TOWARDS WHOLE CELL IMMUNOPROTEOME AND SUBPROTEOMES OF *BORDETELLA PERTUSSIS*

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BURCU EMİNE TEFON

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submitted by **BURCU EMİNE TEFON** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Biology Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Musa Doğan _____
Head of Department, **Biology**

Prof. Dr. Gülay Özcengiz
Supervisor, **Biology Dept., METU** _____

Examining Committee Members:

Prof. Dr. Ufuk GÜNDÜZ _____
Biology Dept., METU

Prof. Dr. Gülay Özcengiz _____
Biology Dept., METU

Assoc. Prof. Dr. Mayda Gürsel _____
Biology Dept., METU

Assist. Prof. Dr. Servet Özcan _____
Biology Dept., Erciyes University

Assist. Prof. Dr. Çağdaş Son _____
Biology Dept., METU

Date: _____

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Burcu Emine Tefon

Signature:

ABSTRACT

TOWARDS WHOLE CELL IMMUNOPROTEOME AND SUBPROTEOMES OF *BORDETELLA PERTUSSIS*

Tefon, Burcu Emine
Ph.D., Department of Biology
Supervisor: Prof. Dr. Gülay Özcengiz
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Bordetella pertussis is a gram-negative, human pathogen and etiologic agent of whooping cough (pertussis), a highly contagious, acute respiratory illness. In this study, the analysis of whole immunproteome and subproteomes of this microorganism was performed. The soluble cytoplasmic proteomes of *B. pertussis* Tohama I strain and a local isolate Saadet were separated by 2DE. By Western blot analysis, we identified 25 immunogenic proteins of three categories. In the first group, there were well-known proteins of the pathogen The second group comprised proteins which were already shown antigenic in certain pathogenic bacteria, but not in *B. pertussis* before. The third group of proteins were those which have not been shown to be immunogenic in any pathogen till the present study such as putative chromosome partition protein, preprotein translocase SecA subunit, carbamoyl-phosphate synthase large chain, PRP synthase, putative substrate-CoA ligase, lysyl-tRNA synthetase, fumaryl acetoacetase, putative peptidyl-prolyl cis-trans isomerase, aspartate-semialdehyde dehydrogenase, putative DNA-binding protein and a putative outer membrane protein.

In our surfaceome study, surface proteins of two strains were identified by 2DE followed by MALDI-TOF-MS/MS analysis and also geLC-MS/MS. With these

techniques 45 proteins were identified by 2DE and 226 proteins by geLC-MS/MS. The immunogenicity of surface proteins on 2DE gels were analyzed by Western blotting and among 11 identified immunogenic proteins glutamine-binding periplasmic protein, leu/ile/val-binding protein, one putative exported protein, and iron-superoxide dismutase were found to be immunogenic for the first time in *Bordetella*. It was also found that 16 proteins were differentially expressed in *B. pertussis* Saadet and Tohama I. Five proteins were expressed only in Saadet (adhesin, chaperone protein DnaJ, fimbrial protein FimX, putative secreted protein Bsp22 and putative universal stress protein), and two (ABC transporter substrate-binding protein and a putative binding protein-dependent transport periplasmic protein) only in Tohama I.

In the secretome study, we identified 40 proteins by 2DE and 357 proteins by geLC-MS/MS. It was found that 12 proteins were immunogenic by Western blot analysis and the immunogenicity of putative secreted protein (BP1047) was shown for the first time in this study. In our study, PT subunit 2 and putative outer protein D (BopD) were more abundant in Saadet while one protein, glutamate synthase subunit beta was expressed at a higher level in Tohama I. Four proteins were expressed only in Saadet (two capsular polysaccharide biosynthesis protein, protein FimX and putative outer membrane permeability protein).

The present study comprehensively covered almost the entire proteome of a crucial pathogen, demonstrated many novel antigens and identified hundreds of membrane-bound proteins, cell surface-associated and extracellular proteins. Thus, it is anticipated to greatly aid in a better understanding of pathogen-host relations, rational design of novel drugs and developing new generation vaccines against *B. pertussis*.

Key words: *Bordetella pertussis*, immunoproteomics, 2-DE, MALDI-TOF-MS, Western Blot, geLC-MS/MS

ÖZ

BORDETELLA PERTUSSIS'İN TÜM HÜCRESEL İMMUNOPROTEOMU VE ALTPROTEOMLARININ DETAYLI ANALİZİ

Tefon, Burcu Emine
Doktora, Biyoloji Bölümü
Tez Yöneticisi: Prof. Dr. Gülay Özcengiz
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Bulaşıcı bir akut solunum yolu hastalığı olan boğmaca etkeni gram-negatif bir insan patojeni olan *Bordetella pertussis*'dir. Bu çalışmada, patojenin tüm hücre immunoproteom ve alt proteom analizleri yapılmıştır. *B. pertussis* Tohama ve yerel izolat Saadet suşlarının çözünür sitoplazmik proteomları 2DE tekniği ile ayrılmıştır. Bakterinin, Western blot analizi ile tanımlanan 25 immunojenik proteini 3 grup altında toplanmıştır: İlk gruba patojenin iyi bilinen proteinleri, ikinci gruba immunojenitesi başka bakterilerde daha önce gösterilmiş, ancak *B. pertussis*'te ilk defa gösterilen dahil edilmiştir. Üçüncü grupta, immunojenik aktiviteleri daha önce hiçbir bakteride gösterilmemiş olan putatif kromozom bölümlenme proteini, preprotein translokaz SecA, karbamoyl-fosfat sintaz, fosfoenolpürivat sintaz, putatif substrat-CoA ligaz, lizil-tRNA syntetaz, fumaril asetoasetaz, putatif peptidil-prolil sis-trans isomeraz, aspartat-semialdehit dehidrogenaz, putatif DNA-bağlanma proteini ve putatif dış zar proteini bulunmaktadır.

