline-rich peptide found in insects [22]. It is predominantly active against Gram-negative bacteria [23-25]. HNP-1 is a 30 amino-acid, β-sheet defensin found in human neutrophils [26, 27]. It has wide-spectrum activity against bacteria (Gram-positive and Gram-negative) [28], yeast [29, 30] and enveloped viruses [31].
2.2.1 Apidaecin IB

Short proline-rich ABPs (less than 21 amino acid residues) refer to a group of linear peptides produced by insects [22, 77, 78]. They include apidaecins isolated from several hymenopteran species, drosocin from *Drosophila melanogaster*, pyrrhocoricin from the hemipteran species *Pyrrhocoris apterus*, formaecins from the bulldog ant *Myrmecia gulosa*, and metalnikowins from the bug *Palomena prasina* [77]. The sequences of this type of peptides are shown in Figure 2.4. Members of this group of ABPs are predominantly active against Gram-negative bacteria, which they kill without bacterial membrane permerbilization [20, 77].

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apidaecin Ia (honey bee)</td>
<td>+GNRPVYIPQPRPPHPRRI-</td>
</tr>
<tr>
<td>Apidaecin Ib (honey bee)</td>
<td>+GNRPVYIPQPRPPHPRRL-</td>
</tr>
<tr>
<td>Apidaecin I (honey bee)</td>
<td>+GNRPVIYIPQPRPPHPRRL-</td>
</tr>
<tr>
<td>Apidaecin (European bumblebee)</td>
<td>+GN RPYIPPRPPHPRRL-</td>
</tr>
<tr>
<td>Drosocin (fruit fly)</td>
<td>+GKPRPYSPRPTSHPRPPIRV-</td>
</tr>
<tr>
<td>Formaecin 1 (red bulldog ant)</td>
<td>+GRPNFPVHNKPTPHPRRL-</td>
</tr>
<tr>
<td>Formaecin 2 (red bulldog ant)</td>
<td>+GRPNFPVNTKPTYPYPRRL-</td>
</tr>
<tr>
<td>Pyrrhocoricin (firebug)</td>
<td>+VDKGSYLPRPTPPPIYNRR-</td>
</tr>
<tr>
<td>Metalnikowin I (green shield bug)</td>
<td>+VDKPDRPRPPFPRNNM-</td>
</tr>
<tr>
<td>Metalnikowin IIA (green shield bug)</td>
<td>+VDKPDYRPRPPFPRPN-</td>
</tr>
<tr>
<td>Metalnikowin IIB (green shield bug)</td>
<td>+VDKPDYRPRPPFPRNM-</td>
</tr>
<tr>
<td>Metalnikowin III (green shield bug)</td>
<td>+VDKPDYRPRPPFPRFM-</td>
</tr>
</tbody>
</table>

**Figure 2.4** Amino acid sequences of short proline-rich ABPs [78].
The ability of short proline-rich ABPs to penetrate into the cytoplasmic membrane is closely associated with their high content of proline residues. Proline is an unusual amino acid, whose amino nitrogen is cyclized with the side chain terminal carbon. This leads to three consequences: the backbone conformation of proline itself is severely restricted; the conformation of the residue preceding proline is also restricted because of the bulkiness of the N-CH₂ group; and proline is unable to act as a hydrogen bond donor because of the amide proton is replaced by a CH₂ group [79]. A sequence of four or more proline residues in a row adapts to a single conformation known as the polyproline II helix, which is an extended structure with three residues per turn [79]. Previous studies showed that short proline-rich ABPs form the polyproline II helix and this structural module promote penetration of these peptides into the membrane [80]

Apidaecins are the largest group of short-proline-rich ABPs known up to now [22]. They generally have 18 to 20 amino acid residues with proline content of 33% or more [23, 25]. Till now, a total of eighteen isoforms of apidaecins were isolated from six different insects including the honey bee *Apis mellifera*, the bumblebee *Bombus terrestris*, the cicada killer *Sphecius speciosus*, the baldfaced hornet *Dolichovespula maculate*, the yellowjacket *Vespula maculitrons*, the german wasps *Paravespula germanica* and the
parasitic wasps *Coccygomimus disparis* (Table 2.1) [24, 81]. Among them, apidaecin IB shows the strongest activity.

**Table 2.1** Sequence alignment of apidaecins [24].

<table>
<thead>
<tr>
<th>Resources</th>
<th>Isoforms</th>
<th>Peptides sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Honey bee</td>
<td>H0Ia</td>
<td>GHNRFYITPFRPHFRL</td>
</tr>
<tr>
<td></td>
<td>H0Ib</td>
<td>GHNRFYITPFRPHFRL</td>
</tr>
<tr>
<td></td>
<td>H0II</td>
<td>GHNRFYITPFRPHFRL</td>
</tr>
<tr>
<td></td>
<td>H0III</td>
<td>GHNRFYITPFRPHFRL</td>
</tr>
<tr>
<td>Bumble bee</td>
<td>Bb + A</td>
<td>ANNEFVYTPFRPHFRL</td>
</tr>
<tr>
<td></td>
<td>Bb - A</td>
<td>-NEFVYTPFRPHFRL</td>
</tr>
<tr>
<td>Cicada killer</td>
<td>CK P</td>
<td>NBPTVYDPFRPHFRL</td>
</tr>
<tr>
<td></td>
<td>CK A</td>
<td>NBPTVYDPFRPHFRL</td>
</tr>
<tr>
<td>Bald-faced hornet</td>
<td>Ho+</td>
<td>GKEFQVCYFRPHFRL</td>
</tr>
<tr>
<td></td>
<td>Ho–</td>
<td>--RFQVDPRPHFRL</td>
</tr>
<tr>
<td>Yellow jackets and German wasps</td>
<td>Yj + S</td>
<td>SNKRPQDVFRPHFRL</td>
</tr>
<tr>
<td></td>
<td>Yj – S</td>
<td>-NSKRPQDVFRPHFRL</td>
</tr>
<tr>
<td><em>C. disparis</em></td>
<td>CdI+</td>
<td>GKNRPFRPAEQFRPHFRL</td>
</tr>
<tr>
<td></td>
<td>CdI–</td>
<td>--NKRPAEQFRPHFRL</td>
</tr>
<tr>
<td></td>
<td>CdZ+</td>
<td>GRPHXERPAKFRPHFRL</td>
</tr>
<tr>
<td></td>
<td>CdZ–</td>
<td>--NKRPAEKFRPHFRL</td>
</tr>
<tr>
<td></td>
<td>CdS+</td>
<td>GRPSXERPAIKFRPHFRL</td>
</tr>
<tr>
<td></td>
<td>CdS–</td>
<td>--SNRPAPIKFRPHFRL</td>
</tr>
</tbody>
</table>

Conserved sequence of all the isoforms: RKFRPHFR

Previous studies proposed a five-step mechanism by which apidaecins and some other short proline-rich ABPs exert their activity against *E. coli* and other Gram-negative bacteria: *i*) binding to negatively charged components (most probably LPS) of the outer membrane (OM) in a non-specific manner; *ii*) self-promoted invading into the periplasmic space; *iii*) irreversible interaction with a hypothetical docking molecule/receptor molecule on the inner membrane (IM); *iv*) translocation across the membrane and into the cytoplasm of the cell; *v*) Binding its target most probably DnaK protein to
mediate cell death (Figure 2.5) [21, 24, 72, 82-84]. However, it is possible that these peptides kill bacteria by other mechanisms not yet identified.

**Figure 2.5** Mechanisms of action of apidaecins [24]. AP, apidaecin; LPS, lipopolysaccharides; DM, docking molecule or a receptor; OM, outer membrane; and IM, inner membrane. (1) binding to negatively charged components (most probably LPS) of the OM in a non-specific manner; (2) self-promoted invading into the periplasmic space; (3) irreversible interaction with a hypothetical docking molecule/receptor molecule on the IM; (4) translocation across the membrane and into the cytoplasm of the cell; (5) binds its ultimate target most probably DnaK protein.

### 2.2.1 HNP-1

Defensins are a family of evolutionarily related vertebrate ABPs with a characteristic triple-stranded antiparallel β-sheet stabilized by three intramolecular disulfide bonds between six cysteines. Typical defensins have been found in all mammals, as well as in chickens and turkeys [30, 85]. The two main defensin subfamilies, α- and β-defensins, differ in the length and the pairing of the cysteine residues. α-defensins contain about...
30 amino acids, and their three disulfide bonds connect cysteines 1 and 6, 2 and 4, and 3 and 5 [85, 86]. By contrast, β-defensins are somewhat larger, and their three disulfide bonds are between cysteines 1 and 5, 2 and 4, and 3 and 6. Representative structures from each of these subfamilies are presented in Figure 2.6.

![Figure 2.6](image)

**Figure 2.6** Structure of α- and β-defensins [85]. (A) The corresponding cysteines in α- and β-defensins are indicated by dotted lines, disulphide bonds by solid lines. Whereas in α-defensins the six cysteines are linked in the 1–6, 2–4, 3–5 pattern, in β-defensins the pattern is 1–5, 2–4, 3–6. (B) β-sheet structures are indicated by flat ribbons and arrows.
HNP-1 is the most active α-defensin, which includes four human neutrophil peptides (HNP-1 to HNP-4) exclusively present in neutrophil granulocytes, and two enteric defensins (HD-5 and HD-6) found in the granules of Paneth cells (Figure 2.7). It has activities against bacteria (Gram-positive and Gram-negative), yeast, and enveloped viruses. It was originally proposed that permeabilization of the target cell membrane was the mechanism of action of HNP-1 [32]. However, there is an increasing body of evidence indicating that targeting the essential cellular processes, such as inhibition of nucleic acid synthesis, plays a more important role in mediating cell death. Further investigations on different aspects of its mechanisms of action are still required.

Figure 2.7 Amino acid sequences of human α-defensins. Cysteine residues in the defensins are underlined.
2.3 Overview of Proteomic Analysis

Proteomics is a new discipline that originated in the mid-1990s and has grown rapidly in a very short time. The best current definition of proteomics is “any large-scale protein-based systematic analysis of the proteome or defined sub-proteome from a cell, tissue, or entire organism” [33-35]. Most proteomic studies fall into one of the three types: i) quantitative protein profiling, ii) protein composition analysis, and iii) protein-protein interaction analysis [36].

Quantitative protein profiling attempts to quantitatively compare changes in the level of proteins between two or more experimental conditions such as normal versus infected cells, time courses after drug treatment, or responses to stimuli or stresses [36]. It can provide highly informative insights into biological processes and diseases.

Protein composition analysis attempts to identify the total protein complement of a proteome such as that of \textit{E. coli}, or all the proteins present in a cellular fraction or compartment such as outer membrane [36]. The difference relative to quantitative protein profiling is that in protein composition studies, the quantitation of protein levels is not involved. The favored analysis methods for this study are various related multidimensional chromatographic methods coupled to tandem mass
spectrometry, an approach that has been referred to as multidimensional protein identification technology [87].

The third type of proteomic studies is systematic analysis of protein-protein interactions [36]. Most cellular processes are mediated by multi-protein complexes or pathways comprising interacting proteins. For this reason, systematic identification of protein-protein interactions is expected to provide new insights into cellular pathways and networks. In the same way, identification of which proteins of known function associate with a protein of unknown function can provide insights into the role of the latter and the underlying molecular mechanisms involved. Hence, protein-protein interaction maps for a number of model organisms are being constructed [88]. The most direct approach for identification of interacting proteins is isolating a macromolecular complex, followed by identification of all components using MS methods [89, 90].

2.4 LC-MS-Based Quantitative Protein Profiling

LC-MS has become a powerful molecular biology tool, and multiple strategies for peptide and protein quantitation by LC-MS have been developed and applied to address a wide range of biological questions. It is being used more and more to quantitatively compare changes in the level of proteins between two or more experimental conditions.
2.4.1 Protein Identification

LC-MS-based protein identification starts with a purified protein complex, a subcellular protein fraction, or a whole cell lysate. The complex mixture of proteins in solution is first denatured and reduced. The mixture of proteins is then cleaved either enzymatically (i.e., digestion) or chemically to generate a mixture of peptide fragments. The complex peptide mixture is fractionated and analyzed by multidimensional LC coupled with electrospray ionization (ESI) and automated tandem MS. The acquired tandem mass spectra are computationally compared to protein sequences in either protein or translated nucleic acid databases. The list of peptides significantly matching sequences in the databases are used to generate a list of proteins in the original protein complex, subcellular compartment, or whole cell lysate. The workflow of LC-MS/MS-based protein identification is shown in Figure 2.8.
2.4.1.1 LC Separation

Analyte molecules must be ionized before MS analysis. The method of producing the ions is termed the ionization technique. The two major types of ionization for proteomic analysis are ESI [92] and matrix-assisted laser desorption/ionization (MALDI) [93]. One of the fundamental differences between the two methods is that MALDI is employed on samples in a solid state, whereas ESI is employed on samples in a liquid state. Hence, interfacing LC with ESI is relatively straightforward, whereas LC–MALDI analysis is an offline process, where fractions are collected and then analyzed by MALDI at a later stage. Partly as a result of this, ESI is the dominant ionization process for analysis of samples separated by LC, and
will be the emphasis of discussion from hereon.

The process of ESI converts a solution into a mist of charged droplets. These droplets shrink as the solvent is evaporated and when the charge density in the droplet reaches a critical level, coulombic repulsion causes desorption of charged gaseous ions from the droplet [94, 95]. This process continues; as solvent continues to evaporate, droplets get smaller and more ions are ejected into the gaseous phase. Unsurprisingly, this conversion of liquid sample into gaseous ions is more efficient when less solvent is present. Hence, electrospray is referred to as a concentration-sensitive process. This means that the smaller the volume of sample introduced, the better the efficiency and sensitivity of the process. Increasing sample concentration can be achieved in two ways using LC. First, use of a narrower column and lower flow rate will cause elution in smaller volumes. Second, by improving the resolution of separation, the same amount of sample will elute in a narrower profile, giving a higher concentration at the maxima of peak elution.

Nanospray is more sensitive than ESI approaches at higher flow rates [96]; so sub-microliter flow rates are typically used for proteomic analyses. Many chromatographic systems cannot natively produce reliable gradients at these low flow rates due to the presence of solvent-mixing chambers in the plumbing that have too large volumes in comparison to the solvent flow rate,
leading to inconsistent solvent mixing and irreproducible gradients. Hence, pressure-based flow-splitting systems are commonly employed and built into the chromatography system. Generally, a split in the range of 1:100 to 1:1,000 is used post-pump but prior to the separation column, allowing efficient mixing of solvents prior to chromatography to produce consistent separations, albeit with large amounts of solvent waste. Recently, some systems have been developed that use air pressure as the pumping mechanism and allow splitless delivery of reproducible nanoliter per minute flow rates.

Different stationary phases in chromatography columns provide variable levels of resolution. Reverse-phase (RF) chromatography is highly compatible with subsequent mass spectrometric analysis due to the lack of salts in the buffers and provides relatively high-resolution separation, so is widely used for proteomic analysis. With the growing need for more powerful and highly resolving separation methods, the use of multidimensional LC in proteomics has thrived. Multidimensional LC combines two or more forms of LC to increase the peak capacity, and thus the resolving power, of separations to better fractionate peptides prior to entering the mass spectrometer [87].
2.4.1.2 Mass Analysis

Mass spectrometer is an analytical technique that measures the mass-to-charge ratio (m/z) of ions based upon their motion in an electric or a magnetic field. Sample molecules are converted into ions in the gas phase and separated according to their m/z; positively and negatively charged ions can be formed. Mass spectrometer typically consists of three components: ionization source, analyzer and detector. Ionization source is the region where the gas phase ions are produced from sample molecules. ESI and MALDI are the two major types of ionization techniques for proteomic analysis, and this has been discussed in above section. Mass analyzer is the component that separates the ions according to their m/z. Detector is the final component which records the signal produced by ions. Although in theory any type of mass analyzer could be used for LC-MS, four types including quadrupole, time-of-flight (TOF), ion trap, Fourier transform-ion cyclotron resonance (FT-ICR) are used most often.

**Quadrupole**

A quadrupole mass analyzer (Figure 2.9) consists of four parallel rods arranged in a square. The analyte ions are directed down the center of the square. Voltages applied to the rods generate electromagnetic fields. These fields determine which m/z of ions can pass through the filter at a given time.
Quadrupoles tend to be the simplest and least expensive mass analyzers. They can operate in two modes: scan mode and selected ion monitoring (SIM) mode (Figure 2.9). In scan mode, the mass analyzer monitors a range of m/z. In SIM mode, the mass analyzer monitors only a few m/z. SIM mode is significantly more sensitive than scan mode but provides information about fewer ions. Scan mode is typically used for qualitative analyses or for quantitation when all analyte masses are not known in advance. SIM mode is used for quantitation and monitoring of target compounds.

**Figure 2.9** Quadrupole mass analyzer. The quadrupole mass analyzer can scan over a range of m/z or alternate between just a few.
**TOF**

In a TOF mass analyzer (Figure 2.10), a uniform electromagnetic force is applied to all ions at the same time, causing them to accelerate down a flight tube. Lighter ions travel faster and arrive at the detector first, so the m/z of the ions is determined by their arrival times. TOF mass analyzers have a wide mass range and can be very accurate in their mass measurements.