Yüzey proteomu çalışmamızda, iki suşa ait proteinler 2DE'yi takiben uygulanan MALDI-TOF-MS/MS ve geLC-MS/MS teknikleri kullanılarak tanımlanmıştır. 2DE tekniği ile 45 protein tanımlanırken, geLC-MS/MS tekniği ile 226 protein

tanımlanmıştır. Western blot analizleriyle ise 11 immunojenik protein belirlenmiştir. Bunlardan glutamin-bağlanma periplazmik proteini, leu/ile/val-bağlanma proteini, bir adet putatif salgı proteini ve demir-superoksit dismutaz, *B. pertussis* için immunojeniteleri ilk defa gösterilmiş proteinlerdir. Ayrıca 16 proteinin *B. pertussis* Saadet ve Tohama I suşlarında farklı miktarlarda ifade edildiği tespit edilmiştir. Adhezin, şaperon protein DnaJ, fimbriyal protein FimX, putatif salgı proteini Bsp22 ve putatif evrensel stres proteininin yalnızca Saadet suşunda ifade edildiği, ABC transporter substrat-bağlanma proteini ve putatif bağlanma proteini bağımlı transport periplazmik proteininin ise yalnızca Tohama I suşunda ifade edildiği bulunmuştur.

Sekretom çalışmamızda ise 2DE tekniği kullanılarak 40 protein ve geLC-MS/MS tekniği kullanılarak 357 protein belirlenmiştir. 12 proteinin immunojenik aktivitesi Western blot analiziyle belirlenmiş ve bunlardan putatif salgı proteininin (BP1047) immunojenitesi literatürde ilk defa gösterilmiştir. Bu çalışmada aynı zamanda PT altünite 2'nin ve putatif dış zar proteini D'nin (BopD) ekspresyonunun Saadet suşunda daha fazla olduğu, glutamat sentazın ise Tohama I suşunda daha fazla ifade edildiği tespit edilmiştir. Dört proteinin (iki kapsular polisakkarit biyosentez proteini, FimX ve putatif dış zar geçirgenlik proteini) ise yalnızca Saadet suşunda ifade edilmektedir..

Bu çalışma, önemli bir patojenin hemen tüm proteomunu derinlemesine kapsamış, bir çok yeni antijeni, membrana ve dış yüzeye bağlı ve hücre dışına salgılanan yüzlerce protein tanımlamıştır. Sonuçlarının, *B. pertussis*-konakçı ilişkilerini daha iyi anlamaya, yeni ilaçların rasyonel tasarımına ve yeni kuşak aşıların geliştirilmesine önemli katkıları olacağı öngörülmektedir.

Anahtar sözcükler: *Bordetella pertussis*, immunoproteomik, 2-DE haritası, MALDITOF-MS, Western Blot, , geLC-MS/MS

To my familiy, Cayenne and
all the people who walked this way
along with me

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TABLE OF CONTENTS

ABSTRACT iv
ÖZ vi
ACKNOWLEDGEMENTS ix
LIST OF TABLES xv
TABLES xv
LIST OF FIGURES xvi
CHAPTERS
1. INTRODUCTION 1
1.1. The Genus *Bordetella* 1
1.2. *Bordetella pertussis* 3
1.3. Pertussis (Whooping Cough) 6
1.4. Virulence Factors 8
1.4.1. Filamentous Hemagglutinin (FHA) 9
1.4.2. Fimbriae (FIM) 10
1.4.3. Pertactin (PRN) 10
1.4.4. Serum-resistance Protein (BrkA) 11
1.4.5. Tracheal Colonization Factor (Tcf) 11
1.4.6. Pertussis Toxin (PT) 11
1.4.7. Adenylate Cyclase Toxin (AC) 12
1.4.8. Dermonecrotic Toxin (DNT) 12
1.4.9. Tracheal Cytotoxin (TCT) 13
1.4.10. Lipopolysaccharide (LPS) 13
1.5. The Resurgence of Pertussis 13
1.6. *B. pertussis* Vaccines 14
1.7. Vaccine Manufactured Against *Bordetella pertussis* in Turkey 18
1.8. Proteomics 20

1.9. Proteomic Strategies 21
1.9.1. Bottom-up Proteomics 22
1.9.2. Top-down Proteomics 22
1.10. Immunoproteomics 23
1.11. Analysis of Hydrophobic Membrane Proteins 25
1.12. Steps in Immunoproteomics 25
1.12.1. Sample Preparation and Protein Solubilization 26
1.12.2. Two Dimensional Gel Electrophoresis (2-DE) 26
1.12.3. Protein Detection 27
1.12.4. Computerized Image Analysis 28
1.12.5. Immunoblotting 29
1.12.6. Mass Spectrometry Analysis 30
1.12.6.1. Ion Source 31
1.12.6.2. Mass Analyzers 34
1.13. Aim of the Study 37
2. MATERIALS AND METHODS 38
2.1. Materials 38
2.2. Bacterial Strains 38
2.3. Culture Media and Growth 38
2.4. Preparation of Cytoplasmic Proteomes 39
2.5. Preparation of Outer Membrane Proteins 39
2.6. Preparation of Secreted Proteins 40
2.7. Determination of Protein Concentration 40
2.8. 1D Gel Electrophoresis 41
2.9. 2D Gel Electrophoresis 41
2.10. Preparation of Antisera against *B. pertussis* 42
2.11. Western Blotting of 2-DE gels 42
2.12. Dot Blotting 43
2.13. MALDI-TOF-MS Analysis 43
2.14. LC-MS Analysis 44
2.15. Protein Identification and Database Searches 45

2.16. Relative abundance of surface proteins 45
3. RESULTS AND DISCUSSION 47
3.1. Theoretical Proteome Map of *B. pertussis* 47
3.2. Master Gels of *B. pertussis* Strains 48
3.3. Cytoplasmic Proteomes of *B. pertussis* Tohama and Saadet Strains 55
3.3.1. Identification of Cytoplasmic Immunoreactive Proteins 57
3.3.2. Epitope Prediction, Subcellular Localization and Signal Peptides 59
3.3.3. Evaluation of Cytoplasmic Immunogenic Proteins 63
3.3.3.1. Putative Chromosome Partition Protein 63
3.3.3.2. Heat Shock Protein 70 (Hsp70) 63
3.3.3.3. Preprotein Translocase SecA Subunit 64
3.3.3.4. Carbamoyl-phosphate Synthase Large Chain 64
3.3.3.5. ATP-dependent Protease, ATPase Subunit 65
3.3.3.6. Phosphoenolpyruvate Synthase (PEP Synthase) 65
3.3.3.7. Serum Resistance Protein (BrkA) 66
3.3.3.8. ATP Synthase Subunit B 66
3.3.3.9. 30S Ribosomal Protein S1 66
3.3.3.10. Pertactin 67
3.3.3.11. Putative Substrate-CoA Ligase 67
3.3.3.12. Heat Shock Protein 60 (Hsp60) 68
3.3.3.13. Serine Protease 68
3.3.3.14. Glutamyl-tRNA Amidotransferase Subunit A 69
3.3.3.15. Lysyl-tRNA Synthetase 69
3.3.3.16. S-Adenosylmethionine Synthetase 70
3.3.3.17. Fumaryl acetoacetase 70
3.3.3.18. RNA polymerase α subunit 70
3.3.3.19. Elongation factor Tu (EF-Tu) 71
3.3.3.20. Ketol-acid Reductoisomerase 72
3.3.3.21. Putative Peptidyl-prolyl Cis-trans Isomerase 72
3.3.3.22. Aspartate-semialdehyde Dehydrogenase 73
3.3.3.23. Putative DNA-binding Protein 73

3.3.3.24. Heat Shock Protein 10 (Hsp10) 73
3.3.3.25. Putative Outer Membrane Protein 74
3.4. Surface Proteome 75
3.4.1. Identification of Immunoreactive Proteins 76
3.4.2. Functional Classes, Protein Localization and Signal Peptides 76
3.4.3. Evaluation of Surface Proteins 91
3.4.3.1. Well-known Virulence Factors of *B. pertussis* 91
3.4.3.2. Adhesin 92
3.4.3.3. FimX 92
3.4.3.4. Type III Secretion System (T3SS) Proteins 93
3.4.3.5. Outer Membrane Protein Q (OmpQ) 94
3.4.3.6. Superoxide Dismutase (SOD) 94
3.4.3.7. ATP Synthase Subunit beta 94
3.4.3.8. Periplasmic Binding Proteins 95
3.5. Secretome 96
3.5.1. Identification of Immunoreactive Proteins 97
3.5.2. Functional classes, Protein Localization and Signal Peptides 98
3.5.3. Evaluation of Secreted Proteins 117
3.5.3.1. Putative Outer Protein D (BopD) 117
3.5.3.2. Glutamate Synthase Subunit Beta (GltS subunit β) 118
3.5.3.3. Capsular Polysaccharide Biosynthesis Protein 118
3.5.3.4. Outer Membrane Permeability Protein 119
3.5.3.5. Outer Membrane Porin Protein Precursor 120
3.5.3.6. Virulence-activated Gene 8 (Vag8) 120
4. CONCLUSION 122
REFERENCES 125
APPENDICES 158
A. COLLOIDAL COOMASSIE BLUE (CCB) STAINING 158
B. CHEMICALS AND THEIR SUPPLIERS 160
C. CULTURE MEDIA COMPONENTS 162
CURRICULUM VITAE 164

LIST OF TABLES

TABLES

Table 1.1. Composition of acellular vaccines and combinations from major manufacturers of acellular vaccines (Storsaeter *et al*., 2007). 16
Table 1.2. Current childhood vaccination schedule in Turkey (http://www.euvac.net/graphics/euvac/vaccination/turkey.html). 20
Table 1.3. Characteristic of mass spectrometers which have been commonly used for proteomic studies (Han et al., 2008)..... 36
Table 3.1. Cytoplasmic immunogenic proteins detected in total soluble proteome of *B. pertussis* Tohama I and Saadet strains (Altındiş et al., 2009).....61
Table 3.2. Surface proteins *of B. pertussis* Tohama I and Saadet strains identified by geLC-MS/MS (Tefon et al., 2011)..... 80
Table 3.3. Immunogenic proteins detected in surface proteome of *B. pertussis* Tohama I and Saadet strains (Tefon et al., 2011). 90
Table 3. 4. Secretome proteins *of B. pertussis* Tohama I and Saadet strains identified by geLC-MS/MS. 102
Table 3. 5. Immunogenic proteins detected in secretome of *B. pertussis* Tohama I and Saadet strains..... 116

LIST OF FIGURES

FIGURES

Figure 1. 1. 16S rRNA sequences based phylogenetic relationships of *Bordetella* species (Ko et al., 2005). 1
Figure 1. 2. Gram staining of *B. pertussis*. 4
Figure 1. 3. Scanning electron micrograph of *B. pertussis* cells 4
Figure 1.4. Scanning electron micrograph showing the colonization of *B. pertussis* to ciliated respiratory tract epithelial cells 6
Figure 1. 5. Virulence factors of *B. pertussis*. 9
Figure 1.6. Pertussis incidence in Turkey between the years 1980 and 2010 (WHO) 18
Figure 1.7. Steps in "bottom up" proteomics and "top down" proteomics strategies (Han, 2008). 23
Figure 1.8. Three important components of a mass spectrometer (Graham et al., 2007). 31
Figure 1.9. ESI process (Graham et al., 2007). 32
Figure 1.10. MALDI process (Graham et al., 2007). 33
Figure 3.1. Theoretical proteome gel of *B. pertussis*. pH 3-10 range was indicated by a rectangle. 47
Figure 3.2. 2-D master gel of the cytoplasmic proteome of *B. pertussis* Tohama I. 49
Figure 3.3. 2-D master gel of the cytoplasmic proteome of *B. pertussis* Saadet. 50
Figure 3.4. 2-D master gel of the surface proteins of *B. pertussis* Tohama I. 51
Figure 3.5. 2-D master gel of the surface proteins of *B. pertussis* Saadet. 52
Figure 3.6. 2-D master gel of the secretome of *B. pertussis* Tohama I. 53