![Figure 2.10 TOF mass analyzer.](image)
**Ion Trap**

An ion trap mass analyzer (Figure 2.11) consists of a circular ring electrode plus two end caps that together form a chamber. Ions entering the chamber are “trapped” there by electromagnetic fields. Another field can be applied to selectively eject ions from the trap. Ion traps have the advantage of being able to perform multiple stages of MS without additional mass analyzers.

![Ion Trap Diagram](image)

**Figure 2.11** Ion trap mass analyzer.

**FT-ICR**

An FT-ICR mass analyzer (also called FT-MS, Figure 2.12) is another type of trapping analyzer. Ions entering a chamber are trapped in circular orbits by powerful electrical and magnetic fields. When excited by a
radio-frequency electrical field, the ions generate a time-dependent current. This current is converted by Fourier transform into orbital frequencies of the ions which correspond to their m/z. Like ion traps, FT-ICR mass analyzers can perform multiple stages of MS without additional mass analyzers. They also have a wide mass range and excellent mass resolution. They are, however, the most expensive of the mass analyzers.

Figure 2.12 FT-ICR mass analyzer.

2.4.1.3 Tandem MS

For identification of peptides in complex mixtures, measurement of peptide mass alone is not sufficiently informative. While the combination of accurate mass and retention time can be employed for identification in well-defined
samples [97], the most flexible and generally applicable approach involves fragmentation analysis of components. In an initial scan, the masses of intact components are measured, and then in a subsequent scan/s, individual components are isolated in the mass spectrometer and then fragmented.

Generally, these experiments are performed successfully on two types of instruments: those where analyzers are in series (tandem in space) such as the triple quadrupole and hybrid quadrupole-TOF configurations; and secondly those instruments which employ ion trapping mechanisms such as the quadrupole ion trap and FT-ICR analyzer (tandem in time). In triple-quadrupole or hybrid quadrupole-TOF instruments (Figure 2.13), the first quadrupole is used to select the precursor ion. Fragmentation takes place in the second stage (quadrupole or octopole), which is called the collision cell. The third stage (quadrupole or TOF) then generates a spectrum of the resulting product ions. It can also perform SIM of only a few product ions when quantitating target compounds. However, in ion trap and FT-ICR MS, all ions except the desired precursor ion are ejected from the trap. The precursor ion is then energized and collided to generate product ions. The product ions can be ejected to generate a mass spectrum, or a particular product ion can be retained and collided to obtain another set of product ions (Figure 2.14). This process can be sequentially automated so
that the most abundant ion(s) from each stage of MS are retained and collided.

**Figure 2.13** MS/MS in a triple-quadrupole mass spectrometer.

**Figure 2.14** MS/MS in an ion trap mass spectrometer.
Collision induced dissociation (CID) is the major fragmentation approach. Peptide molecular ions are allowed to collide with neutral gas molecules (helium, nitrogen or argon) within a cell in the mass spectrometer. As a result of the collision, some of the kinetic energy possessed by the molecular ion is converted into internal energy which results in bond breakage and the fragmentation of the molecular ion into smaller fragments. Fragmentation within triple quadrupole, quadrupole ion trap and hybrid quadrupole TOF analyzers occurs at collisional energies in the order of 10–100 eV range, whilst fragmentation within FT-ICR analyzer occurs at collisional energies at least an order of magnitude higher in the keV range. The former is described as low-energy CID whilst the latter is described as high-energy CID. As a result of the low-energy collisional fragmentation, the peptide precursor ion fragments predictably at each peptide amide bond along the peptide backbone yielding a distribution of product ions in two complementary ion series forming a ladder which is indicative of the peptide sequence. This fragmentation nomenclature was described by Roepstorff and Fohlman [98] (Figure 2.15). The two complementary ion series are: N-terminal ion series, or b-ion series. The ions of the n-terminal ion series will contain the N-terminal amino acid and extensions from this residue. At higher collisional energies, the peptide is additionally fragmented at the amino acid side-chains [99], the subsequent fragmentation pattern can be used to differentiate the isobaric residues, leucine and isoleucine.
2.4.2 Protein Quantification

LC-MS protein quantification strategies generally involve protein/peptide labeling (known as differential mass tagging or isotopic labeling). Labeling strategies permit the mixing of samples prior to LC-MS, and in some cases upstream of any fractionation. Thus, multiple specimens can be run simultaneously with the same peptides (or proteins) being identically separated and co-eluted into the mass spectrometer with ion intensities being directly compared in the same MS or MS/MS scans. The labeling strategies thus improve throughput and quantitative accuracy.

Differential labeling approaches for quantitative LC-MS in proteomics fall into two main categories: those which use chemical derivatization or enzymatic modification of proteins or peptides after sample collection, and those which use incorporation of isotope-labeled amino acids in vivo. The chemical labeling approaches make use of tags with the same (isobaric labeling) or different masses (isotopic labeling). Isobaric labeling,
exemplified by iTRAQ [100], consists of peptide tags which generate specific fragment ions by MS/MS. Samples are differentially labeled and then combined and concurrently analyzed by LC-MS/MS, with relative quantitation performed by comparison of intensities of the ‘reporter’ fragments in the MS/MS spectra. In contrast, isotopic labeling methods, such as isotope-coded affinity tags (ICATs) [101] or proteolytic $^{18}$O labeling [102], generate pairs (or more) of peptides with a mass difference introduced by the label. The ion intensities of the isotopic forms of the labeled peptides which should have identical LC elution profiles are then compared to give a peptide ratio of the ‘heavy’-labeled versus ‘light’-labeled peptide. In a similar manner, differential isotopic labeling in vivo allows quantification of peptides following incorporation of ‘light’ ($^{12}$C, $^{14}$N, $^1$H) and ‘heavy’ stable isotope-labeled ($^{13}$C, $^{15}$N, $^2$H) amino acids and is exemplified by the stable isotope labeling of amino acids in culture (SILAC) strategy [103]. In all of these methods, the ratios of detected ‘reporter’ fragments or isotopically labeled peptides are computed and integrated into protein ratios which can then be evaluated statistically. Multiple software solutions for the analysis of quantitative information using these labeling strategies are available. Although many of these solutions are instrument, data or tag dependent, they all work on the same principle whereby isotopically labeled peptide pairs (or reporter ions) are extracted on the basis of their characteristic mass differences and successful MS/MS
peptide assignments. Ratios of the extracted isotopic pairs are then computed and statistical evaluation performed. It is important to note that the smaller the mass difference of the tags is, then the more difficult it becomes to interpret the data and perform accurate quantification, since the isotope envelopes of the differentially labeled peptides may overlap. When using any labeling approach for LC-MS, the labels are best introduced at the earliest point in the workflow to minimize differences introduced into the samples by handling or quantitative differences between LC-MS runs. In this sense, the in vivo labeling strategies outperform the chemical and enzymatic labeling strategies in terms of accuracy of quantitation; however, as will be discussed below, multiplexing using in vivo labeling is presently more limited than chemical labeling, where 12-plex strategies have been reported [104]. Another important difference in these tagging strategies is in the analysis of primary tissues and clinical samples such as tissues, body fluids and urine, which are only amenable to the in vitro labeling approaches.

2.4.2.1 ICAT

Although stable isotope labeling for protein quantitation had been previously reported, ICAT was the first robust and universal differential labeling strategy to be developed for quantitative LC-MS and is based on cysteine thiol group modification using iodoacetamide tags. In the first
report, ICAT was used to examine the expression profiles of yeast growing on either galactose or ethanol in a single analysis [101]. Stable isotopes were incorporated into intact proteins after lysis by selective alkylation of cysteines with either a heavy (deuterium D₈) or a light (deuterium D₀) reagent bearing a biotin tag. Prior to LC-ESI-MS/MS analysis, the protein mixture was digested with trypsin and the ICAT labeled (cysteine-containing) peptides enriched on monomeric avidin–agarose. This had the advantage of simplifying the peptide mixture for downstream LC-MS/MS-based identification. Outline of a protocol for ICAT-based quantification of proteins in two biological samples is shown in Figure 2.16.
Figure 2.16 Outline of a protocol for ICAT-based quantification of proteins in two biological samples [105]. (A) Steps involved in ICAT labeling and purification. One of the samples may be a pooled reference if more than two samples are compared. (B) Targeted MS analysis of ICAT samples for quantification and identification of differentially expressed proteins. Steps involving computerized data analysis are indicated with a computer icon.
Several technical problems were reported with the original deuterated ICAT reagents. Prominent of these was the differential elution of ICAT-labeled peptide pairs in RP-LC leading to errors in the quantification [106, 107]. A further problem was the relatively low efficiencies of labeled peptide CID, which was speculated to be due to the relatively large size of the ICAT moiety [108]. Poor recovery of tagged peptides from avidin may also contribute to reduced proteomic coverage. To improve these issues, a new generation of ICAT reagents were developed where the deuterium atoms were substituted for nine $^{12}\text{C}$ (light) or $^{13}\text{C}$ (heavy) atoms and an acid-cleavable linker added. These new generation cleavable ICAT reagents gave more precise co-migration of the light- and heavy-tagged peptides in RP-LC, whilst the cleavage strategy eliminated undesired residual fragmentation of biotinylated peptides and improved recovery [109]. However, an additional clean-up step was required for removal of the cleaved biotin moiety prior to LC-MS analysis.

2.4.2.2 Differential Proteolytic $^{16}\text{O}/^{18}\text{O}$ Labeling

Differential stable isotopic labeling can also be achieved enzymatically. The major method relies on the oxygen atom exchange that takes place at the C-terminal carboxyl group of peptides during proteolytic digestion. Here, one or two $^{16}\text{O}$ atoms are replaced by one or two $^{18}\text{O}$ atoms through enzyme-catalyzed exchange in the presence of $\text{H}_2^{18}\text{O}$. The method was
first suggested as a protein quantification tool when $^{18}$O-labelled internal standards were generated for absolute quantification by MALDI–MS [110]. This was followed by the reporting of conditions for protein labeling [111] and then the first proteomic application of the method, where trypsin was used to incorporate two $^{18}$O atoms into the C termini of all tryptic peptides and was applied to compare proteins from two serotypes of adenovirus [102]. Subsequent work by the same group showed that Glu-C could also be used for labeling, that $^{18}$O-labelled and unlabelled ($^{16}$O) peptide pairs co-eluted in RP-LC and measurements of isotope ratios by LC-MS were accurate and precise [112]. Schematic representation of the enzyme-catalyzed $^{18}$O-labeling strategy is shown in Figure 2.17.
Figure 2.17 Schematic representation of the enzyme-catalyzed 18O-labeling strategy for comparative proteomics with digestion and labeling steps decoupled [113].

Several drawbacks of the proteolytic labeling strategy are apparent. Only two samples can be compared simultaneously, C-terminal peptides of proteins cannot be quantified and variable incorporation of $^{18}$O into peptides can occur [114]. There is also a lack of computational tools for accurate quantification of peptide differences and this is exacerbated by
the overlap of isotopic envelopes for $^{16}\text{O}$- and $^{18}\text{O}_1$- and $^{18}\text{O}_2$-labelled peptides.

### 2.4.2.3 iTRAQ

Isobaric tagging is a multiplex peptide labeling method that relies on the introduction of stable isotope tags that are chemically identical but distinguishable by MS/MS due to their fragmentation into reporter ions of different masses. The most commonly used method of this type has been the 4-plex iTRAQ reagents, which are N-hydroxysuccinimidyld esters for the labeling of primary amino groups. A specific reporter group in each tag based on N-methylpiperazine generates ions of 114, 115, 116 and 117 m/z upon CID fragmentation (Figure 2.18 A). These appear in an ion-sparse region of MS/MS spectra and their relative intensities provide the relative abundance of labeled peptides between the samples. The reporter groups in each tag are mass-balanced with a linker group making the tags isobaric. The major advantage of this MS/MS-dependent strategy is that the multiplex labeling does not increase the mass complexity of the samples and only peptides subjected to CID fragmentation are quantified. In addition, higher signal-to-noise ratios can be achieved with MS/MS-based detection versus MS-mode measurements. In the first reported use of iTRAQ, Ross et al. [100] compared global protein expression of wild-type and mutant yeast strains defective in the nonsense-mediated and 5' to 3'
mRNA decay pathways using 2D-LC linked to MALDI- and ESI-MS/MS. Under optimized labeling conditions, there was an estimated 97% labeling of N termini and lysine-amino groups, with a minimal degree of unlabelled or tyrosine-labeled peptides. Lysinederivatised peptides were more frequently identified, possibly due to their higher ionization efficiency versus arginine-terminated peptides. Peptide ratios were averaged for each protein and 685 proteins were quantified in all three yeast strains using two or more significant scoring peptides. A high degree of reproducibility for individual peptides contributing to any given protein was reported. This study also determined the absolute levels of a target protein after spiking with a synthetic peptide standard labeled with one of the isobaric tags. An 8-plex version of iTRAQ has also been commercialized, generating a spectrum of eight unique reporter ions at 113, 114, 115, 116, 117, 118, 119 and 121 m/z increasing sample throughput for complex differential analyses [115-117]. An overview of an iTRAQ workflow is shown in Figure 2.18 B.
Figure 2.18 A generic iTRAQ experiment [118]. (A) A schematic of two iTRAQ tags, showing three distinct groups, a peptidereactive group, the reporter group, which allows relative quantification upon MS/MS, and a balance group, which maintains the isobaric nature of the tags. (B) An overview of an iTRAQ workflow. Peptides from multiple samples are labeled with iTRAQ tags. These samples are pooled, generating a single ion for each peptide. One putative peptide ion (marked with a * in the figure) is then selected and fragmented. A typical MS/MS spectrum is shown, demonstrating sequence identification, with the reporter ion region expanded to demonstrate how peptide-relative quantification is determined.

There are several drawbacks to the isobaric tagging methods when compared to other labeling strategies. The iTRAQ reagents are expensive, difficult to synthesize and show signals only when peptides are subjected to fragmentation. Thus, the strategy misses peptides not selected for MS/MS, thus lowering proteomic coverage. Dedicated software must also be used for data analysis, although as well as commercially available software (Mascot, Proteome Discoverer, Protein Pilot), free computational tools for iTRAQ quantification and protein identification with details of
statistical considerations when analyzing iTRAQ data have been reported [119-127]. It is also evident that the low collision energies used in ion traps and some Q-TOF platforms can result in low iTRAQ reporter ion abundances and hence less accurate quantification data. Thus, higher energy CID methods have been employed, such as pulsed Q dissociation available on the popular LTQ linear ion-trap instruments or ‘higher energy CID’ available on the LTQ-Orbitrap. However, it is apparent that careful tuning is required for optimal fragmentation [128-130]. Finally, isotope purity correction in measured peak areas needs to be applied for each batch of reagents used and there is reported evidence of the compression of the dynamic range of ratios determined by the iTRAQ technique [131].

2.4.2.4 SILAC

As mentioned previously, SILAC is an in vivo stable isotope labeling method which uses heavy and light versions of essential amino acids that are added to the growth media of metabolically active cells. The idea behind the strategy originates from comparative proteomic experiments of simple model organisms [132-135] and plants [135] which can be grown in either medium containing $^{14}\text{N}$ at natural abundance (99.6%) or the same medium enriched in $^{15}\text{N}$. The method was adapted for the analysis of mammalian cell culture systems where deuterated leucine ([D3]Leu) was supplemented into the growth media of cells in one state for comparison with unlabelled
cells in another state [103]. Cells were harvested and equal amounts of
cells or protein lysate mixed prior to fractionation and LC-MS. As mentioned
above, this has the benefit of reducing technical variability since the
samples for comparison are mixed at the earliest possible stage in the
workflow and are thus treated as one sample and subjected to the same
downstream manipulations. The intensities of all peptides ions containing
the label can be compared with their unlabelled counterparts for relative
quantification.

The method was improved with the introduction of $[^{13}C_6]$Lys and $[^{13}C_6]$Arg,
providing a larger mass difference between light and heavy peptides,
giving predominantly C-terminal tagging of tryptic peptides and improving
co-elution of heavy and light peptides by RP–LC. Various labels have
subsequently been combined for 3-, 4- and 5-plex comparisons ($[^{13}C_6]$Lys
(+6 Da); $[^{13}C_6]$Arg (+6 Da); $[^{15}N_4]$Arg (+4 Da); $[D_4]$Lys (+4 Da);
$[^{13}C_6,^{15}N_2]$Lys (+8 Da); $[^{13}C_6,^{15}N_4]$Arg (+10 Da); $[^{13}C_6,^{15}N_2,D_9]$Lys (+17 Da);
$[^{13}C_6,^{15}N_4,D_7]$Arg (+17 Da)), with the method shown to give reproducible
quantitative information [136-138]. Software platforms for SILAC-based
quantification have also been described, including MaxQuant for
LTQOrbitrap- acquired data [139]. Example of Arg6-SILAC labeling and
assessment of proline conversion is shown in Figure 2.19.
Figure 2.19 Example of Arg6-SILAC labeling and assessment of proline conversion [140]. (A) Peptides from cells passaged in either Arg0- or Arg6-containing medium can be distinguished by MS because they are separated in mass by 6 Da as a consequence of the different isotopic labeling. (B) Too low concentrations of arginine in the labeling media cause incomplete incorporation (top panel, unfilled circle) while too high concentrations can lead to proline conversion (bottom panel, filled circle). In between a range of optimal arginine concentrations can be established where the incorporation is complete and the degree of proline conversion is negligible (middle panel).

The major drawback of the SILAC method is that it can be used only for samples where in vivo labelling is possible. This has restricted its use to simple organisms or cultured cell models, and so the quantitative comparison of proteins derived from tissues or body fluids is not possible. An additional drawback of the SILAC method is the cellular conversion of isotopelabelled arginine to proline, resulting in dilution of heavy peptide ion signals and hence inaccuracies in quantification. This is of particular concern as it can effect up to half of all peptides in a proteomic experiment.
The problem can however be alleviated by reducing the L-arginine concentration [141], by supplementing L-proline into the SILAC media [142] or by mathematical correction.

2.4.3 Role in Mechanistic Studies of ABPs

Antibacterial agents act by interfering with essential cellular functions or structures of bacteria. The bacterial proteome is dynamic in nature and quickly adjusts in response to antibacterial-agent attacks on physiological homeostasis. The cellular responses are highly specific for the physiological impairment encountered and usually directed at either compensating for the loss of a particular function or counteracting the inflicted damage. The cellular response to antibacterial-agent treatment therefore virtually mirrors the antibacterial mechanism of action. As a result, quantitatively comparing changes in the level of proteins between control and antibacterial-agent-treated conditions may aid mechanistic studies of antibacterial agents such as ABPs.
Chapter 3: LC-MS/MS Analysis of Cytoplasmic Protein Profile of *E. coli* in Response to Apidaecin IB Challenge

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3.1 Introduction

Apidaecins, 18- to 20-residue peptides produced by insects, are the largest group of proline-rich ABPs known to date. They are predominantly active against Gram-negative bacteria including a wide range of plant-associated bacteria and some human pathogens [22]. Previous studies suggested that the antibacterial mechanism of apidaecins was based on their ability to bind the chaperone DnaK and inhibit its function of assisting the polypeptides folding. However, it is possible that these peptides inactivate bacteria by other mechanisms not yet identified. Therefore, identifying new targets, exploring the underlying molecular mechanisms involved, and thus improving the understanding of the mechanisms of action of these peptides are needed.

In this chapter, an iTRAQ-coupled 2D LC-MS/MS technique was utilized to identify cytoplasmic proteins of *E. coli* altered in response to apidaecin IB
challenge. Levels of 60 kDa charperonin (GroEL) and 10 kDa charperonin (GroES), which together form the only essential chaperone system in *E. coli* cytoplasm under all growth conditions, were decreased in cells incubated with apidaecin IB. The reduction in the amount of the GroEL/GroES chaperon team was further found to be involved in a new antibacterial mechanism of these peptides.

### 3.2 Materials and Methods

#### 3.2.1 Bacterial Strain and Culture

The bacterial strain used in this work was *E. coli* ATCC 25922 obtained from the American Type Culture Collection (Rockville, MD). Frozen *E. coli* stock was streaked onto Mueller-Hinton (MH) agar plates and grown at 37 °C. Cells from a single colony were inoculated into MH broth and cultured overnight at 37 °C with shaking at 225 rpm for subsequent experiments.

#### 3.2.2 Minimal Inhibitory Concentration (MIC) Assay

The MIC of apidaecin IB was determined as described previously [143]. An aliquot of fresh overnight culture was inoculated into MH broth and incubated at 37 °C with shaking at 225 rpm until the optical density at
600nm (OD$_{600}$) of the undiluted culture was between 0.2 and 0.4. Cell suspension was diluted to obtain a concentration of $5 \times 10^5$ colony-forming units (CFUs)/ml. Apidaecin IB (AnaSpec, Inc., USA) was diluted in 0.01% acetic acid buffer to obtain a concentration of 1280 µg/ml. The diluted cell suspension (100 µl) and the serial two-fold dilution of the peptide solution (11 µl) were distributed in each well of round-bottomed, 96-well microtiter plate. Growth of cells in the plate was determined by visual inspection after 16–20 hr incubation at 37 °C. The MIC was defined as the lowest concentration that inhibited visible growth of the tested isolate.

3.2.3 Growth Kinetics of *E. coli* Incubated with Apidaecin IB

An aliquot of fresh overnight culture was inoculated into MH broth and incubated at 37 °C with shaking at 225 rpm until OD$_{600}$ of the undiluted culture was between 0.2 and 0.4. Cell suspension was diluted to obtain a concentration of $5 \times 10^5$ CFUs/ml and then incubated without and with $\frac{1}{10}$ MIC of apidaecin IB. Cell growth was checked by measuring OD$_{600}$ at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 hr.

3.2.4 Cytoplasmic Protein Isolation

*E. coli* cells ($5 \times 10^5$ CFUs/ml) were incubated with $\frac{1}{10}$ MIC of apidaecin IB for 1 and 2 hr. Cells were then harvested by centrifugation at 3000 × g for
10 min at 4 °C and lysed in lysis buffer (50 mM NaCl, 5 mM DTT, 1 mM PMSF and 50 mM Tris·Cl, pH 8.0) by intermittent sonication. Unbroken cells were removed by centrifugation at 3000 × g for 10 min at 4 °C. The supernatants containing the cytoplasmic proteins were collected by centrifugation at 15,000 × g for 30 min at 4 °C. The concentration of the proteins was determined by Bradford assay (Bio-Rad Laboratories, Inc., USA). Standard curves were made using γ-globulin as a control.

3.2.5 iTRAQ Labeling

Proteins from each sample (100 µg) were precipitated by the addition of four volumes of cold acetone at -20 °C for 2 hr. After centrifugation at 15,000 × g for 30 min at 4 °C, the precipitated pellets were reduced, denatured, cysteine blocked, digested and labeled with respective isobaric tags using iTRAQ reagent Multiplex kit (Applied Biosystems Inc., CA, USA) according to manufacturer's protocol. Briefly, each sample was dissolved in 20 µl dissolution buffer and denatured by adding 1 µl of Denaturant in the kit. After being completely dissolved, the sample was reduced by adding 2 µl of Reducing Reagent and incubated at 60 °C for 1 hr. The cysteine residues of each sample were blocked by incubation with 1 µl of Cysteine-Blocking Reagent at room temperature for 10 min. After that, proteins of the sample were digested by incubation with 20 µl of 0.25 mg/ml sequence-grade-modified trypsin solution (Promega Corp., USA) at
37 °C overnight. The sample was then labeled with iTRAQ reagents as follows: iTRAQ tags 114, Control 2 hr; iTRAQ tags 115, apidaecin IB-incubated 2 hr; iTRAQ tags 116, Control 1 hr; iTRAQ tags 117, apidaecin IB-incubated 1 hr. Samples were then pooled for LC-MS/MS analysis.

3.2.6 LC-MS/MS Analysis

The analysis was performed on a combined of an Agilent 1200 nanoflow LC system (Agilent Technologies Inc., USA) and a 6530 Q-TOF mass spectrometer (Agilent Technologies Inc., USA). Peptide mixture was separated by 2D LC, i.e. the combination of strong-cation exchange (SCX) with RP chromatography. The combined peptide mixture (3 μl) was loaded onto the PolySulfoethyl A SCX column (0.32 × 50 mm, 5 μm, PolyLC Inc., USA) and was eluted stepwise by injecting 10 different molar concentrations (10, 20, 30, 40, 50, 60, 80, 100, 300, and 500 mM) of KCl solution. The sequentially eluted peptides from the SCX column were trapped onto the enrichment HPLC chip (Agilent Technologies Inc., USA) and further eluted with buffer A (0.1% formic acid) and buffer B (a nanoflow gradient of 5-80% acetonitrile plus 0.1% formic acid) at a flow rate of 300 nl/min. The mass spectrometer was operated at a nanospray voltage of 2.2 kV. Data were acquired in the positive ion mode with a selected mass range of 300–1500 m/z. Up to two precursor peptides with +2 to +4
charges and 100–2000 m/z were selected for MS/MS using dynamic exclusion. The automatic rolling collision energy was used to promote fragmentation. The peak areas of the iTRAQ reporter ions reflect the relative abundance of the proteins in the sample.

3.2.7 Data Analysis

The identification and quantification of the proteins were performed using Spectrum Mill MS Proteomics Workbench (Agilent Technologies, Software Revision A.03.03.084 SR4). Each MS/MS spectrum was searched for species of \textit{E. coli} against the UniProt_sprot_20070123 database. The searches were run using the following parameters: fixed modification of methylmethanethiosulfate-labeled cysteine and fixed iTRAQ modification of free amine in the amino terminus and lysine. Other parameters such as tryptic cleavage specificity, precursor ion mass accuracy, and fragment ion mass accuracy are built-in functions Spectrum Mill software. The protein profile results were filtered with protein score of 11 or greater in combination with peptide score of 7 or greater and % SPI (the percentage of the spectral peak-detected ion current explained by the search interpretation) of 60% or greater. Relative quantification of proteins in the case of iTRAQ was performed on the MS/MS scans and displayed as the ratio of the areas under the peaks at 114, 115, 116 and 117 Da, which were the masses of the tags that correspond to the iTRAQ reagents.
Sequence coverage was calculated by dividing the number of amino acids observed by the protein amino acid length. The following criteria were required to consider a protein for further statistical analysis: two or more distinct peptides had to be identified and the average ratio had to be greater than 1.2 or less than 0.8.

3.2.8 Western Blot Analysis

Western blotting was performed as described previously [144]. Briefly, equal amount of total protein (20 µg) were resolved using 7.5%- to 12%-sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were semi-dry electro-transferred to Polyvinylidene Fluoride (PVDF) membrane (Bio-Rad Laboratories Inc., USA). The membrane was then blocked in 1 × PBST (3.2 mM Na₂HPO₄, 0.5 mM KH₂PO₄, 1.3 mM KCl, 135 mM NaCl and 0.05% Tween 20, pH 7.4) at room temperature for 1 to 2 hr and then probed with primary antibody, either anti-groEL or anti-groES (Abcam, UK), at 4 °C overnight. Horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody (Santa Cruz Biotechnology Inc., USA) was used as the secondary antibody. The membrane was then incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., USA) for 3 to 5 min in dark, and exposed to CL-XPosure Film (Thermo Fisher Scientific Inc., USA) for a few seconds. The X-ray film was then developed.
sequentially. The expression of DNA-directed RNA polymerase subunit alpha (RpoA) was used as a loading control.

3.2.9 Growth Kinetics of Gene-overexpression Strains in Response to Apidaecin IB Challenge

Plasmid that permits controlled expression of GroEL–GroES or DnaK–DnaJ–GrpE chaperone team (purchased form Takara, Japan) was transformed into *E. coli* BL21 (DE3) cells. Expression of the two chaperone teams is induced by the addition of L-arabinose (1 mg/ml) for 2 hr. Cell suspensions were then diluted to obtain a concentration of $5 \times 10^5$ CFU/ml and incubated without and with $\frac{1}{10}$ MIC of apidaecin IB. Cell growth was checked by measuring $OD_{600}$ in interval of 1 hr.

3.2.10 Statistical Analysis

Unless indicated in the figure legends, all experiment were replicated three times. The statistical significance was assessed by Student’s t-tests. A $p$-value of 0.05 or less was considered significant.
3.3 Results

3.3.1 MIC of Apidaecin IB

MIC is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. MIC is important in diagnostic laboratories to monitor the activity of new antimicrobial agents. In this study, MIC of apidaecin IB was evaluated by the broth micro-dilution assay. Apidaecin IB showed obvious antibacterial activity and its MIC was found to be 16 µg/ml.

3.3.2 Growth Kinetics of E. coli in Response to Apidaecin IB Challenge

In our work of monitoring the growth kinetics of E. coli cells in the presence of different concentrations of apidaecin IB, we found that apidaecin IB with any concentrations higher than 1/10 MIC could have inhibition effects on the growth of E. coli although the effect was not as strong as that by using MIC of apidaecin IB. As a result, the concentration of 1/10 MIC was chosen for all the following experiments.

The growth kinetics of E. coli incubated with 1/10 MIC of apidaecin IB is shown in Figure 3.1. Apidaecin IB started to inhibit E. coli growth at 0.5 h
after its incubation. The inhibition in the growth of *E. coli* was probably the result of the interference in essential cellular functions or structures of cells by apidaecin IB. The bacterial proteome is dynamic in nature and quickly adjusts in response to antimicrobial attacks on physiological homeostasis. Therefore, quantitatively comparing changes in the level of proteins between control and apidaecin IB-incubated conditions when the inhibition effect occurs may aid mechanistic studies of this peptide. Two time points (1 hr and 2 hr) were thus chosen for the quantitative protein profiling analysis.

![Figure 3.1](image)

**Figure 3.1** Growth kinetics of *E. coli* incubated with $\frac{1}{10}$ MIC of apidaecin IB. Each value represents the mean optical density (OD) readings from two cultures.
3.3.3 Cytoplasmic Proteins Altered in Response to Apidaecin IB Challenge

To establish the biological difference between apidaecin IB-incubated cells and control cells, the protein profiles in *E. coli* cells incubated with and without apidaecin IB were analyzed. Proteins from cells were collected, lysed, and labeled prior to 2D LC-MS/MS. Combining three independent experiments, a total of 391 distinct proteins were identified based on the following criteria: protein score of 11 or greater, peptide score of 7 or greater and % SPI of 60% or greater. The criteria give a confidence value of the identified protein of 99% or greater. All information about the 391 proteins was shown in Appendix 1.

Among the identified 391 proteins, 18 proteins displayed significant changes (*p*<0.05), and the trend of the changes in 2 hr-apidaecin IB-incubated cells was in accordance with that in 1 hr-apidaecin IB-incubated cells (Table 3.1). These proteins were subsequently categorized into seven groups based on their cellular functions: protein folding, oxidation-reduction process, tRNA processing, transcription regulation, and lipid, amino acid and carbohydrate metabolism.
Table 3.1  Cytoplasmic proteins of *E. coli* altered in response to apidaecin IB challenge

<table>
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<tr>
<th>Accession Number</th>
<th>Protein Name</th>
<th>Distinct Peptides</th>
<th>% AA Cov</th>
<th>Avg A:C (±S.D.)&lt;sup&gt;a&lt;/sup&gt; 1 hr</th>
<th>Avg A:C (±S.D.)&lt;sup&gt;a&lt;/sup&gt; 2 hr</th>
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<td><strong>Protein Folding</strong></td>
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<tr>
<td>P0A6F5</td>
<td>60 kDa chaperonin</td>
<td>25</td>
<td>59</td>
<td>0.70±0.01</td>
<td>0.62±0.01</td>
</tr>
<tr>
<td>P0A6H1</td>
<td>ATP-dependent Clp protease</td>
<td>4</td>
<td>15</td>
<td>1.20±0.16</td>
<td>1.33±0.18</td>
</tr>
<tr>
<td>P0A6F9</td>
<td>10 kDa chaperonin</td>
<td>4</td>
<td>50</td>
<td>0.80±0.01</td>
<td>0.61±0.01</td>
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<tr>
<td><strong>Lipid Metabolism</strong></td>
<td></td>
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<tr>
<td>P0A715</td>
<td>2-dehydro-3-deoxyphosphoheptonate aldolase</td>
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<td>26</td>
<td>0.70±0.00</td>
<td>0.50±0.00</td>
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<tr>
<td>P0AAI9</td>
<td>Malonyl CoA-acyl carrier protein transacylase</td>
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<td>0.74±0.06</td>
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<td>P0A6A8</td>
<td>Acyl carrier protein</td>
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<td>P13029</td>
<td>Catalase-peroxidase</td>
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<td>2</td>
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<tr>
<td>P00805</td>
<td>L-asparaginase</td>
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<td>Aspartate aminotransferase</td>
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<td>P0AEI1</td>
<td>(Dimethylallyl)adenosine tRNA methylthiotransferase miaB</td>
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<td>0.70±0.01</td>
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<td>P0A9Y6</td>
<td>Cold shock-like protein CspC</td>
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<td>40</td>
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<td><strong>Carbohydrate Metabolism</strong></td>
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<td>P0A715</td>
<td>2-dehydro-3-deoxyphosphoheptonate aldolase</td>
<td>4</td>
<td>26</td>
<td>0.70±0.00</td>
<td>0.50±0.00</td>
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<td>P09147</td>
<td>UDP-glucose 4-epimerase</td>
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<td>Triosephosphate isomerase</td>
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<td>0.42±0.00</td>
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<tr>
<td>P0AFG6</td>
<td>Dihydrolipolysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex</td>
<td>3</td>
<td>8</td>
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<tr>
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<td>KHG/KDPG aldolase</td>
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<td>15</td>
<td>0.55±0.00</td>
<td>0.52±0.00</td>
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<td>P0AC47</td>
<td>Fumarate reductase iron-sulfur subunit</td>
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<td>11</td>
<td>0.68±0.00</td>
<td>0.38±0.00</td>
</tr>
</tbody>
</table>

* The ratio of proteins in the apidaecin IB-incubated cells relative to control cells.
3.3.4 Western Blot Analysis on GroEL and GroES

Western blot analysis was then performed to validate the changes in the level of GroEL and GroES identified by LC-MS/MS analysis. Equal amount of cell lysates from apidaecin IB-incubated cells and control cells were tested with antibodies to GroEL and GroES respectively. Results shown in Figure 3.2 indicated that levels of both GroEL and GroES were decreased in cells incubated with apidaecin IB for both 1 h and 2 h, and the reduction in the amount of both proteins was higher in 2h-apidaecin IB-incubated cells.