Figure 3.7. 2-D master gel of the secretome of *B. pertussis* Saadet..... 54
Figure 3.8. Dual channel 2-D imaging of *B. pertussis* strains Tohama I (green) and Saadet (red) (Altındiş et al., 2009). 56
Figure 3.9. 2-D Western blot analysis of the total soluble proteome of *B. pertussis* strain Tohama I against antiserum Th (sc) (Altındiş et al., 2009). 58
Figure 3. 10. 2-D Western blot analysis of the total soluble proteome of *B. pertussis* strain Saadet against antiserum Sa (sc) (Altındiş et al., 2009)..... 59
Figure 3. 11. Dual channel 2D imaging of *B. pertussis* strains Tohama I (green) and Saadet (red) (Tefon et al., 2011)..... 77
Figure 3. 12. Fused 2D Western blot analysis of the surface proteins of *B. pertussis* strains Tohama and Saadet (Tefon et al., 2011)..... 78
Figure 3.13. Functional classes of the surface proteins identified from *B. pertussis* (Tefon et al., 2011)..... 79
Figure 3.14. Dual channel 2D secretome imaging of *B. pertussis* strains Tohama I (green) and Saadet (red) for secretome. 99
Figure 3.15. Fused 2-D Western blot analysis of the secreted proteins of *B. pertussis* strains Tohama and Saadet..... 100
Figure 3.16. Functional classes of the secreted proteins identified from *B. pertussis*. 101

LIST OF ABBREVIATIONS

2DE	Two Dimensional Gel Electrophoresis
B. pertussis	*Bordetella pertussis*
BrkA	Serum Resistance Protein
ESI	Electrospray Ionization
FHA	Filamentous Hemagglutinin
LC	Liquid Chromatography
IEF	Isoelectric Focusin
kDa	kilo Dalton
MALDI-TOF	Matrix-assisted Laser Desorption Ionization Time of Flight
MS	Mass Spectrometry
p*I*	Isoelectric Point
PT	Pertussis Toxin
PRN	Pertactin
SDS	Sodium Dodecyl Sulfate

CHAPTER I

INTRODUCTION

1.1. The Genus *Bordetella*

Bordetella is a genus that belongs to the phylum proteobacteria. The members of this genus are Gram-negative, small coccobacilli which are obligate aerobes. It currently consists of ten species with the new identified one, *Bordetella ansorpii*, *B. pertussis*, *B. parapertussis*$_{ov}$ (ovine-adapted), *B. parapertussis*$_{hu}$ (human-adapted), *B. bronchiseptica*, *B. avium*, *B. hinzii*, *B. trematum*, *B. petrii*, *B. holmesii* (Figure 1.1) (Ko et al., 2005).

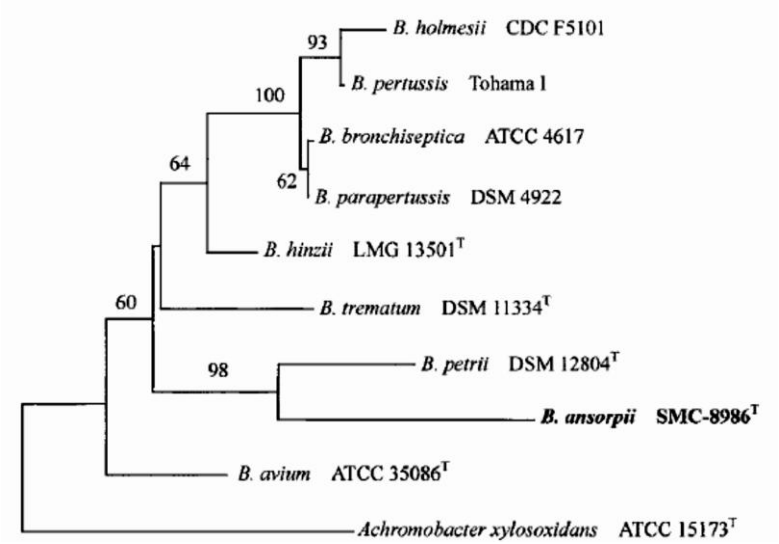

Figure 1. 1. 16S rRNA sequences based phylogenetic relationships of *Bordetella* species (Ko et al., 2005).

The GC content of these bacteria is in the range of 65-68 mol% and their optimal growth temperature is between 35 and 37 °C. Most members have modified to live in close connection with higher organisms, either as primary pathogens or in commensal associations, which generally result in opportunistic diseases (Gerlach et al., 2001; Weiss, 2006).

The three main species of *Bordetella*, *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, are respiratory pathogens of mammals and have an important economical impact on both human health and agriculture. Historically, the classification of species was based on host range and severity of clinical disease. However, it is now apparent that by true genetic criteria, they actually comprise a single group of highly related subspecies. *B. pertussis* and *B. parapertussis* appear to be more host-adapted and differentiated and are less representative of the group as a whole than *B. bronchiseptica* strains (Weiss, 2006). *B. pertussis* strains show little genetic variation, indicating that the species derived from a common ancestor in the recent past, perhaps only a few thousand years ago. *B. parapertussis* infects both humans and sheep; in human infants it causes whooping cough. Phylogenetic analyses have shown that *B. parapertussis* strains isolated from humans (*B. parapertussis*$_{hu}$) are distinct from those isolated from sheep (*B. parapertussis*$_{ov}$). It has been suggested that *B. parapertussis*$_{hu}$ and *B. parapertussis*$_{ov}$ evolved independently from a common ancestor (*B. bronchiseptica*), and there is little or no transmission between the two reservoirs (sheep and human) (Parkhill, 2003). The incidence of human *B. parapertussis* infections may have been underestimated and several surveys indicate that 5 to 25% of all pertussis cases may be caused by this pathogen. *B. bronchiseptica* has a broad host range, causing chronic and often asymptomatic respiratory infections in a wide range of animals including dogs, monkeys, rabbits, swine and horses, but only occasionally in humans. All three species cause upper respiratory disease which involves the interaction of the bacteria with ciliated tracheal epithelial cells, resulting in ciliastasis and killing of the ciliated cells (Mallory and Hornor, 1912; Gerlach, 2001). *B. avium* is a bird pathogen which is most distantly related to the

B. bronchiseptica cluster industry (Gentry-Weeks et al., 1988; Mattoo and Cherry, 2005). *B. trematum* is a causative agent of ear and wound infections in humans (Vandamme et al., 1996). *B. hinzii* colonizes the respiratory tracts of poultry and this microorganism is also pathogenic on immunosuppressed patients. It is also found that this microorganism is the causative agent of fatal septicemia (Cookson et al., 1994; Vandamme et al., 1995 and Cherry, 2005). *B. holmesii* also causes septicemia in humans (Weyant et al., 1995). Environment-isolated member of this genus *Bordetella petrii* can grow at anaerobic conditions (von Wintzingerode et al., 2001; Gross et al., 2008). *B. ansorpii* is the most recently proposed species of the genus which was isolated from an epidermal cyst (Ko et al., 2005; Gross et al., 2010) (Table 1).