![Figure 3.2](image)

**Figure 3.2** Western blot analysis of GroEL (A) and GroES (B) in *E. coli* incubated with apidaecin IB.
3.3.5 Growth Kinetics of Gene-overexpression Strains

To construct the GroEL–GroES and DnaK–DnaJ–GrpE chaperone overexpression strains, plasmid that permits controlled expression of GroEL–GroES or DnaK–DnaJ–GrpE chaperone team was transformed into *E. coli* BL21 (DE3) cells, separately. Expressions of these two chaperone teams were validated by western blot analysis on GroEL and DnaK (Figure 3.3). The growth of the cells in which GroEL-GroES or DnaK–DnaJ–GrpE chaperone team was overexpressed in response to apidaecin IB challenge was subsequently checked. The results shown in Figure 3.4 indicated that either overexpression of GroEL-GroES chaperone team or overexpression DnaK–DnaJ–GrpE chaperone team can alleviate the inhibition effect of apidaecin IB on cells; however, the alleviation effect of DnaK-DnaJ-GrpE overexpression was less than that of GroEL-GroES overexpression.

![Figure 3.3](image)

**Figure 3.3** Validation the expression of GroEL (A) and DnaK (B) by western blot analysis. Lane 1, mock transformation; lane 2, transformation of empty plasmid; and lane 3, transformation of plasmid that permits controlled expression of GroEL–GroES or DnaK–DnaJ–GrpE chaperone team.
Figure 3.4 Effect of GroEL-GroES and DnaK-DnaJ-GrpE overexpression on the growth of *E. coli* incubated with apidaecin IB. (A) Growth curve of control, GroEL-GroES overexpression and DnaK-DnaJ-GrpE overexpression cells without apidaecin IB incubation. (B) Growth curve of control, GroEL-GroES overexpression and DnaK-DnaJ-GrpE overexpression cells with apidaecin IB incubation. (C) OD_{600} ratio from 6 h of culture. OD_{600} ratio of control cells was adjusted to 1 and those of cells overexpressing GroEL-GroES and DnaK-DnaJ-GrpE chaperone teams were normalized accordingly. Asterisk indicates *p*<0.05.
3.4 Discussion

Apidaecins are good representatives of non-membrane-permeabilizing ABPs which can translocate across the membrane and accumulate in the cytoplasm, where they kill bacteria by targeting different essential cellular processes. Previous studies proposed a five-step mechanism by which apidaecins exert their activity against *E. coli* and other Gram-negative bacteria: *i*) binding to negatively charged components (most probably LPS) of the OM in a non-specific manner; *ii*) self-promoted invading into the periplasmic space; *iii*) irreversible interaction with a hypothetical docking molecule/receptor molecule on the IM; *iv*) translocation across the membrane and into the cytoplasm of the cell; *v*) binding its target most probably DnaK protein to mediate cell death (Figure 2.5) [21, 24, 72, 82-84]. However, it is possible that these peptides kill bacteria by other mechanisms not yet identified.

In this study, an iTRAQ-coupled 2D LC–MS/MS approach was utilized to analyze the cytoplasmic protein profile of *E. coli* in response to apidaecin IB challenge in aim to identify target proteins whose alterations are involved in the antibacterial mechanism of action of apidaecin IB. A total of 18 proteins showed differential changes, and the trend of the changes in 2 hr-apidaecin IB-incubated cells was in accordance with that in 1 hr-apidaecin IB-incubated cells (Table 3.1). Two of the altered proteins,
GroEL and GroES, captured our attention. Levels of the two proteins were decreased in both 1 hr and 2 hr-apidaecin-incubated cells, with the decrease in the latter greater than that in the former (Table 3.1). The changes of GroEL and GroES were further validated by western blot analysis (Figure 3.2).

As we know, folding of polypeptides in the cell typically requires the assistance of a set of proteins termed molecular chaperones [145]. Chaperones are necessary for cell viability under both normal and stress conditions [146]. Chaperones can assist in the efficient folding of newly-translated proteins as these proteins are being synthesized on the ribosome and can maintain pre-existing proteins in a stable conformation [146]. Chaperones can also promote the disaggregation of preformed protein aggregates [147]. There are several chaperone systems in E. coli which carry out a multitude of functions all aiming towards insuring the proper folding of target proteins [148]. However, the chaperonin GroEL with its cofactor GroES form the only essential chaperone system in E. coli cytoplasm under all growth conditions [149]. About 300 proteins, representing 10-15% of total cytoplasmic E. coli proteins, utilize GroEL for de novo folding under normal growth conditions and about twice as much under stress conditions [150-152]. As a result, the decrease in the level of GroEL and GroES could be correlated with the antibacterial activity of
The growth of the cells in which GroEL-GroES chaperone team was overexpressed in response to apidaecin IB incubation was therefore checked. The results showed that overexpression of GroEL-GroES chaperone team significantly alleviated the inhibition effect of apidaecin IB on cells (Figure 3.4). As previous studies showed that apidaecins could inhibit another chaperone-DnaK (a component of DnaK/DnaJ/GrpE chaperone system) to mediate cell death [21], the alleviation of the inhibition effect of apidaecin IB on the GroEL-GroES overexpression cells could be the result of the GroEL/GroES chaperone system partially compensating for the loss of DnaK’s function, but not the result of the decrease in the level of GroEL and GroES in response to apidaecin IB. The growth of the cells in which DnaK-DnaJ-GrpE chaperone team was overexpressed in response to apidaecin IB incubation was further checked. The results showed that overexpression of DnaK-DnaJ-GrpE chaperone team can also alleviate the inhibition effect of apidaecin IB on cells; however the alleviation effect of DnaK-DnaJ-GrpE overexpression was less than that of GroEL-GroES overexpression (Figure 3.4). Thus, the alleviation of the inhibition effect of apidaecin IB on the GroEL-GroES overexpression cells could be at least partially the result of the decrease in the level of GroEL and GroES in response to apidaecin IB. Collectively, the
data suggest that, besides inhibiting DnaK’s function, apidaecin IB can also cause the decrease in the level of GroEL-GroES chaperone team, which could be involved in a new antibacterial mechanism of the peptides.

Except locating in the cytoplasm of bacteria, some proteins are attached to, or associated with the inner or outer bacterial membrane. These proteins are called membrane proteins and account for about one quarter to one third of all bacterial proteins. As membrane proteins perform essential physiological functions, they may be the target of antibacterial agents. Further analysis of the membrane protein profile is required to comprehensively investigate the changes in the global proteome of *E. coli* in response to the challenge apidaecin IB and thus infer the possible mechanisms of action of these peptides.
Chapter 4: LC-MS/MS Analysis of Membrane Protein

Profile of *E. coli* in Response to Apidaecin IB Challenge

*(Part of this section was from our paper published in PLoS One. 6(6): e20442. Permission to use the article in this dissertation was obtained from publisher.)*

4.1 Introduction

Our previous work focused on analysis of changes in the cytoplasmic proteins in response to the challenge of apidaecin IB. However, except cytoplasmic proteins, about one quarter to one third of all bacterial genes encode proteins of inner or outer bacterial membrane. These membrane proteins perform essential physiological functions, such as the import or export of metabolites, the homeostasis of metal ions, the extrusion of toxic substances, and the generation or conversion of energy. For this reason, these proteins may be targeted by antibacterial agents. It is thus necessary to analyze changes of membrane proteins in response to apidaecins challenge, with of hope of identifying new targets of these peptides.

In this chapter, the iTRAQ-coupled 2D LC-MS/MS technique was utilized to identify membrane proteins of *E. coli* altered in response to the challenge of apidaecin IB. Cell division protease ftsH (FtsH), an essential regulator in maintenance of membrane lipid homeostasis, was overproduced in cells incubated with apidaecin IB for both 1 hr and 2 hr. Its overproduction intensified the degradation of cytoplasmic protein
UDP-3-O-acyl-N-acetylglucosamine deacetylase (LpxC), which catalyzes the first committed step in the biosynthesis of the lipid A moiety of LPS, and thus led to the further unbalanced biosynthesis of LPS and phospholipids. These findings suggested a new antibacterial mechanism of apidaecins.

4.2 Materials and Methods

4.2.1 Bacterial Strain and Culture

Bacterial strain and its culture procedure was the same as described in Section 3.2.1 in Chapter 3.

4.2.2 Membrane and Cytoplasmic Protein Isolation

_E. coli_ cells (5 × 10^5 CFUs/ml) were incubated with 1/10 MIC of apidaecin IB for 1 and 2 h. The membrane proteins were isolated as described previously with a slightly modification [28]. Briefly, the cells were harvested by centrifugation at 3000 × g for 10 min at 4 °C and lysed in lysis buffer (50 mM NaCl, 5 mM DTT, 1 mM PMSF and 50 mM Tris·Cl, pH 8.0) by intermittent sonication. Unbroken cells were removed by centrifugation at 3000 × g for 10 min at 4 °C. The supernatants containing the cytoplasmic proteins were collected by centrifugation at 120,000 × g for 60 min at 4 °C. The resulting pellets were resuspended in IM solubilization buffer (1% Sarkosyl, 100 mM NaCl and 50 mM Tris·Cl, pH 8.0) and incubated at room temperature for 60 min with gentle shaking. The supernatants containing the solubilized inner membrane proteins were collected by centrifugation at 120,000 × g for 60 min at 4 °C again. The pellets were resuspended in Milli-Q water and
centrifuged for up to three times. The resulting pellets were resuspended in OM solubilization buffer (3% n-octylpolyoxyethylene, 150 mM NaCl, 50 mM EDTA, 10 mM DTT, 0.1 mg/ml lysozyme and 50 mM Tris-Cl, pH 8.0) and incubated at room temperature for 60 min with stirring. The supernatants containing the solubilized outer membrane proteins were collected by centrifugation as above. The concentration of cytoplasmic, inner and outer membrane proteins were determined by Bradford assay (Bio-Rad Laboratories, Inc., USA). Standard curves were made using γ-globulin as a control.

4.2.3 iTRAQ Labeling

iTRAQ Labeling was performed using the same procedure as described in Section 3.2.5 in Chapter 3.

4.2.4 LC-MS/MS analysis

iTRAQ-labeled peptide mixtures were analyzed by 2D nanoflow LC system (Agilent Technologies Inc., USA) interfaced with a QSTAR XL mass spectrometer (Applied Biosystems Inc., USA), as described previously [144, 153-156]. The peptide mixture was loaded into a PolySulfoethyl A SCX column (50 × 0.32 mm, 5 mm, PolyLC Inc., USA) and fractionated by ten salt steps (10, 20, 30, 40, 50, 60, 80, 100, 300, and 500 mM KCl) in the first dimension. The peptides eluted from the SCX column were concentrated and desalted in a ZORBAX 300SB C18 RP column (5 × 0.3 mm, 5 mm, Agilent Technologies Inc., USA). The second dimensional chromatographic separation was carried out with a ZORBAX 300SB C18 RP column (50 ×
0.075 mm, 3.5 mm, Agilent Technologies Inc., USA) directly into a PicoFrit nanospray tip (New Objective, USA) operating with gradient starting from 5% up to 80% of acetonitrile at a flow rate of 500 nl/min over 100 min. The mass spectrometer was operated at a nanospray voltage of 2.2 kV. Data were acquired in the positive ion mode with a selected mass range of 300–1500 m/z. Up to two precursor peptides with +2 to +4 charges and 100–2000 m/z were selected for MS/MS using dynamic exclusion. The automatic rolling collision energy was used to promote fragmentation. The peak areas of the iTRAQ reporter ions reflect the relative abundance of the proteins in the sample.

4.2.5 Data Analysis

The identification and quantification of the proteins were performed using ProteinPilot Software 3.0 (Applied Biosystems Inc., USA). The Paragon Algorithm in the ProteinPilot software was used for the peptide identification and further processed by Pro Group Algorithm where isoform-specific quantification was adopted to trace the differences between expressions of various isoforms. The defined parameters were as follows: (i) Sample Type, iTRAQ 4-plex (Peptide Labeled); (ii) Cysteine alkylation, MMTS; (iii) Digestion, Trypsin; (iv) Instrument, QSTAR ESI; (v) Special factors, None; (vi) Species, E. coli; (vii) Specify Processing, Quantitate, Bias correction (viii) ID Focus, Biological modifications; (ix) Database, UniProt_sprot_20070123; (x) Search effort, thorough. Protein confidence for a detected protein is measured by Unused ProtScore. It calculated from the peptide confidence for peptides from spectra that are not already completely “used” by higher
scoring winning proteins. A protein with Unused ProtScore of 2.0, which corresponds to 99% confidence, is generally true. The peptide for quantification was automatically selected by Pro Group Algorithm, with the criterion that the peptide was usable for quantitation, identified with good confidence, and not shared with another protein identified with higher confidence, to calculate the reporter peak area, $p$-value and error factor (EF). $p$-value is a measure of the certainty that the average ratio randomly differs from 1. The smaller the $p$-value, the more likely any differential expression is real. EF is a statistic that was expressed to reflect the true value for the average ratio that was used for the quantification of differentially expressed proteins (EF= $10^{95\% \text{ confidence interval}}$, in which 95% confidence interval= (ratio $\times$ EF) - (ratio/EF)). The average ratio is far from the actual value when the EF value is more than 2. The following criteria were required to consider a protein for further statistical analysis: the EF had to be of 2 or less and the average ratio had to be greater than 1.1 or less than 0.9.

4.2.6 Western Blot Analysis

Rabbit antisera to FtsH and LpxC were produced by Invitrogen Corp., USA. The specificity of anti-FtsH and LpxC was validated. These antisera were used as the primary antibodies. Western blotting was performed using the same procedure as described in Section 3.2.8 in Chapter 3.

4.2.7 LPS and Phospholipids Analysis

LPS was extracted by using LPS extraction kit (iNtRON Biotechnology,
Korea) according to manufacturer’s protocol. Briefly, cells were lysed in 1 ml of Lysis Buffer and then mixed with 200 µl of chloroform for 5 min. After centrifuging at 4 °C, 13,000 rpm for 10 min, supernatant was transferred to a new tube and mixed with 800 µl of Purification Buffer at -20 °C for 10 min. The mixture was centrifuged again at 4 °C, 13,000 rpm for 5 min. The LPS pellet was washed with 1 ml of 70% ethanol and dissolved in 10 mM Tris·Cl, pH 8.0. The LPS was then quantified by measuring 2-keto-3-deoxyheptonic acid (KDO) as described previously [157]. Phospholipids were extracted by a method described previously with a slight modification [158]. In brief, the cell pellets were resuspended in 2 volumes of Milli-Q water and mixed with 7.5 volumes organic solvent mixture (methanol-chloroform 2:1, v/v). This suspension was incubated at room temperature for 2 hr with periodic vortexing. After centrifuging at 3000 × g for 10 min, extract from the top was removed and mixed with half volume of chloroform and Milli-Q water. After thorough vortexing, the mixture was centrifuged again. Extract from the lower chloroform phase was removed and qualified by phosphate assay as described previously [159].

4.2.8 Gene Cloning

**Whole genome extraction**

Whole genome of *E. coli* was purified by using DNeasy Blood & Tissue Kit (QIAGEN, Germany). Briefly, cells were harvested in a microcentrifuge tube by centrifuging at 5000 × g for 10 min. Pellet was resuspended in Buffer ATL (180 µl). After mixing with proteinase K (20 µl), the mixture was
incubated at 56 °C until the cells were completely lysed. Buffer AL (200 µl) and ethanol (200 µl, 96-100%) were then sequentially added. The mixture was transferred to the DNeasy Mini spin column and centrifuged at ≥6000 × g for 1 min. The column was then washed by Buffer AW1 (500 µl) and Buffer AW2 (500 µl). Finally, the DNA was eluted from the column by Buffer AE (100 µl).

**DNA quantification**

The ratio of the readings at 260 nm and 280 nm (\(A_{260}/A_{280}\)) provides an estimate of the purity of DNA with respect to contaminants that absorb UV, such as protein. Pure DNA has an \(A_{260}/A_{280}\) ratio of 1.8-2.0. DNA concentration was calculated according to the formula below, as an \(A_{260}\) value of 1 (with a 1 cm detection path) corresponds to 50 µg DNA per ml water. DNA concentration (µg/ml) = \(A_{260} \times 50 \times\) dilution factor

**Gene amplification**

The gene encoding *E. coli* FtsH and LpxC was amplified by PCR, using the appropriate forward and reverse oligonucleotide primers designed with the help of online free software Primer 3 (http://frodo.wi.mit.edu/primer3/) (Table 4.1). The forward primer introduced an EcoRI site and the reverse primer introduced an XhoI site downstream from the stop codon. Three extra bases were introduced into the 5’ end of each primer as protecting bases to ensure correct digestion of the restriction endonucleases.
Table 4.1 Primers used for plasmid construction.