1.2. *Bordetella pertussis*

B. pertussis is highly labile, catalase- and oxidase-positive and urease-negative, very small Gram-negative, aerobic coccobacilli which can be identified by specific antiserum via agglutination or fluorescence (Kerr and Matthews, 2000; Mattoo and Cherry, 2005; Guiso, 2009) (Figure 1.2 and 1.3). This microorganism infects the respiratory route of the host and generally localize in the upper respiratory tract (Locht, 2001).

B. pertussis strains can be isolated only from the human respiratory tract. This very slow growing microorganism doubles once every 4 hours under optimal conditions and so obtaining logarithmic growth can be difficult. The bacteria are nutritionally fastidious and usually cultivated on rich media supplemented with blood. *B. pertussis* isolates are sensitive to fatty acids and they can be grown in blood agar and in synthetic medium which contains buffer, salts, an amino acid energy source, and growth factors such as nicotinamide. Even on blood agar, the organism grows slowly and requires 3-6 days to form colonies (Weiss, 2006).

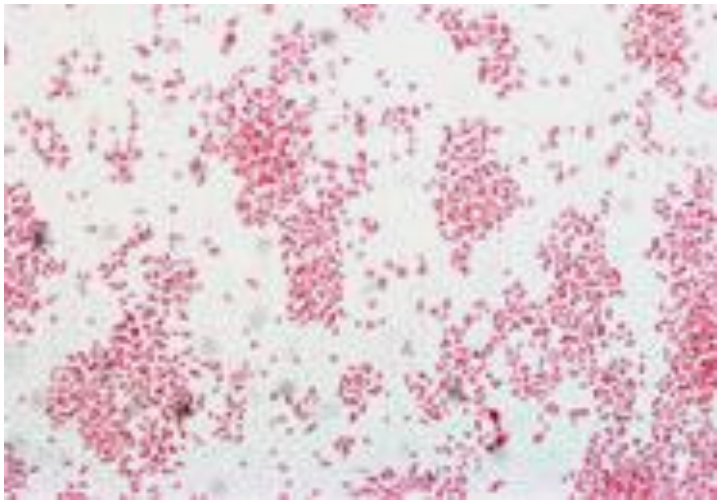

Figure 1. 2. Gram staining of *B. pertussis* (http://www.cdc.gov/pertussis/clinical/disease-specifics.html).

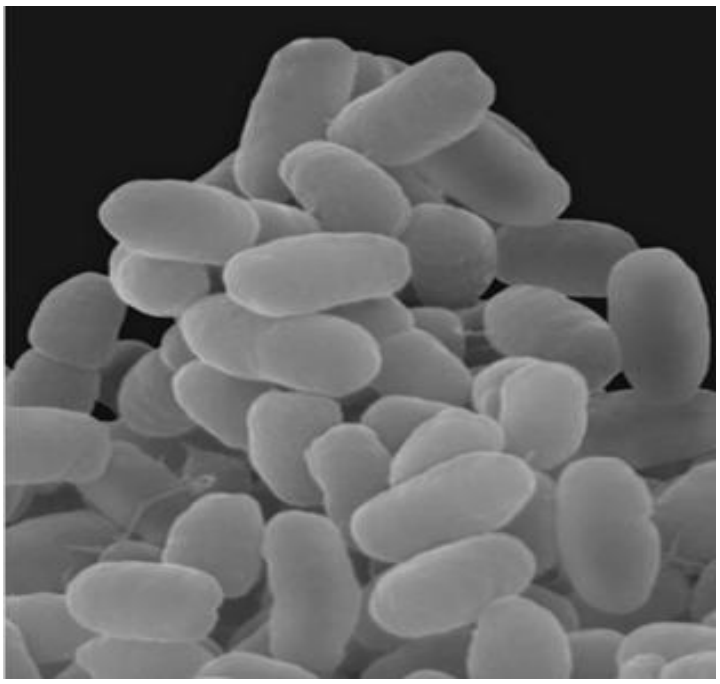

Figure 1. 3. Scanning electron micrograph of *B. pertussis* cells (http://www.historyofvaccines.org/content/sem-photograph-bordetella-pertussis).

This microorganism contains all the virulence factors which are important for attaching to respiratory tract, evading host defenses, causing damage to the host's respiratory system (Mattoo and Cherry, 2005; Guiso, 2009). Infection is initiated by the attachment of *B. pertussis* organisms to the cilia of epithelial cells of the upper respiratory tract (Mattoo and Cherry, 2005) (Figure 1.4). This interaction with ciliated epithelial cells occurs via bacterial adhesins; filamentous haemagglutinin (FHA), pertactin (PRN), fimbriae, pertussis toxin (PT) and tracheal colonization factor (TCF). The next step in pathogenesis is the paralysis of the cilia and death of ciliated cells, probably mediated by a synergistic effect of tracheal cytotoxin (TCT) and lipopolysaccharide (LPS) via induction of interleukin-1 (IL-1) and nitric oxide. This leads to defective mucociliary clearance, allowing the bacteria to establish in, and move down, the respiratory tract. Expression of the *bvg* regulated toxins, PT and adenylate cyclase-haemolysin, may damage the respiratory tract further and may also interfere with immune responses. PT has been suggested as a primary cause of the prolonged cough associated with pertussis. At this point the infection is established and the patient exhibits the symptoms characteristic of whooping cough. Depending on the response of the patient, the infection will be cleared over time, or may progress, in some cases; it will turn into pneumonia and possibly results in death (Van Den Berg et al., 1999). The pathogenesis of pertussis infection may also involve an intracellular stage. The organism can invade and survive within phagocytes (including dendritic cells) and non-phagocytic cells in vitro and can be seen inside alveolar macrophages in children with AIDS. Intracellular survival may explain the prolonged nature of the cough in some cases of whooping cough. Cell mediated immunity is likely to be required to combat intracellular infection, distinct from the antibody responses previously thought to be sufficient for protection (Maskell and Preston, 2002).

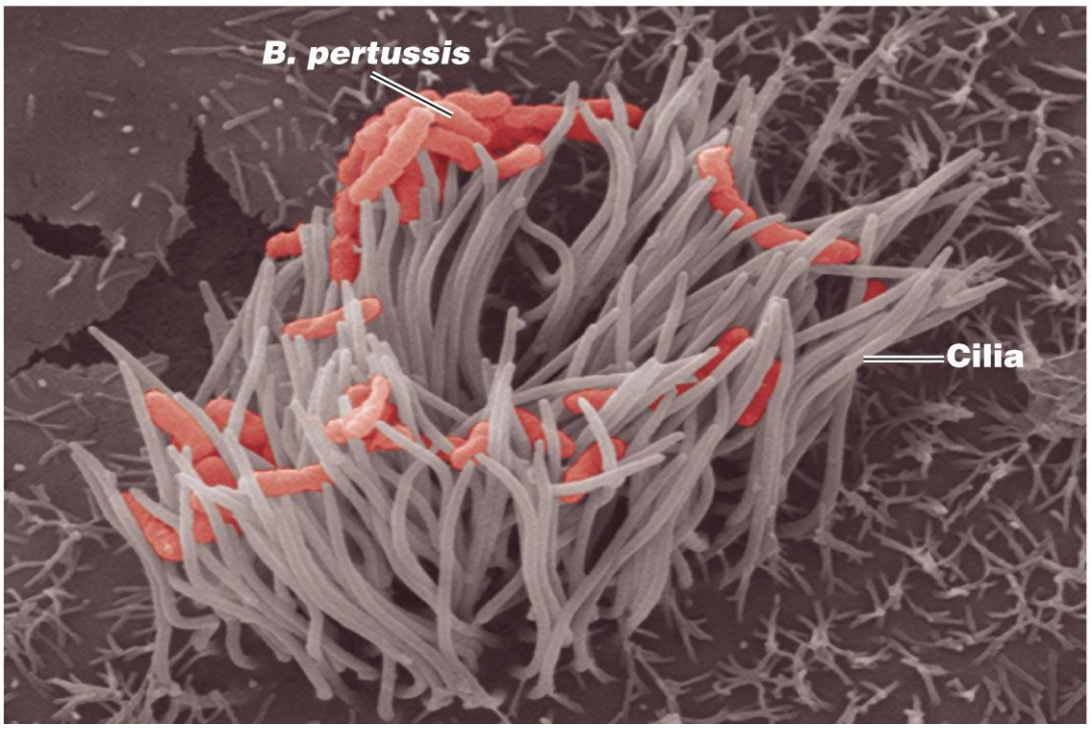

Figure 1.4. Scanning electron micrograph showing the colonization of *B. pertussis* to ciliated respiratory tract epithelial cells (http://www2.raritanval.edu/departments/Science/full-time/Weber/Microbiology%20Majors/SoftChalkeCoursesubmission/chapter11sub/chapter11sub_print.html).

1.3. Pertussis (Whooping Cough)

Pertussis, a highly contagious respiratory illness caused by *B. pertussis*, is marked by a paroxysmal cough that can last for several weeks. This disease can occur at any age, it's most severe in unimmunized children and in infants less than 1 year of age. According to World Health Organization data, 30–50 million disease cases are reported and about 300,000 of this cases result in death per year (http://www.who.int/immunization/topics/pertussis/en/index1.html). The bacteria spread from person to person through tiny drops of fluid from an infected person's noise or mouth. The onset of most cases occurs 7 to 10 days after exposure and

the illness lasts for 6 to 12 weeks and has three stages: catarrhal, paroxysmal, and convalescent (Watanabe and Nagai, 2004).

Yet in the last 2 decades, the number of cases of pertussis, or whooping cough, has been increasing in countries with high vaccination rates, particularly among 10- to 19-year-olds and infants younger than 5 months. Pertussis cases peak every 3 to 5 years, and the United States and other countries are in the midst of another upswing. According to the US Centers for Disease Control and Prevention (CDC), more than 21 000 cases were reported in 2010, the highest number since the 1950s (Friedrich, 2011).

In the catarrhal stage, there are rhinorrhea, lacrimation, and mild cough, similar to the events which occur with rhinovirus infections. Over a 7 to 14 day period, the cough worsens in both frequency and degree. The temperature is normal or occasionally mildly elevated. The paroxysmal stage is characterized by repeated coughing fits with 5 to 10 more forceful coughs during a single expiration (paroxysm). At the end of paroxysm, there is a massive inspiratory effort during which the classic whoop occurs. In conjunction with a paroxysm, cyanosis, bulging eyes, protrusion of the tongue, salivation, lacrimation, and distention of neck veins may occur. The paroxysmal stage lasts for 2 to 8 weeks. The transition to the convalescent stage is gradual and is associated with an initial decrease in the frequency of the paroxysms and subsequently a decrease in the severity of the events as well. The convalescent stage usually lasts for 1 to 2 weeks (Watanabe and Nagai, 2004; Mattoo and Cherry, 2005).

Common complications of classic pertussis include pneumonia, otitis media, seizures, and encephalopathy. The pneumonia may be a primary event in response to *B. pertussis* infections or may be due to a secondary infection with other pathogens. Seizures and encephalopathy are most probably due to cerebral hypoxia related to severe paroxysms (Mattoo and Cherry, 2005). The severity of

the disease tends to decrease with age. The cases of infection by *B. pertussis* are associated with lymhocytosis.

1.4. Virulence Factors

B. pertussis is a complicated bacterium that expresses many bacterial factors with immunomodulating functions. The expression of these proteins depends on *Bordetella* pathogenesis which is regulated as a reaction to environmental stimuli by a two-component regulatory system encoded by the *bvgAS* locus. BvgS is an inner membrane protein that senses changes in the environment. It contains a large periplasmic domain attached to a cytoplasmic domain and a cytoplasmic linker (Locht 2001). After some intramolecular phosphorylation cascade, the phosphate group is transferred to the transcriptional activator BvgA. *bvgAS* expression can be activated by growth at 37°C in the relative lack of $MgSO_4$ or nicotinic acid. *B. pertussis* grown under such "nonmodulating" conditions are referred to as Bvg^+-phase-specific bacteria (Mattoo and Cherry, 2005). When phosphorylated by BvgS, BvgA protein's affinity for the promoter regions of *B. pertussis* virulence-activated genes (*vag*) increases (Boucher, 1994). For the transcription process, the BvgA–RNA polymerase interaction may vary, which results in differential expression of the *vag* genes. *vrg* (for "*vir*-repressed genes) is also expressed under the control of *bvgAS* locus (Boucher, 1994; Locht, 2001).