<table>
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<th>Primer Name</th>
<th>Sequence</th>
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<tr>
<td>ftsH forward</td>
<td>5’-CCGGAATTCATGGCGAAAAACCTAATAC-3’</td>
</tr>
<tr>
<td>lpxC forward</td>
<td>5’-CCGGAATTCATGGATCAAAAACAGGACAC-3’</td>
</tr>
<tr>
<td>ftsH reverse</td>
<td>5’-CCGCTCGAGTTACTTTGTCGCCCTAACTGC-3’</td>
</tr>
<tr>
<td>lpxC reverse</td>
<td>5’-CCGCTCGAGTTATGCCAGTACAGCTGAAGG-3’</td>
</tr>
</tbody>
</table>

**Vector used for cloning**

The vector used in this study was pET-24a (EMD Chemicals, USA), which carry an N-terminal T7•Tag sequence plus an optional C-terminal His•Tag sequence. The map of the vector is shown in Figure 4.1.

---

**Figure 4.1** Feature map of pET-24a vector. The pET-24a (+) vector carry an N-terminal T7•Tag® sequence plus an optional C-terminal His•Tag® sequence.
**PCR purification**

The desired DNA fragment was directly purified with QIAquick PCR Purification Kit (QIAGEN, Germany). Briefly, five volumes of Buffer PB were added to one volume of the PCR sample. The mixture was then transferred to a QIAquick spin column and centrifuged at 10,000 rpm for 60 sec. The column was then washed with 0.75 ml of Buffer PE. Finally the DNA was eluted from the column with 30-50 µl Buffer EB.

**Endonucleases digestion and CIP treatment**

The purified PCR fragments and empty vectors were digested respectively by EcoRI and XhoI (NEW ENGLAND BioLabs Inc., USA). Generally, the purified PCR fragments or vector plasmids (3-5 µg) were digested in an endonuclease mixture (1 × NEB buffer, 5-10 units of endonuclease, and 1 × BSA) at 37 °C for 1-2 hr. Vector DNA was further dephosphorylated with calf intestinal alkaline phosphatase (CIP) (NEW ENGLAND BioLabs Inc., USA) at 37 °C for 30-60 min. This treatment could release the 5’ phosphate group of DNA fragments and significantly reduced the rate of self-ligation of vectors in the subsequent ligation reaction.

**DNA purification by agarose gel extraction**

After digestion, PCR fragments and vectors were extracted by agarose gel electrophoresis and further purified by QIAquick Gel Extraction kit (QIAGEN, Germany). In brief, DNA band slice was excised from the gel. Three volumes of Buffer QG were added to one volume of gel. The mixture was
then incubated at 50 °C for 10 min until the gel was completely dissolved. Then the mixture was mixed with one gel volume of isopropanol and transferred to QIAquick column. After centrifuging, the column was washed with Buffer QG (0.5 ml) and Buffer PE (0.5 ml). Finally, DNA was eluted from the column by Buffer EB (50 µl).

**Ligation**

Purified PCR fragment and linear vector were ligated with T4 DNA ligase (NEW ENGLAND BioLabs Inc., USA). For each ligation, a 10 µl of reaction mixture was prepared by adding 1 µl of T4 DNA ligase, 1 µl of 10 × T4 DNA ligase buffer, 1µl of vector (50-100 ng) and 7 µl of PCR fragment. The mixture was incubated at room temperature for 1-2 hr.

**Preparation of competent E. coli cells**

*E. coli* DH5α stock stain was streaked onto a fresh LB agar plate and incubated at 37 °C overnight. A single clone was picked out from the agar plate and inoculated into 5ml of LB medium and incubated at 37 °C overnight with shaking at 225 rpm. The next day, 1 ml of the amplified bacteria was re-inoculated into fresh 100 ml of LB medium. The culture was incubated at 37 °C with shaking at 225 rpm until OD$_{600}$ of the undiluted culture was between 0.3-0.4, which corresponded to the exponential growth phase. A total of 50 ml of this culture was transferred to 50-ml pre-chilled centrifuge tubes and incubated on ice for 10 min. After centrifuging for 5 min at 4 °C, 5000 rpm, cell pellets were then resuspended in 30 ml of 0.1 M ice-cold MgCl$_2$. After centrifuging again at 4 °C, 5000 rpm for 5 min, the cell
pellets were resuspended in 20 ml 0.1 M ice-cold CaCl₂ and incubated on ice for 30 min. After centrifuging once again at 4 °C, 5000 rpm for 5 min, the cell pellets were resuspended in 1.5 ml of solution (0.1 M ice-cold CaCl₂ and 15% glycerol). The resulting competent cells could be used immediately or aliquoted into autoclaved fresh tubes and stored at -80 °C.

**Transformation**

Ligation reaction mixture (10 µl) was added into above competent cells (100 µl). The cells were sequentially incubated on ice for 60 min, at 42 °C for 90 sec, and on ice again for another 90 sec. LB medium without antibiotics (800 µl) was then added into the cell mixture. Cells were recovered by incubating at 37 °C, 225 rpm for 1 hr, and then pelleted by quick spin. Approximately 100 µl of supernatant were left in the tube to resuspend the cell pellet. After that, cell suspension was spread onto a selective LB agar plate. The plate was then incubated at 37 °C for 16-18 hr.

**Mini-preparation of plasmids**

After overnight incubation, clones on the plate were selected and inoculated into 5 ml LB medium for another overnight incubation. Plasmids were prepared by QIAprep plasmid miniprep kit (QIAGEN, Germany). Briefly, cells from overnight culture were collected by centrifuging at 10,000 rpm for 1 min. Cell pellet was resuspended in Buffer P1 (250 µl, with RNase A). This mixture was first mixed with Buffer P2 (250 µl) and then mixed with Buffer P3 (350 µl). After turning into a homogeneous colorless suspension, the mixture was centrifuged for 10 min at 13,000 rpm. The supernatant was
transferred to the QIAprep spin column. After centrifuging, the column was washed with Buffer PB (0.5 ml) and Buffer PE (0.75 ml). Finally, Buffer EB (50 µl) was added to elute the plasmids.

**DNA sequencing and sequence analysis**

Plasmids containing genes or promoters were validated by sequencing. Sequences were analyzed and aligned with the software Vector NTI Suite (v.6.0, InfoMax Inc.) according to the manual of the software. After importing the sequences of interest from NCBI Genbank database (http://www.ncbi.nlm.nih.gov/genbank/) into the software, all the restriction enzymes’ cutting sites and other sequence elements (including 3’-UTR, 5’-UTR, coding regions, poly A tails and so on) could be viewed under the Analyze function of the software. Once the DNA has been sequenced, the sequencing results can also been imported into the software. By using the Align function of the software, the correction of the cloning can be checked.

**4.2.9 Growth Kinetics of Gene-overexpression Strains in Response to Apidaecin IB Challenge**

After verifying the DNA sequence, plasmid DNA was transformed into *E. coli* BL21 (DE3) cells. Expression of the two proteins (FtsH and LpxC) is induced by the addition of IPTG (1 mM) for 3 hr. which provides a tightly regulated bacterial expression system. Cell suspensions were then diluted to obtain a concentration of 5×10⁵ CFU/ml and incubated without and with ¹/₁₀ MIC of apidaecin IB. Cell growth was checked by measuring OD₆₀₀ in interval of 1 hr.
4.2.10 Statistical Analysis

Statistical analysis was performed using the same procedure as described in Section 3.2.10 in Chapter 3.

4.3 Results

4.3.1 Membrane Proteins Altered in Response to Apidaecin IB Challenge

To investigate how bacterial membrane proteins changed in response to apidaecin IB challenge, IM and OM proteins were extracted and identified by iTRAQ-coupled 2D LC-MS/MS analysis, respectively. Combining three independent experiments, a total of 75 distinct membrane proteins, including 38 IM proteins, 28 OM proteins, and 9 M proteins (M means whether the protein is found in or associated with the inner or outer cell membrane is unknown), were identified. The Unused ProtScore of those proteins was more than 2 which corresponds to more than 99% confidence. Among them, 5 IM proteins, 1 OM protein and 2 M proteins showed significant changes ($p<0.05$), and the trend of the changes in 2 hr-apidaecin IB-incubated cells was in accordance with that in 1 hr-apidaecin IB-incubated cells (Table 4.2).
Table 4.2 Membrane proteins of *E. coli* altered in response to apidaecin IB challenge

<table>
<thead>
<tr>
<th>Accession Number</th>
<th>Protein Name</th>
<th>Unused&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cov&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>Avg A:C (±S.D.)&lt;sup&gt;c&lt;/sup&gt; 1 h</th>
<th>Avg A:C (±S.D.)&lt;sup&gt;c&lt;/sup&gt; 2 h</th>
<th>Function</th>
<th>Subcellular Location&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0AAI3</td>
<td>Cell division protease ftsH</td>
<td>18.03</td>
<td>53.26</td>
<td>1.67±0.16</td>
<td>2.23±0.37</td>
<td>Metalloprotease</td>
<td>IM</td>
</tr>
<tr>
<td>P31224</td>
<td>Acriflavine resistance protein B</td>
<td>6.54</td>
<td>29.17</td>
<td>0.87±0.02</td>
<td>0.77±0.01</td>
<td>Drug efflux</td>
<td>IM</td>
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<td>P09127</td>
<td>Putative uroporphyrinogen-III C-methyltransferase</td>
<td>6</td>
<td>27.23</td>
<td>1.14±0.14</td>
<td>1.44±0.01</td>
<td>Porphyrin biosynthesis</td>
<td>IM</td>
</tr>
<tr>
<td>P20966</td>
<td>PTS system fructose-specific EIIBC component</td>
<td>5.8</td>
<td>28.6</td>
<td>0.87±0.04</td>
<td>0.64±0.00</td>
<td>Fructose transport</td>
<td>IM</td>
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<tr>
<td>P15877</td>
<td>Quinoprotein dehydrogenase</td>
<td>3.56</td>
<td>27.39</td>
<td>1.45±0.01</td>
<td>1.53±0.09</td>
<td>Energy conservation</td>
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<td>P0A935</td>
<td>Membrane-bound lytic murein transglycosylase A</td>
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<td>1.17±0.07</td>
<td>Murein degradation</td>
<td>OM</td>
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<tr>
<td>P0ADA5</td>
<td>Uncharacterized lipoprotein yajG</td>
<td>9.68</td>
<td>53.13</td>
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<td>1.66±0.25</td>
<td>Unknown</td>
<td>M</td>
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<tr>
<td>P11557</td>
<td>Protein damX</td>
<td>6</td>
<td>25.23</td>
<td>1.00±0.01</td>
<td>1.27±0.06</td>
<td>Interferes with cell division</td>
<td>M</td>
</tr>
</tbody>
</table>

<sup>a</sup> Unused is Unused ProtScore, a measure of the protein confidence for a detected protein, calculated from the peptide confidence for peptides from spectra that are not already completely "used" by higher scoring winning proteins. A protein with Unused ProtScore of 2.0, which corresponds to 99% confidence, is generally true.  
<sup>b</sup> The percentage of matching amino acids from identified peptides having confidence greater than 0, divided by the total number of amino acids in the sequence.  
<sup>c</sup> The ratio of proteins in the apidaecin IB-incubated cells relative to in the control cell.  
<sup>d</sup> Subcellular location: IM, inner membrane; OM, Outer membrane; M, membrane with whether the protein is found in or associated with the inner or outer cell membrane is not known.
4.3.2 Western Blot Analysis on FtsH and LpxC

Western blot analysis was then performed to validate the change in the level of FtsH identified by LC-MS/MS analysis. Equal amount of cell lysates from apidaecin IB-incubated cells and control cells were tested with antibodies to FtsH respectively. Results shown in Figure 4.2 A indicated that the level of FtsH was increased in both 1 hr and 2 hr-apidaecin-incubated cells, and the increase was greater in 2 hr-apidaecin-incubated cells.

The level of cytoplasmic protein LpxC was also analyzed in both apidaecin IB-incubated cells and control cells. Results showed that no significant changes occurred in the amount of LpxC in cells incubated with apidaecin IB for 1 hr; however, the amount of LpxC markedly decreased in cells incubated with apidaecin IB for 2 hr (Figure 4.2 B).

**Figure 4.2** Western blot analysis of FtsH (A) and LpxC (B) in *E. coli* incubated with apidaecin IB.
4.3.3 LPS and Phospholipids Analysis

LPS and phospholipids in cell membranes were extracted, and their amounts were analyzed by KDO assay [157] and phosphate assay respectively. Results shown in Figure 4.3 indicated that no significant changes occurred in the amount of LPS and phospholipids in cells incubated with apidaecin IB for 1 hr; however, the amount of LPS and phospholipids was markedly decreased and increased respectively in cells incubated with apidaecin IB for 2 hr.

Figure 4.3 Analysis of LPS (A) and phospholipids (B) in *E. coli* incubated with apidaecin IB. LPS and phospholipids were determined by measuring KDO and phosphate respectively. Values for cells without incubation of apidaecin IB were adjusted to 1 and values for those incubated with apidaecin IB were normalized accordingly. Asterisk indicates \( p<0.05 \).
4.3.4 Growth Kinetics of Gene-overexpression Strains

ftsH or lpxC was cloned into a pET-24a vector and expressed in E. coli BL21 (DE3) cells. The expression of these two genes was validated by western blot analysis (Figure 4.4). The growth of the cells containing pET-24a, pET-24a/ftsH or pET-24a/lpxC plasmid was then checked separately. The results shown in Figure 4.5 indicated that overexpression of FtsH enhanced the inhibition effect of apidaecin IB on cells; in contrast, overexpression of LpxC significantly alleviated this effect.

Figure 4.4 Validation the expression of FtsH (A) and LpxC (B) by western blot analysis. Lane 1, mock transformation; lane 2, transformation of empty plasmid; and lane 3, transformation of plasmid that permits controlled expression of FtsH or LpxC.
Figure 4.5 Effect of FtsH and LpxC overexpression on the growth of *E. coli* incubated with apidaecin IB. (A) Growth curve of *E. coli* separately harboring pET-24a, pET-24a/ftsH and pET-24a/lpxC plasmids without apidaecin IB incubation following IPTG induction. (B) Growth curve of *E. coli* separately harboring pET-24a, pET-24a/ftsH and pET-24a/lpxC plasmids with apidaecin IB incubation following IPTG induction. (C) $\text{OD}_{600}$ ratio from 6 hr of cultures. $\text{OD}_{600}$ ratio of *E. coli* harboring pET-24a plasmid was adjusted to 1 and those of cells harboring pET-24a/ftsH and pET-24a/lpxC plasmids were normalized accordingly. Asterisk indicates $p<0.05$. 
4.4 Discussion

Membrane proteins are attached to, or associated with the inner or outer bacterial membrane. In order to isolate these proteins, membranes (include both IM and OM) were firstly enriched by ultracentrifuge, proteins of the different membranes were then separately isolated by using two different detergents and analyzed by iTRAQ-coupled 2-D LC–MS/MS. A total of 5 IM proteins, 1 OM protein and 2 M proteins showed differential changes, and the trend of the changes in 2 hr-apidaecin IB-incubated cells was in accordance with that in 1 hr-apidaecin IB-incubated cells (Table 4.2). One of the altered membrane proteins, cell division protease ftsH, captured our attention. The level of FtsH was increased in both 1 hr and 2 hr-apidaecin-incubated cells, with the increase in the latter greater than that in the former (Table 4.3). The change in FtsH was further validated by western blot analysis (Figure 4.2).

Gram-negative bacteria have two membranes—IM and OM. The IM is a phospholipid bilayer, and the OM is an asymmetrical bilayer consisting of phospholipids and LPS in the inner and outer leaflet, respectively. The synthesis of LPS and phospholipids must be properly balanced, which is critical for cell viability. The same reaction precursor (R-3-hydroxymyristoyl-ACP) is used by LpxC for the biosynthesis of the lipid A moiety of LPS (LpxC catalyzes the first committed step) and by (3R)-hydroxymyristoyl-[acyl-carrier-protein] dehydratase (FabZ) for the synthesis of fatty acid (Figure 4.6) ([160-165]). Thus the balance of these enzymes is important to maintain a proper LPS/phospholipids ratio. FtsH is
the sole, ATP-dependent, growth-essential protease of *E. coli* [166]. Its essentiality lies in its function in keeping a proper LpxC/FabZ ratio by degrading LpxC ([167, 168]. The increase in FtsH in this study would probably lead to the change in the cellular level of LpxC. We therefore analyzed LpxC by Western blotting. The results showed no significant changes in the amount of LpxC in cells incubated with apidaecin IB for 1 hr; however, the amount of LpxC was markedly decreased in cells incubated with apidaecin IB for 2 hr (Figure 4.2). We then did LPS and phospholipids analysis. The results showed that the amount of LPS was markedly decreased in cells incubated with apidaecin IB for 2 hr; in contrast, the amount of phospholipids was significantly increased (Figure 4.3).

![Figure 4.6 Schematic representations of biosynthetic pathways of membrane lipid components. Functions of FtsH in the regulation of biosynthesis of LPS and phospholipids are drawn. ACP, acyl carrier protein; GlcNAc, N-acetylg glucosamine; and GlcN, glucosamine.](image)

Moreover, we investigated the characterization of FtsH and LpxC in
response to apidaecin IB challenge by using gene-overexpression strains. *ftsH* or *lpxC* was separately cloned into a pET-24a vector and expressed in *E. coli* BL21 (DE3) cells. Cells containing the pET-24a, pET-24a/*ftsH* or pET-24a/*lpxC* plasmid were then separately cultured in LB-kanamycin medium. After IPTG induction for 3 hr, cell suspensions were diluted to obtain a concentration of $5 \times 10^5$ CFUs/ml and then incubated without and with $1/10$ MIC of apidaecin IB. Cell growth was checked by measuring OD$_{600}$ in interval of 1 hr (Figure 4.7 A, B). The inhibition on the bacteria separately harboring pET-24a, pET-24a/*ftsH* or pET-24a/*lpxC* was obtained by comparing the OD$_{600}$ of the 6 hr cultures. The results showed that overexpression of FtsH enhanced the inhibition effect of apidaecin IB on cells; in contrast, overexpression of LpxC significantly alleviated this effect (Figure 4.7 C). Cellular proteins of the 6 hr cultures were also isolated; the LpxC level in ftsH overexpression cells was further analyzed by western blotting. The results indicated that only with the incubation of apidaecin IB, the overexpression of ftsH can cause the decrease in the cellular level of LpxC (Figure 4.8). Collectively, the data suggested that apidaecin IB acted against *E. coli* by overexpressing FtsH to intensify the degradation of LpxC. As R-3-hydroxymyristoyl-ACP is used by both LpxC for the synthesis of LPS and by FabZ for the synthesis of phospholipids, the over-degradation of LpxC left more R-3-hydroxymyristoyl-ACP to FabZ, and ultimately led to an unbalanced LPS phospholipids ratio and the loss of membrane lipid homeostasis.
Apidaecins and other short proline-rich ABPs attract particular interest because of their special antibacterial mechanism that is non-membrane-permeabilizing [24]. They can translocate across cell membrane, penetrate into the cytoplasm, and target essential cellular processes to mediate cell death [24]. Previous studies on the antibacterial mechanism of apidaecins and other short proline-rich ABPs identified that they killed bacteria by inhibiting heat shock protein DnaK’s two major functions: the ATPase activity and refolding of misfolded proteins [21]. However, it is possible that these peptides inactivate bacteria by other mechanisms. Overproduction of FtsH and the resulting intensified degradation of LpxC in response to apidaecin challenge found in this study could be involved in a new antibacterial mechanism of apidaecins. However, further studies are required to identify the reason of the increase in FtsH and other specific targets of FtsH (i.e. excepting LpxC) to fully understand this new mechanism.

**Figure 4.7** Effect of FtsH overexpression on the production of LpxC in *E. coli* incubated with apidaecin IB.
Chapter 5: LC-MS/MS Analysis of Cytoplasmic Protein Profile of *E. coli* in Response to HNP-1 Challenge

*(Part of this section was from our paper published in Rapid Commun. Mass Spec. 24(18):2787-90. Permission to use the article in this dissertation was obtained from publisher.)*

5.1 Introduction

HNP-1, a 30 amino-acid, β-sheet antibacterial peptide found in human neutrophils [26, 27], is another representative of non-membrane-permeabilizing ABPs. It has activity against bacteria (Gram-positive and Gram-negative) [28], yeast [29, 30] and enveloped viruses [31].

In this chapter, the iTRAQ-coupled LC-MS/MS-based quantitative protein profiling platform established in chapter 3 was utilized to analyze the cytoplasmic protein profile of *E. coli* in response to HNP-1 challenge. Our data indicated that levels of a number of glycolytic enzymes were decreased; in contrast, levels of several proteins were significantly increased, and these proteins were probably involved in a compensatory response to the suppression effect.
5.2 Materials and Methods

5.2.1 Bacterial Strain and Culture

5.2.2 MIC Assay

5.2.3 Growth Kinetics of *E. coli* Incubated with HNP-1

5.2.4 Cytoplasmic Protein Isolation

5.2.5 iTRAQ Labeling

Section 5.2.1 to 5.2.5 was performed using the same procedure as described in Section 3.2.1 to 3.2.5 in Chapter 3.

5.2.6 LC-MS/MS Analysis

5.2.7 Data Analysis

Section 5.2.6 to 5.2.7 was performed using the same procedure as described in Section 4.2.4 to 4.2.5 in Chapter 4.

5.2.8 RNA Extraction and Quantification

Total RNA of *E. coli* cells was isolated using RNeasy Mini kit (QIAGEN, Germany) by following the manufacturer’s protocol. Briefly, cell pellet was dissolved in Buffer RTL. After mixing with 1 volume of 70% ethanol, the mixture was transferred to RNeasy mini column and centrifuged. The column was then washed with Buffer Rw1 and Buffer RPE. Finally, RNA was eluted from the column by RNase-free water. The ratio of the readings at 260 nm and 280 nm (\(A_{260}/A_{280}\)) provides an estimate of the purity of RNA.
with respect to contaminants that absorb UV, such as protein. Pure RNA has an $A_{260}/A_{280}$ ratio of 1.8-2.0. RNA concentration was calculated according to the formula below, as an $A_{260}$ value of 1 (with a 1 cm detection path) corresponds to 40 µg DNA per ml water. RNA concentration (µg/ml) = $A_{260} \times 40 \times$ dilution factor.

### 5.2.9 Real-time RT-PCR

Real-time RT-PCR was performed by utilizing IQ5 multicolor realtime PCR detection system (Bio-Rad Laboratories, Inc., USA) with iScript Onestep RT-PCR kit (Bio-Rad Laboratories, Inc., USA). Primer sequences used were shown in Table 3.1. Reaction mixtures were initially incubated for 10 min at 50 °C and 5 min at 95 °C, followed by 40 cycles of 10 sec at 95 °C and 30 sec at 60 °C. The disassociation analysis was routinely carried out by acquiring fluorescent readings for 1 °C increases from 55 to 95 °C. Microsoft Excel formatted data including amplification analysis, experimental report, melting curve analysis and threshold cycle number were provided automatically by IQ5 optical system software version 2.0 (Bio-Rad Laboratories, Inc., USA). The fold changes were calculated using the following formula: Sample $\Delta Ct = Ct_{\text{sample}} - Ct_{\text{rsD}}$; $\Delta \Delta Ct = \text{Sample} \Delta Ct - \text{control} \Delta Ct$; the fold of sample versus control = $2^{-\Delta \Delta Ct}$. 
Table 5.1 Primers used for real-time RT-PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense primer sequence (5’-3’)</th>
<th>Antisense primer sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
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<td>pfkA</td>
<td>TATTTATGACGGCTATCTGGG</td>
<td>CGCTCTACAACGGTGCT</td>
</tr>
<tr>
<td>gapA</td>
<td>CGGCTAACC TGA AAT GGG</td>
<td>CGG TAG AGG ACG GGA TGA</td>
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<td>eno</td>
<td>CTG TGG AAG CCG AAG T</td>
<td>CCA CCG TTG ATG ATG TT</td>
</tr>
<tr>
<td>pgk</td>
<td>GCG AGA AGA AAG ACG ACG</td>
<td>ACC AAC GAT AGC CAC CAT</td>
</tr>
<tr>
<td>pykF</td>
<td>ATC GGC AAC AGC GAA ATG</td>
<td>CTG GCA GAG CAA TGG AAA</td>
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<td>rrsG</td>
<td>TCAAGGGCAACACCTCCAAGTC</td>
<td>GGTGTAGCGGTGAAATGCGTAG</td>
</tr>
</tbody>
</table>

5.2.10 ATP assay

The cellular ATP level was measured by using a colorimetric assay kit (BioVision, Inc., USA) according to the manufacturer’s instructions. Briefly, cells were lysed in ATP assay buffer. After centrifuging at 15,000 × g for 2 min, supernatant was collected and added to a 96-well plate. Then, 50 µl of reaction mixture was added into each well. After 30 min in the dark, the plate was read at 570 nm wavelength on a microplate reader (Benchmark Plus, USA).

5.2.11 Statistical Analysis

Statistical analysis was performed using the same procedure as described in Section 3.2.10 in Chapter 3.
5.3 Results

5.3.1 MIC of HNP-1

MIC of HNP-1 was evaluated by using the same procedure as described in Section 3.2.2. HNP-1 showed obvious antibacterial activity and its MIC was found to be 25 µg/ml, which was slightly lower than that of apidaecin IB.

5.3.2 Growth Kinetics of E. coli in Response to HNP-1 Challenge

The growth kinetics of cells was subsequently assayed in the presence of $\frac{1}{10}$ MIC of HNP-1. Compared to no HNP-1 control, $\frac{1}{10}$ MIC of HNP-1 decreased E. coli growth starting at 2 hr post-inoculation (Figure 5.1).

![Figure 5.1](image)

**Figure 5.1** Growth kinetics of E. coli incubated with $\frac{1}{10}$ MIC HNP-1. Each value represents the mean optical density (OD) readings from two cultures.
5.3.3 Cytoplasmic Proteins Altered in Response to HNP-1 Challenge

To establish the biological difference between HNP-1-incubated cells and control cells, the protein profiles in *E. coli* cells incubated with and without HNP-1 were analyzed. Proteins from cells were collected, lysed, and labeled prior to 2D LC-MS/MS. Combining three independent experiments, a total of 313 and 396 distinct proteins were identified in cells incubated with HNP-1 for 1 hr and 2 hr based on the criterion that the Unused ProtScore of these proteins was more than 2, which corresponds to more than 99% confidence.

Among the identified 313 and 396 distinct proteins, 20 and 35 proteins, respectively, displayed significant changes (*p*<0.05, Table 5.2 and 5.3). These proteins were subsequently categorized into four groups according to their cellular functions: carbohydrate metabolism, defense, amino acids and protein metabolism, and nucleoside metabolism. All information about the 313 and 396 proteins was shown in Appendix 2.
Table 5.2 Altered cytoplasmic proteins of *E. coli* incubated with HNP-1 for 1 hr.

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Protein Name</th>
<th>Unused&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cov&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>Avg. H:C&lt;sup&gt;c&lt;/sup&gt; (± SD)</th>
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<tr>
<td><strong>Carbohydrate metabolism</strong></td>
<td></td>
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<td>P0AFG8</td>
<td>Pyruvate dehydrogenase component</td>
<td>E1</td>
<td>51.3</td>
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<td>P09373</td>
<td>Formate acetyltransferase 1</td>
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<td>36.1</td>
<td>58.4</td>
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<td>P0A9B2</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase A</td>
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<td>29.0</td>
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<td>P0A6P9</td>
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<td>Aldehyde-alcohol dehydrogenase</td>
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<td>26.3</td>
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<td>Aconitate hydratase 2</td>
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<td>8.05</td>
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<td>20.3</td>
<td>0.81 ± 0.10</td>
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<td>59.5</td>
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<td>14.1</td>
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<td>50S ribosomal protein L29</td>
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<td>5.4</td>
<td>79.4</td>
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<sup>a</sup> Unused is Unused ProtScore, a measure of the protein confidence for a detected protein, calculated from the peptide confidence for peptides from spectra that are not
already completely “used” by higher scoring winning proteins. A protein with Unused ProtScore of 2.0, which corresponds to 99% confidence, is generally true. The percentage of matching amino acids from identified peptides having confidence greater than 0, divided by the total number of amino acids in the sequence. The ratio of proteins in the apidaecin IB-incubated cells relative to in the control cell.
Table 5.3 Altered cytoplasmic proteins of *E. coli* incubated with HNP-1 for 2 hr.

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Protein Name</th>
<th>Unused (^a) (%)</th>
<th>Cov(^b) (%)</th>
<th>avg. H:C (^c) (± SD)</th>
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</thead>
<tbody>
<tr>
<td><strong>Carbohydrate metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P0AFG8</td>
<td>Pyruvate dehydrogenase E1 component</td>
<td>61.9</td>
<td>70</td>
<td>1.25 ± 0.01</td>
</tr>
<tr>
<td>P09373</td>
<td>Formate acetyltransferase 1</td>
<td>62.2</td>
<td>61.2</td>
<td>0.50 ± 0.09</td>
</tr>
<tr>
<td>P0A9Q7</td>
<td>Aldehyde-alcohol dehydrogenase</td>
<td>45.2</td>
<td>60.5</td>
<td>0.62 ± 0.14</td>
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<td>P0A9B2</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase A</td>
<td>35.6</td>
<td>78.2</td>
<td>0.35 ± 0.11</td>
</tr>
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<td>P0A6P9</td>
<td>Enolase</td>
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<td>75.7</td>
<td>0.45 ± 0.25</td>
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<td>Phosphoglycerate kinase</td>
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<tr>
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<td>46.1</td>
<td>0.49 ± 0.08</td>
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<tr>
<td>P0A9M8</td>
<td>Phosphate acetyltransferase</td>
<td>26.4</td>
<td>51.5</td>
<td>0.61 ± 0.01</td>
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<tr>
<td>P36683</td>
<td>Aconitate hydratase 2</td>
<td>24.7</td>
<td>42.5</td>
<td>1.81 ± 0.13</td>
</tr>
<tr>
<td>P0A6A3</td>
<td>Acetate kinase</td>
<td>24.3</td>
<td>67.5</td>
<td>0.67 ± 0.04</td>
</tr>
<tr>
<td>P08200</td>
<td>Isocitrate dehydrogenase</td>
<td>22.0</td>
<td>44.2</td>
<td>1.62 ± 0.15</td>
</tr>
<tr>
<td>P0AD61</td>
<td>Pyruvate kinase I</td>
<td>17.4</td>
<td>63.2</td>
<td>0.71 ± 0.07</td>
</tr>
<tr>
<td>P0A6T1</td>
<td>Glucose-6-phosphate isomerase</td>
<td>16.0</td>
<td>38.3</td>
<td>0.62 ± 0.07</td>
</tr>
<tr>
<td>P0AFG6</td>
<td>Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex</td>
<td>13.3</td>
<td>45.4</td>
<td>2.09 ± 0.18</td>
</tr>
<tr>
<td>P0A858</td>
<td>Triosephosphate isomerase</td>
<td>8.7</td>
<td>49.8</td>
<td>0.47 ± 0.04</td>
</tr>
<tr>
<td>P0A796</td>
<td>6-phosphofructokinase isozyme</td>
<td>13.54</td>
<td>25.6</td>
<td>0.84 ± 0.03</td>
</tr>
<tr>
<td><strong>Defense</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>0AE08</td>
<td>Alkyl hydroperoxide reductase subunit C</td>
<td>20.1</td>
<td>75.4</td>
<td>0.44 ± 0.12</td>
</tr>
<tr>
<td>P0A862</td>
<td>Thiol peroxidase</td>
<td>13.0</td>
<td>58.9</td>
<td>1.76 ± 0.11</td>
</tr>
<tr>
<td><strong>Amino acid and protein metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P0A6N1</td>
<td>Elongation factor Tu</td>
<td>89.9</td>
<td>85.3</td>
<td>1.21 ± 0.11</td>
</tr>
<tr>
<td>P0AC38</td>
<td>Aspartate ammonia-lyase</td>
<td>37.1</td>
<td>65.7</td>
<td>0.31 ± 0.12</td>
</tr>
<tr>
<td>Accession No.</td>
<td>Protein Name</td>
<td>Unused^a</td>
<td>Cov^b (%)</td>
<td>avg. H:C^c (± SD)</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
<td>----------</td>
<td>-----------</td>
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</tr>
<tr>
<td>P0A853</td>
<td>Tryptophanase</td>
<td>26.5</td>
<td>55.4</td>
<td>0.73 ± 0.07</td>
</tr>
<tr>
<td>P0A6P1</td>
<td>Elongation factor Ts</td>
<td>24.7</td>
<td>72.8</td>
<td>1.40 ± 0.06</td>
</tr>
<tr>
<td>P15288</td>
<td>Aminoacyl-histidine dipeptidase</td>
<td>13.5</td>
<td>43.9</td>
<td>0.50 ± 0.02</td>
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<tr>
<td>P00805</td>
<td>L-asparaginase 2 precursor</td>
<td>10.2</td>
<td>36.2</td>
<td>0.52 ± 0.07</td>
</tr>
<tr>
<td>P0A9H3</td>
<td>Lysine decarboxylase, inducible</td>
<td>8.7</td>
<td>29.1</td>
<td>0.28 ± 0.10</td>
</tr>
<tr>
<td>P0AG67</td>
<td>30S ribosomal protein S1</td>
<td>52.6</td>
<td>71.5</td>
<td>1.31 ± 0.05</td>
</tr>
<tr>
<td>P0A7L0</td>
<td>50S ribosomal protein L1</td>
<td>29.3</td>
<td>85.0</td>
<td>1.35 ± 0.23</td>
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<tr>
<td>P62399</td>
<td>50S ribosomal protein L5</td>
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<td>56.4</td>
<td>2.04 ± 0.48</td>
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<td>P60422</td>
<td>50S ribosomal protein L2</td>
<td>21.2</td>
<td>61.5</td>
<td>1.28 ± 0.13</td>
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<td>P0A7V3</td>
<td>30S ribosomal protein S3</td>
<td>19.7</td>
<td>54.5</td>
<td>1.43 ± 0.20</td>
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<td>P02359</td>
<td>30S ribosomal protein S7</td>
<td>18.3</td>
<td>77.7</td>
<td>1.31 ± 0.10</td>
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**Nucleoside metabolism**

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<th>Accession No.</th>
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<th>Cov^b (%)</th>
<th>avg. H:C^c (± SD)</th>
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</thead>
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<tr>
<td>P0A763</td>
<td>Nucleoside diphosphate kinase</td>
<td>6.2</td>
<td>60.8</td>
<td>2.92 ± 0.28</td>
</tr>
</tbody>
</table>

**Others**

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Protein Name</th>
<th>Unused^a</th>
<th>Cov^b (%)</th>
<th>avg. H:C^c (± SD)</th>
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<tbody>
<tr>
<td>P61517</td>
<td>Carbonic anhydrase 2</td>
<td>4.0</td>
<td>17.7</td>
<td>1.42 ± 0.07</td>
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<tr>
<td>P33025</td>
<td>Hypothetical protein yeiN</td>
<td>6.1</td>
<td>29.2</td>
<td>0.37 ± 0.18</td>
</tr>
</tbody>
</table>

^a Unused is Unused ProtScore, a measure of the protein confidence for a detected protein, calculated from the peptide confidence for peptides from spectra that are not already completely “used” by higher scoring winning proteins. A protein with Unused ProtScore of 2.0, which corresponds to 99% confidence, is generally true. ^b The percentage of matching amino acids from identified peptides having confidence greater than 0, divided by the total number of amino acids in the sequence. ^c The ratio of proteins in the apidaecin IB-incubated cells relative to in the control cell.
5.4 Discussion

HNP-1 is the most active α-defensin. It has activities against bacteria (Gram-positive and Gram-negative), yeast, and enveloped viruses. There is an increasing body of evidence indicating that targeting the essential cellular processes, such as inhibition of nucleic acid synthesis, plays an important role in mediating cell death. Further investigations on different aspects of its mechanisms of action are required.