At low temperatures or in the existence of nicotinic acid or sulfate ions, the phosphorylation of BvgA is arrested and the *vag* genes are silent. This phenomenon is termed as phenotypic modulation (Stibitz, 1989). When there is not functional BvgA in the cell, the *vag* genes are silent but this time the virulence-repressed genes (*vrg*) are derepressed. BvgA regulation of the *vrg* genes is controlled by BvgR repressor protein (Boucher, 1997).

During the disease, bacteria produce various factors related with the symptoms (Locht, 1999). These factors can be divided into toxins and adhesins. Toxins (e.g.,

tracheal cytotoxin; pertussis toxin) can damage ciliated epithelial cells and alveolar macrophages and cause hyperlymphocytosis and adhesins (e.g., filamentous hemagglutinin, pertactin, and 2 fimbrial proteins) take place in the attachment of the microorganism to tracheal cells. *B. pertussis* possesses all the tools needed for attaching to host cells, escaping from host defenses, and causing damage to the respiratory tract of the host (Figure 1.3) (Guiso, 2009).

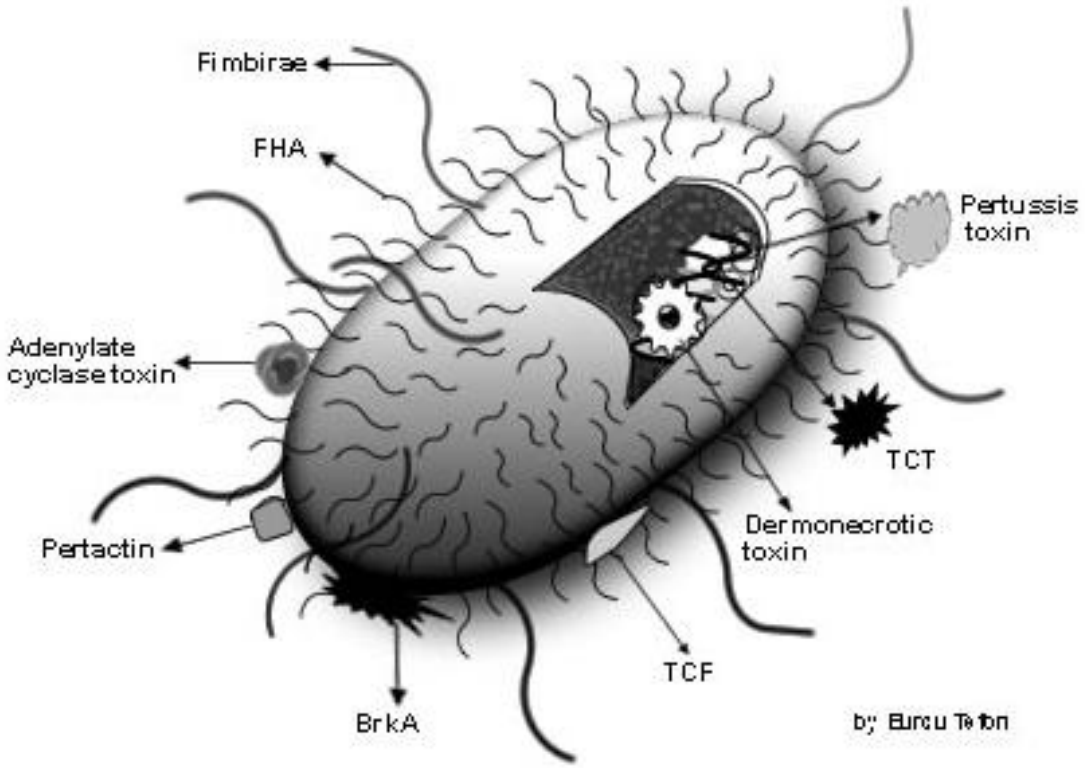

Figure 1. 5. Virulence factors of *B. pertussis*.

1.4.1. Filamentous Hemagglutinin (FHA)

A primary component of acellular pertussis vaccines, filamentous hemagglutinin is a highly immunogenic 220 kDa protein which can be found as secreted or

surface-associated (Renauld-Mongenie et al., 1996; de Gouw, 2011). It is a dominant attachment factor required for tracheal colonization and synthesized as a 367 kDa precursor FhaB, and only after N- and C-terminal modifications becoming the mature 220 kDa FHA protein. 220 kDa FHA is secreted from the cell by SphB1 (Piatti, 1999). It is also known that FHA proteins from *B. pertussis* and *B. bronchiseptica* are almost identical when their masses, configurations, hemagglutination properties and immunogenic epitopes are considered (Mattoo and Cherry, 2005).

1.4.2. Fimbriae (FIM)

B. pertussis fimbriae antigens play an important role in the attachment of the cell and and induce generation of protective host antibodies (Atakan-Ablay and Özcengiz, 2007). They contain major fimbrial subunits (Fim2 or Fim3) and a minor subunit, FimD (Blom et al., 1983). Fim2, Fim3, and FimD have molecular weights of 22.5 kDa, 22 kDa, and 40 kDa, respectively (Crowcroft et al., 2002). Chaperone proteins FimB and FimC and FimD assist the correct fimbrial structure formation and its outer membrane translocation (Willems et al., 1992). The Fim2 and Fim3 subunits have two heparin-binding regions, which play role in attachment of the pathogen to the extracellular matrix of epithelial cells (de Gouw, 2011).

1.4.3. Pertactin (PRN)

As member of the autotransporter family pertactin (PRN) is a non-fimbrial outer membrane protein, maturing from 93 kDa precursor can also direct its own secretion via Sec machinery. This protein plays a role in attachment of microorganism (Locht, 1999; de Gouw, 2011). PRN can be found as 68 kDa protein in *B. bronchiseptica*, a 69 kDa protein in *B. pertussis*, and a 70 kDa protein in *B. parapertussis* $_{hu}$. All PRNs contain Arg-Gly-Asp (RGD) motifs and

proline rich regions which are needed for binding to eukaryotic cells (Matoo and Cherry, 2005).