In this study, the iTRAQ-coupled 2-D LC–MS/MS approach was utilized to analyze the cytoplasmic protein profile of E. coli in response to HNP-1 challenge in aim to identify target proteins whose alterations are involved in the antibacterial mechanism of action of this peptide. A total of 313 and 396 distinct proteins were identified in cells incubated with HNP-1 for 1 hr and 2 hr; Among them, 20 and 35 proteins, respectively, displayed significant changes (p<0.05, Table 5.2 and 5.3). A further analysis of these proteins suggested that central metabolism was involved in the response of E. coli to HNP-1 challenge. Particularly, levels of a number of enzymes in glycolysis were decreased, including 6-phosphofructokinase isozyme 1 (PfkA), glyceraldehyde-3-phosphate dehydrogenase A (GapA), phosphoglycerate kinase (Pgk), enolase (Eno), and pyruvate kinase I (PykF). Their levels were found to be reduced by 20–60%. The reduction would result in a lower concentration of pyruvate. At the same time, HNP-1
appeared to cause an increase in pyruvate conversion into isocitrate, which was reflected by the increase in the level of pyruvate dehydrogenase (AceE) and aconitate hydratase 2 (AcnB). The disturbance in the metabolism of pyruvate, an important intermediate of respiration, would lower the metabolism rate of cells. This was strengthened by the results of the ATP assay and the growth kinetics assay. The cellular ATP level was significantly decreased in cells incubated with HNP-1 for 2 hr (Figure 5.2) and the growth of *E. coli* culture started to slow down from 2 hr post-inoculation (Figure 5.1).

![Figure 5.2 ATP level in cells incubated with HNP-1 for 1 hr and 2 hr. Ratios between the HNP-1 incubated and control cells are shown. Asterisks indicate significant difference at \( p < 0.05 \).]
Catabolite repressor/activator (Cra) is a helix-turn-helix DNA-binding protein [169]. It acts as a global regulator of genes encoding enzymes of central carbohydrate metabolism [170]. The unliganded form of Cra binds to the operator regions of target operons, causing either activation or inhibition of transcription. The presence of glucose or other phosphotransferase system sugars produces glycolytic catabolites, such as fructose-1-phosphate, which bind to the Cra protein and cause it to dissociate from the target DNA, resulting in either catabolite repression or catabolite activation. Based on the Regulon DB (http://regulondb.ccg.unam.mx/) database, five of the total nine glycolytic enzymes are known to be under the repressive regulation of the Cra. Interestingly, levels of these five enzymes were all decreased in cells incubated with HNP-1 for both 1hr and 2 hr. The mRNA level of these enzymes was further analyzed. The results shown in Figure 5.3 indicated that the mRNA level of the five enzymes was decreased in cells incubated with HNP-1 for both 1hr and 2 hr, and the trend of the changes was in accordance with that in the protein level. It was thus likely that the transcriptional regulator Cra participated in the cellular response to HNP-1 challenge.
Figure 5.3 mRNA expression analyses of five proteins repressed by transcriptional regulator Cra. pfkA: 6-phosphofructokinase isozyme 1; gapA: glyceraldehyde-3-phosphate dehydrogenase A; pgk: phosphoglycerate kinase; eno: enolase; and pykF: pyruvate kinase. Ratios between the HNP-1 incubated and control cells were shown. Asterisks indicate significant difference at \( p < 0.05 \).

Further studies are still required on the following two aspects: the role of the Cra protein in the response of *E. coli* cells to the challenge of HNP-1; and those proteins whose levels were changed in response to HNP-1 challenge but playing functions other than regulating carbohydrate metabolism.
Chapter 6: Conclusions and Limitations

6.1 Conclusions

To overcome the continuous growth in the emergence of bacterial resistance to current antibiotics, novel antibacterial drugs are in urgent need. Non-membrane-permeabilizing ABPs, which generally exhibit a broad range of activity, act by specific mechanisms other than direct membrane disrupting, do not easily induce antibacterial-drug resistance, are bacteriocidal as opposed to bacteriostatic, and require a short contact time to induce killing [19-21], are believed as excellent candidates for development as novel antibacterial drugs. Systematic and comprehensive understanding of the mechanism of action of this type of ABPs is thus urgently required. Quantitative protein profiling, which attempts to quantitatively compare changes in the level of proteins between two or more experimental conditions, may aid mechanistic studies of ABPs. However, the platform for such studies has not been established. Confronted by this problem, the aim of this Ph.D. project was to develop a global protein profiling platform to analyze changes in the level of proteins in *E. coli* in response to the challenge of apidaecin IB and HNP-1, two representatives of non-membrane-permeabilizing ABPs, and thus improve the understanding of their mechanisms of action.
The first part of this project, which was reported in Chapter 3 and 4, focused on the development of an iTRAQ-coupled LC-MS/MS platform to investigate changes in the global proteome (cytoplasmic and membrane protein profiles) of *E. coli* in response to apidaecin IB challenge. A number of proteins which take essential roles in cellular protein quality control were found to be significantly changed. Levels of GroES and GroEL, which together form the only essential chaperon system in *E. coli* cytoplasm under all growth conditions, were decreased; in contrast, levels of ATP-dependent proteases ClpX and FtsH, which locate in cytoplasm and IM respectively, were increased. The increase in the proteases was probably involved in a compensatory response to the suppression effect. However, the overproduction of FtsH further intensified the degrading of LpxC, an enzyme catalyzing the first committed step in the biosynthesis of the lipid A moiety of LPS. As the same reaction precursor (R-3-hydroxymyristoyl-ACP) is used by LpxC for the biosynthesis of the lipid A moiety of LPS and by FabZ for the synthesis of fatty acid, the reduction in LpxC led to further unbalanced synthesis of LPS and phospholipids and the loss of membrane lipid homeostasis. These findings suggested a new antibacterial mechanism of apidaecin IB. As most of short-proline-rich ABPs show very similar antibacterial activity, this new antibacterial mechanism may also exists in other short-proline-rich ABPs. Proposed mechanisms of action of apidaecin IB are indicated in Figure 6.1.
The iTRAQ-coupled LC-MS/MS platform for investigation of changes in the global proteome of *E. coli* in response to the challenge of ABPs had been established and the flow diagram is presented in Figure 6.2.

**Figure 6.1** Proposed mechanisms of action of apidaecin IB.
Figure 6.2 Flow diagram of the iTRAQ-coupled LC-MS/MS platform established for investigation of changes in the global proteome of *E. coli* in response to the challenge of ABPs.

Chapter 5 analyzed changes in cytoplasmic proteins of *E. coli* in response to HNP-1 challenge by utilizing the platform established in chapter 3. Levels of a number of enzymes in glycolysis were decreased, including PfkA, GapA, Pgk, Eno and PykF; in contrast, levels of enzymes (AceE and AcnB) which regulate the conversion of pyruvate into isocitrate were increased. In concert with the decreasing in cellular ATP and the slowing down in the growth of *E. coli* culture, central metabolism was suggested to be involved in the *E. coli* response to HNP-1 challenge. In addition, all the five glycolytic enzymes, which were found to be decreased, are known to be under the repressive regulation of the Cra protein. It was thus likely that Cra participated in the cellular response of *E. coli* to HNP-1 challenge.
Proposed mechanisms of action of HNP-1 are indicated in Figure 6.3.

Figure 6.3 Proposed mechanisms of action of HNP-1.

In summary, the results presented in this study provide new insights into the antibacterial mechanism of action of apidaecin IB and HNP-1. Although both of the peptides are membrane-permeabilizing ABPs, the protein profiles of *E. coli* in response to them were quite diverse. This is probably because of the differences of the two peptides in the amino acid
composition and/or structure. This study therefore opens the way for the mechanistic studies of all other non-membrane-permeabilizing ABPs with different amino acid composition and/or structure. The identified altered proteins may be used as novel targets for more effective antibacterial intervention.

6.2 Limitations

The prominent limitation lies in the analysis of membrane protein profile reported in Chapter 4. Membrane proteins are of low abundance and easily masked by predominant cytoplasmic proteins. For this reason, bacterial membranes were enriched by alkaline pH washes and ultracentrifuge separation. However, even after enrichment, a high percentage of proteins (about 20%) identified are still not membrane proteins. Moreover, the transmembrane regions of membrane proteins are either $\alpha$-helical or $\beta$-barrels. The $\alpha$-helicals are predominantly present in IM; in contrast, the $\beta$-barrels are found only in OM. Membrane proteins with these diverse structures cannot be fully isolated by using one single detergent. As a result, after enrichment, IM and OM proteins were separated isolated by using two different detergents. However, even by using two detergents, not all membrane proteins were completely solubilized and the total number of membrane proteins identified was still not high (around 100). Further optimization of membrane protein isolation is still required. Proposed
method is to isolate the protein population from membrane as an unresolved band on a 1D SDS-PAGE gel. IM and OM are separately isolated by Sucrose gradient isolation. Samples are then solubilized in strong detergent and concentrated at the interface of the 4% acrylamide stacking gel and the 20% acrylamide resolving gel. Each membrane protein sample for comparison by quantitative proteomics was then confidently excised as a single band, digested in-gel, and labelled with iTRAQ reagents for quantitative 2D LC-MS/MS. The workflow scheme of this method is shown in Figure 6.4. The results from our repeating experiments showed that quantitative comparison of samples processed by this gel-based method versus a typical in-solution digestion method revealed a good improvement in the total number of membrane proteins identified.

Figure 6.4 Proposed protocol workflow of membrane protein isolation.
Chapter 7: Future Work

7.1 Mechanism Studies on Proteins Identified in Proteomic Analysis

In this study, changes in both cytoplasmic and membrane proteins in *E. coli* in response to apidaecin IB challenge were successfully investigated by utilizing an iTARQ-coupled LC-MS/MS technique. Proteins which play essential roles in cellular protein quality control displayed significant changes, including decreases in GroEL and GroES and an increase in FtsH. The increase in FtsH further intensified the degradation of LpxC and thus led to the loss of membrane lipid homeostasis. Further studies on how apidaecin IB leads to the changes in GroEL, GroES and FtsH, and why the overproduction of FtsH causes the intensified degrading of LpxC are needed.

By utilizing the same platform developed in the analysis of protein profile of *E. coli* in response to apidaecin IB challenge, the cytoplasmic protein profile of *E. coli* in response to HNP-1 challenge was subsequently analyzed. Levels of five essential glycolytic enzymes, which are known to be under the repressive regulation of DNA-binding protein Cra, were decreased. Further studies are required to investigate whether the Cra protein is involved in the response of *E. coli* to the challenge of HNP-1.
This can be achieved by adding glycolytic catabolites, such as fructose-1-phosphate, into the culture medium. Fructose-1-phosphate can bind to the Cra protein and cause it to dissociate from the target DNA, resulting in the release of target DNA from the repressive regulation of the Cra. If the decrease in the five essential glycolytic enzymes is alleviated in HNP-1-incubated cells after adding fructose-1-phosphate, the Cra could be believed to participate in the cellular response to HNP-1 challenge. In addition, analysis of membrane protein profile of *E. coli* in response to HNP-1 challenge is needed to acquire the whole picture of changes in the global protein level and fully explore the underlying mechanisms of action.

### 7.2 Protein Profile Analysis on Other Pathogenic Bacteria

Bacterial species can be categorized into two large groups, Gram-positive and Gram-negative, based on the chemical and physical properties of their cell walls. Gram-positive bacterium has a thick peptidoglycan layer where the individual peptidoglycan molecules are cross-linked by pentaglycine chains by a DD-transpeptidase enzyme; In contrast, Gram-negative bacterium has a thin peptidoglycan layer, which is much thinner than in Gram-positive bacteria, and the transpeptidase creates a covalent bond directly between peptidoglycan molecules, with no intervening bridge (Figure 7.1). In addition, Gram-negative bacterium has an outer membrane containing LPS in its outer leaflet and phospholipids in the inner leaflet.
(Figure 7.1). In this project, only one Gram-negative bacterium, *E. coli*, was used as a control for the mechanistic studies of both apidaecin IB and HNP-1. In order to establish the consistency or universality of the bacteriocidal targets identified or mechanisms proposed, it is necessary to repeat the studies on other Gram-negative bacteria such as *Pseudomonas aeruginosa*. Furthermore, as Gram-positive and Gram-negative bacteria have different structures, the antibacterial mechanism of action on Gram-negative bacteria may be different from that on Gram-positive bacteria, it is thus required to repeat the studies on Gram-positive bacteria, such as *Staphylococcus aureus* and *Enterococcus faecalis*, to thoroughly understand the mechanism of action of these two ABPs.

**Figure 7.1** Gram-positive and -negative cell wall structures.
7.3 Protein Profile Analysis on Other Non-Membrane-Permeabilizing ABPs

In this study, the same platform was applied to study the cytoplasmic protein profile of *E. coli* in response to the challenge of either HNP-1 or apidaecin IB. Although both of the peptides are non-membrane-permeabilizing ABPs, the protein profiles of *E. coli* in response to them were quite diverse. This is probably because of the differences of the two peptides in the amino acid composition and/or structure. The platform can thus be expanded into the analysis of protein profile of *E. coli* in response to other representatives of non-membrane-permeabilizing ABPs with different amino acid composition and/or structure, and further improve the understanding of their antibacterial mechanism of action. The proteomic response reference compendium established on all the representative non-membrane-permeabilizing ABPs can be used to rapidly formulate a hypothesis on the target area or mechanism of the newly identified or structurally modified ABPs by means of pattern matching. For example, if the protein profile of *E. coli* in response to the challenge of a newly identified antibacterial peptide matches that in response to apidaecin IB attack, such as decreases in GroEL and GroES and an increase in FtsH, this newly identified peptide probably has the same mechanism of action.
as apidaecin IB. Moreover, the specific altered proteins in response to different representative non-membrane-permeabilizing ABPs can be used as novel targets for more effective antibacterial intervention.
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Appendix

Appendix 1: Cytoplasmic proteins identified in *E. coli* incubated with apidaecin IB

Appendix 2: Cytoplasmic proteins identified in *E. coli* incubated with HNP-1
<table>
<thead>
<tr>
<th>Group</th>
<th>Distinct Peptides</th>
<th>Coverage % AA</th>
<th>1 h Avg A:C</th>
<th>2 h Avg A:C</th>
<th>Database Accession</th>
<th>Protein Name</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>32</td>
<td>59</td>
<td>1.03</td>
<td>1.03</td>
<td>P0A6M8</td>
<td>Elongation factor G OS=Escherichia coli (strain K12) GN=fusA PE=1 SV=2</td>
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<tr>
<td>2</td>
<td>34</td>
<td>45</td>
<td>0.97</td>
<td>0.99</td>
<td>P0AFG8</td>
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<td>3</td>
<td>34</td>
<td>34</td>
<td>0.92</td>
<td>1.24</td>
<td>P0A8T7</td>
<td>DNA-directed RNA polymerase subunit beta OS=Escherichia coli (strain K12) GN=rpoC F</td>
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<td>4</td>
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<td>79</td>
<td>1.04</td>
<td>0.86</td>
<td>P0CE48</td>
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<td>P0A6F5</td>
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<td>7</td>
<td>22</td>
<td>37</td>
<td>0.86</td>
<td>0.48</td>
<td>P09373</td>
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<td>56</td>
<td>1.13</td>
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<tr>
<td>9</td>
<td>20</td>
<td>62</td>
<td>1.12</td>
<td>0.78</td>
<td>P0ABB4</td>
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</tr>
<tr>
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<td>22</td>
<td>38</td>
<td>0.98</td>
<td>1.04</td>
<td>P06959</td>
<td>Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex</td>
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<td>11</td>
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<td>42</td>
<td>0.8</td>
<td>1.16</td>
<td>P0A6Y8</td>
<td>Chaperone protein dnaK OS=Escherichia coli (strain K12) GN=dnaK PE=1 SV=2</td>
</tr>
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<td>19</td>
<td>37</td>
<td>0.97</td>
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<td>0.94</td>
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<td>0.85</td>
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<td>77</td>
<td>1.1</td>
<td>1.02</td>
<td>P0A7V0</td>
<td>30S ribosomal protein S2 OS=Escherichia coli (strain K12) GN=rpsB PE=1 SV=2</td>
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**Legend:**
- OS: Organism
- GN: Gene Name
- PE: Protein Entity
- SV: Structural Variation
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| 30    | 16  | 53.2| 0.95  | P0A7D4|PUR1_ECOLI Adenylosuccinate synthetase (EC 6.3.4.4) (IMP--aspartate ligase) (AdSS) (AMPSynase 
| 31    | 15.8| 79  | 0.99  | P0A7V1|RS5_ECOLI 30S ribosomal protein S5 - Escherichia coli 
| 32    | 15.6| 42.9| 1.04  | P0A8B0|ATPA_ECOLI ATP synthase subunit alpha (EC 3.6.3.14) (ATPase subunit alpha) (ATP synthase F1) 
| 33    | 15.2| 60.5| 1.05  | P0AG55|RL6_ECOLI 50S ribosomal protein L6 - Escherichia coli 
| 34    | 15.2| 60.1| 0.98  | P0A7V3|RS3_ECOLI 30S ribosomal protein S3 - Escherichia coli 
| 35    | 14.9| 61.3| 1.01  | P02359|RS7_ECOLI 30S ribosomal protein S7 - Escherichia coli 
| 36    | 14.7| 72.5| 1.04  | P06959|ODP2_ECOLI Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase 
| 37    | 14.5| 38.4| 1.02  | P0AFF6|NUSA_ECOLI Transcription elongation protein numA (N utilization substance protein A) (L factor) - E 
| 38    | 14.5| 59.9| 0.71  | P0AE08|AHPC_ECOLI Alkyl hydroperoxide reductase subunit C (EC 1.11.1.15) (Peroxisperoxidin) (Thioredoxin 
| 39    | 14.2| 69.5| 0.98  | P0A8S7|LS13_ECOLI 30S ribosomal protein S13 - Escherichia coli 
| 40    | 14.1| 89.3| 2.03  | P0A862|TPX_ECOLI Thiol peroxidase (EC 1.11.1.1) (Scavengase P20) - Escherichia coli 
| 41    | 14.1| 34.6| 0.92  | P15289|PEPD_ECOLI Aminoacyl-histidine dipeptidase (EC 3.4.13.3) (Xaa-His dipeptidase) (X-His dipeptidase 
| 42    | 13.8| 53  | 0.93  | P17169|GLMS_ECOLI Glucosamine--fructose-6-phosphate aminotransferase [isomerizing] (EC 2.6.1.16) (His 
| 43    | 13.7| 63.1| 1.02  | P0A7V8|RS4_ECOLI 30S ribosomal protein S4 - Escherichia coli 
| 44    | 13.5| 54.6| 0.97  | P0A7W7|RS8_ECOLI 30S ribosomal protein S8 - Escherichia coli 
| 45    | 13.3| 48.7| 1.03  | P0AB84|ATPB_ECOLI ATP synthase subunit beta (EC 3.6.3.14) (ATPase subunit beta) (ATP synthase F1) 
| 46    | 13.1| 45.5| 1.02  | P0A9P6|DEAD_ECOLI Cold-shock DEAD box protein A (EC 3.6.1.1) (ATP-dependent RNA helicase deAD) - Escherichia coli 
| 47    | 13.1| 80.9| 1.00  | P06438|RL3_ECOLI 50S ribosomal protein L3 - Escherichia coli 
| 48    | 12.2| 72.2| 0.93  | P0A7A9|IPYR_ECOLI Inorganic pyrophosphatase (EC 3.6.1.1) (Pyrophosphate phospho-hydrolase) (PPase 
| 49    | 12.1| 72.9| 1.31  | P02413|RL15_ECOLI 50S ribosomal protein L15 - Escherichia coli 
| 50    | 12.1| 69.1| 0.92  | P0A87I|ALF_ECOLI Fruacto-bisphosphate aldolase class 2 (EC 4.1.2.13) (Fruacto-bisphosphate aldola 
| 51    | 11.6| 73.2| 1.03  | P0AA20|RL13_ECOLI 50S ribosomal protein L13 - Escherichia coli 
| 52    | 11.1| 61.7| 1.06  | P60723|RL22_ECOLI 50S ribosomal protein L22 - Escherichia coli 
| 53    | 11  | 71.8| 0.95  | P61175|RL22_ECOLI 50S ribosomal protein L22 - Escherichia coli 
| 54    | 11  | 52.1| 0.99  | P16659|SYP_ECOLI Prolyl-tRNA synthetase (EC 6.1.1.15) (Proline--tRNA ligase) (ProRS) (Global RNA sy 
| 55    | 10.8| 32.6| 0.99  | P0AGD3|SODF_ECOLI Superoxide dismutase [Fe] (EC 1.15.1.1) - Escherichia coli 
| 56    | 10.7| 49.1| 0.93  | P0A7J3|RL10_ECOLI 50S ribosomal protein L10 (50S ribosomal protein L8) - Escherichia coli 
| 57    | 10.6| 45.2| 0.94  | P0ABP8|DEOD_ECOLI Purine nucleoside phosphorylase deoD-type (EC 2.4.2.1) (PNP) (Inosine phosphoryl 
| 58    | 10.4| 48.6| 1.09  | P0A870|TALB_ECOLI Transaldolase B (EC 2.2.1.2) - Escherichia coli 
| 59    | 10.4| 63.9| 0.90  | P69783|PTGA_ECOLI Glucose-specific phosphotransferase enzyme II A component (EC 2.7.1.1) (PTS syste 
| 60    | 10.4| 48.8| 0.97  | P0A910|OMPA_ECOLI Outer membrane protein A precursor (Outer membrane protein II*) - Escherichia coli
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**Notes:**
- The table lists various proteins and enzymes associated with Escherichia coli, including transporters, enzymes, and regulatory factors.
- Each entry includes the protein ID and the assigned function or role within the bacterial system.
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**Note:** The table represents a selection of proteins and their associated Enzyme Commission (EC) numbers, along with their abbreviations and corresponding organism (ECOLI). Each entry includes the Protein ID (P0 prefix), the protein name, and the EC number where applicable. The table highlights a variety of enzymes involved in different cellular processes, from metabolism to translation and energy generation.
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Translation initiation factor IF-3 [Contains: IF-3L; IF-3S] - Escherichia coli
Threonine dehydratase catalytic (EC 4.3.1.19) (Threonine deaminase) - Escherichia coli
Pyruvate kinase II (EC 2.7.1.40) (PK-2) - Escherichia coli
Pyruvate formate-lyase 1-activating enzyme (EC 1.97.1.4) (PFL-activating enzyme) (I)
Tryptophanyl-tRNA synthetase (EC 6.1.1.2) (Tryptophan--tRNA ligase) (TrpRS) - Escherichia coli
Phosphoglucomutase (EC 5.4.2.10) - Escherichia coli
Anaerobic C4-dicarboxylate transporter dcuC - Escherichia coli
Glutamate-1-semialdehyde 2,1-aminomutase (EC 5.4.3.8) (GSA) (Glutamate-1-semialdehyde 2,1-aminomutase) - Escherichia coli
Out membrane lipoprotein slyB precursor - Escherichia coli
Protein-export membrane protein secD - Escherichia coli
Methionyl-tRNA synthetase (EC 6.1.1.10) (Methionine--tRNA ligase) (MetRS) - Escherichia coli
Chaperone clpB (Heat-shock protein F84.1) - Escherichia coli
ATP-dependent protease La (EC 3.4.21.53) - Escherichia coli
Phosphoenolpyruvate carboxylase (EC 4.1.1.31) (PEPCase) (PEP) - Escherichia coli
Ribonuclease R (EC 3.1.14.23) (RNase R) (Protein vacB) - Escherichia coli
GTP-binding protein lepA - Escherichia coli
Inner membrane protein yjiY - Escherichia coli
Xaa-Pro dipeptidase (EC 3.4.13.9) (X-Pro dipeptidase) (Proline dipeptidase) (Prolidase) - Escherichia coli
Valyl-tRNA synthetase (EC 6.1.1.9) (Valine--tRNA ligase) (ValRS) - Escherichia coli
ABC transporter ATP-binding protein up - Escherichia coli
Periplasmic beta-glucosidase precursor (EC 3.2.1.21) (Gentiobiose) (Cellobiose) (Bel)
Putative ATP-dependent RNA helicase rhlE (EC 3.6.1.2) - Escherichia coli
2-amino-3-ketobutyrate coenzyme A ligase (EC 2.3.1.29) (AKB ligase) (Glycine acetyltransferase) - Escherichia coli
UPF0234 protein yajQ - Escherichia coli
Hypothetical protein ygdH - Escherichia coli
Protein ydjA - Escherichia coli
Nitrite reductase [NAD(P)H] large subunit (EC 1.7.1.4) - Escherichia coli
Peptide chain release factor 2 (RF-2) - Escherichia coli
Lipoyl synthase (EC 2.8.1.1) (Lipoic acid synthetase) (Lipoate synthetase) (Lipoyl-acetyl-CoA synthetase) - Escherichia coli
Nucleoside permease nupC (Nucleoside-transport system protein nupC) - Escherichia coli
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<td>Alky hydroperoxide reductase subunit C (EC 1.1.1.15) (Peroxiredoxin)</td>
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<td>Lysyl-tRNA synthetase, heat inducible (EC 6.1.11.11) (Serine--tRNA ligase)</td>
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<td>ATP synthase subunit alpha (EC 3.6.3.14) (ATPase subunit alpha)</td>
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<td>Chaperone HtpG (Heat-shock protein)</td>
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<td>P06649</td>
<td>AK (Acetyl-CoA kinase) - Escherichia coli</td>
<td>EC 2.7.2.4</td>
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**Notes:**
- Protein names and EC numbers are based on the listed entries in UniProt.
- The entries are organized by their accession numbers and proteins are listed in alphabetical order.
| P0ABU5|ELBB_ECOLI | Enhancing lycopene biosynthesis protein 2 (Sigma cross-reacting protein) |
| P0AB77|KB1_ECOLI  | 2-amino-3-ketobutyrate coenzyme A ligase (EC 2.3.1.29) (AKB ligase) (C |
| P0AB46|YMGD_ECOLI | Hypothetical protein ymgD precursor - Escherichia coli |
| P0AAD6|SDAC_ECOLI | Serine transporter - Escherichia coli |
| P0A6L0|DEOC_ECOLI | Deoxyribose-phosphate aldolase (EC 4.1.2.4) (Phosphodeoxyriboaldolase) |
| P09323|PTW3C_ECOLI | PTS system N-acetylglucosamine-specific EIIICBA component (EIICBA-N |
| P07004|PROA_ECOLI | Gamma-glutamyl phosphate reductase (EC 1.2.1.41) (GPR) (Glutamate- |
| P06805|PTND_ECOLI | Mannose permease IID component (PTS system mannose-specific EIID |
| P64581|YQJD_ECOLI | Hypothetical protein yqjD - Escherichia coli |
| P63417|YHBS_ECOLI | Hypothetical acetyltransferase yhbS (EC 2.3.1.-) - Escherichia coli |
| P39396|YJIY_ECOLI | Inner membrane protein yjiY - Escherichia coli |
| P39286|ENGHC_ECOLI | Probable GTPase engC precursor (EC 3.6.1.-) - Escherichia coli |
| P37903|USPF_ECOLI | Universal stress protein F - Escherichia coli |
| P36767|RDGC_ECOLI | Recombination-associated protein rdgC - Escherichia coli |
| P28248|DCD_ECOLI | Deoxycytidine triphosphate deaminase (EC 3.5.4.13) (dCTP deaminase) |
| P24169|DCS_ECOLI | Ornithine decarboxylase, inducible (EC 4.1.1.17) - Escherichia coli |
| P23827|ECOT_ECOLI | Ecton precursor - Escherichia coli |
| P0AFU8|RISA_ECOLI | Riboflavin synthase alpha chain (EC 2.5.1.9) - Escherichia coli |
| P0ADW3|YHCB_ECOLI | Putative cytochrome d ubiquinol oxidase subunit III (EC 1.10.3.-) (Cytoch |
| P0ADW6|YGG_ECOLI | Hypothetical protein yggE - Escherichia coli |
| P0ACR3|MPRA_ECOLI | Transcriptional repressor mprA (Protein emrR) - Escherichia coli |
| P0A968|CSPD_ECOLI | Cold shock-like protein cspD (CSP-D) - Escherichia coli |
| P0A610|KYW_ECOLI | Cytidine kinase (EC 2.7.4.14) (CK) (Cytidine monophosphate kinase) (t |
| P09158|SPEE_ECOLI | Spermidine synthase (EC 2.5.1.16) (Putrescine aminopropyltransferase) |
| P07014|DSB_ECOLI | Succinate dehydrogenase iron-sulfur protein (EC 1.3.99.1) - Escherichia |
| P65556|YFD_ECOLI | Putative Nudix hydrolase yfcD (EC 3.6.-.-) - Escherichia coli |
| P39199|YFCB_ECOLI | Hypothetical adenine-specific methylase yfcB (EC 2.1.1.72) - Escherichia |
| P0AE07|FKB_ECOLI | FKB-type 16 kDa peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8) (PPIa |
| P0AEB7|YOAB_ECOLI | UPF0076 protein yoaB - Escherichia coli |
| P0AC62|GLRX3_ECOLI | Glutaredoxin-3 (Grx3) - Escherichia coli |
| P0AC62|GST_ECOLI | Glutathione S-transferase (EC 2.5.1.18) - Escherichia coli |
| P0A912|PAL_ECOLI | Peptidoglycan-associated lipoprotein precursor - Escherichia coli |
|      | 2.0 | 17.4 | 0.80 | P0A7P5|RL34_ECOLI | 50S ribosomal protein L34 - Escherichia coli |
|------|-----|------|------|----------|---------------------------------------------|
| 381  | 2.0 | 17.8 | 0.94 | P0A6T9|GCSH_ECOLI | Glycine cleavage system H protein - Escherichia coli |
| 382  | 2.0 | 20.9 | 1.03 | P0A6E6|ATPE_ECOLI | ATP synthase epsilon chain (EC 3.6.3.14) (ATP synthase F1 sector epsilon) |
| 383  | 2.0 | 31.1 | 0.99 | P0ACE7|YCFF_ECOLI | HIT-like protein ycfF - Escherichia coli |
| 384  | 2.0 | 25.9 | 1.07 | P52061|HAM1_ECOLI | HAM1 protein homolog - Escherichia coli |
| 385  | 2.0 | 18.1 | 1.09 | P0AD10|YECJ_ECOLI | Hypothetical protein yecJ - Escherichia coli |
| 386  | 2.0 | 19.4 | 1.09 | P0AC41|DHSA_ECOLI | Succinate dehydrogenase flavoprotein subunit (EC 1.3.99.1) - Escherichia coli |
| 387  | 2.0 | 16.8 | 0.89 | P04825|AMPN_ECOLI | Aminopeptidase N (EC 3.4.11.2) (Alpha-aminoacylpeptide hydrolase) - E |
| 388  | 2.0 | 28.3 | 0.91 | P07658|FDHF_ECOLI | Formate dehydrogenase H (EC 1.2.1.2) (Formate-hydrogen-lyase-linked) |
| 389  | 2.0 | 20.1 | 1.00 | P0AAA1|YAGU_ECOLI | Inner membrane protein yagU - Escherichia coli |
| 390  | 2.0 | 34.4 | 0.87 | P23830|PSS_ECOLI | CDP-diacylglycerol--serine O-phosphatidyltransferase (EC 2.7.8.8) (Phos |
| 391  | 2.0 | 8.8  | 0.97 | P0A722|LPXA_ECOLI | Acyl-[acyl-carrier-protein]--UDP-N-acetylglucosamine O-acyltransferase |
| 392  | 2.0 | 29.5 | 0.87 | P0A6W9|GSH1_ECOLI | Glutamate--cysteine ligase (EC 6.3.2.2) (Gamma-glutamylcysteine synth |
| 393  | 2.0 | 18.6 | 0.95 | P23843|OPPA_ECOLI | Periplasmic oligopeptide-binding protein precursor - Escherichia coli |
| 394  | 2.0 | 21.6 | 0.89 | P0A884|TYSY_ECOLI | Thymidylate synthase (EC 2.1.1.45) (TS) (TSase) - Escherichia coli |
| 395  | 2.0 | 22.4 | 0.91 | P69503|APT_ECOLI | Adenine phosphoribosyltransferase (EC 2.4.2.7) (APRT) - Escherichia coli |
| 396  | 2.0 | 16.8 | 0.98 | P0A9C3|GALM_ECOLI | Aldose 1-epimerase (EC 5.1.3.3) (Mutarotase) - Escherichia coli |