1.4.4. Serum-resistance Protein (BrkA)

Serum resistance protein (BrkA) is an important virulence factor of *B. pertussis* which provides resistance against complement-dependent killing by human serum and also takes part in the adherence of *B. pertussis* to tracheal cells (Fernandez and Weiss, 1994). It is an outer membrane protein and an autotransporter. This protein harbors two RGD sequences. BrkA is produced as a 103 kDa precursor and processed into a 73 kDa amino-terminal domain and a 30 kDa carboxy-terminal domain (Shannon and Fernandez, 1999). The latter domain is able to form a pore through which the amino-terminal domain is thought to be transported to the surface (Locht, 2001).

1.4.5. Tracheal Colonization Factor (Tcf)

Tracheal colonization factor is a 60-kDa secreted protein which is a virulence associated factor encoded by the *tcfA* gene and expressed by *B. pertussis* but not by *B. parapertussis* or *B. bronchiseptica*. It is also known that it plays a role in colonization in murine model (Finn and Stevens, 1995; Locht, 2001). Finn and Stevens (1995) showed that the C-terminal 30 kDa of this protein showed 50% identity to the 30 kDa C-terminus of the pertactin precursor.

1.4.6. Pertussis Toxin (PT)

Pertussis toxin (PT) is a virulence factor, strong adjuvant and one of the major components of acellular pertussis vaccines, and important for the colonization. It can be attached to the cell surface or can be secreted (Locht, 1999). This 117 kDa toxin shows a globular structure and has five dissimilar subunits; PtxA–E or S1–S5 (Locht, 2001; Özcengiz et al., 2005).

It is transported across the bacterial outer membrane by the Ptl type IV secretion system. A part consists of S1 subunit which has enzymatic activity. S2 to S5 subunits are responsible for the receptor binding and mediating S1 subunit translocation. In the cytosol, S1 subunit expresses its ADP-ribosyltransferase activity. ADP-ribosylated G-proteins lose their signal-transducing ability, which is also known as the main cause of most PT actions (Locht and Antione, 1995). PT is known to cause most of the systemic symptoms associated with pertussis disease, such as the profound leukocytosis that may be a predictor of poor outcome in infants (Tonon et al., 2002; Carbonetti, 2011). It has been demonstrated that PT and tracheal cytotoxin (TCT) can inhibit the immune cell trafficking and also can affect chemotaxis by inhibiting the release of chemokines from cells (Meade et al., 1985).

1.4.7. Adenylate Cyclase Toxin (AC)

Adenylate cyclase (AC) is a 200 kDa secreted toxin which has both adenylate cyclase and hemolytic activities. The two activities are on different domains of the protein, with the hemolytic domain enabling the entry of the enzymatic domain into the cell (Babu *et al*., 2002). This toxin forms small transient cation-selective pores in cell membranes, which shows its hemolytic activity on erythrocytes. It is an immunogenic protein and can elicit a protective immune response, but it has not been included as a component of acellular pertussis vaccines (Carbonetti 2010; de Gouw, 2011). It is known that after AC enters cells, it causes an increase in the cAMP synthesis and so inhibits phagocytotic activity of immune effector cells (Kerr and Matthews, 2000).

1.4.8. Dermonecrotic Toxin (DNT)

Dermonecrotic toxin (DNT), also called lethal toxin, is a 160 kDa heat-labile secreted toxin which induces inflammation and dermonecrotic lesions and around the colonization area of *B. pertussis* (Iida and Okonogi, 1971). The toxin activates

intracellular Rho GTPases by polyamination or deamidation. As a result of this, the GTPases become permanently active and cause the invaded host cells to express the Rho-dependent phenotypes. The intracellular Rho GTPases regulate the actin skeleton reorganization, cell motility and cell differentiation (Matsuzawa et al., 2004).

1.4.9. Tracheal Cytotoxin (TCT)

Tracheal cytotoxin (TCT) provides adhesion of the *B. pertussis* cells to the respiratory tract. It is 921 Da disaccharide-tetrapeptide and released from *B. pertussis* cell wall at high amounts (Goldman, 1986; de Gouw, 2011). It is known that *Bordetella* spp. also secrete this protein. TCT causes loss of ciliated cells and cell blebbing (Locht, 1999; Mattoo and Cherry, 2005).

1.4.10. Lipopolysaccharide (LPS)

It is known that lipopolysaccharide (LPS) endotoxins from Gram negative bacteria, are pyrogenic, mitogenic, toxic and can induce tumor necrosis factor production in macrophages. Other than these features, *B. pertussis* LPS does not contain a O-antigen (Ayme et al., 1980; Mattoo and Cherry, 2005). The LPS of *B. parapertussis* has O-chains (Locht, 1999). The host has various receptors to sense this molecule. By its LPS, *B. pertussis* prevents surfactant-mediated recognition and clearance (De Gouw, 2011).

1.5. The Resurgence of Pertussis

It is known that pertussis was one of the major infant death causes before 1940s. Introduction of widespread vaccination succeeded in reducing illness and death; however, pertussis remains one of the top ten causes of death in children worldwide (Crowcroft et al., 2003).

According to World Health Organization data, developing countries in Asia, Africa and South America have the highest disease burden (Crowcroft et al., 2003; Altunaiji et al., 2011). In developed countries, such as the United States, Australia and Canada, pertussis is well controlled by vaccination programs; however, there are reports about the resurgence of this microorganism especially among adolescent and adults (Crowcroft and Britto, 2002; de Carvalho and Pereira, 2006). News media announced a global resurgence of whooping cough in April 2002 following a session on pertussis at the 12th European Congress of Clinical Microbiology and Infectious Diseases in Milan, Italy. Subsequently, the European Union sent an alert to member states (Crowcroft *et al*., 2002). The possible reasons for resurgence of pertussis, even in developed countries with well-established vaccination programs can be explained by the decrease in vaccine-induced immunity, suboptimal vaccines, strain polymorphism and improved diagnostics (Wood and McIntyre, 2008).

1.6. *B. pertussis* Vaccines

With the isolation of *B. pertussis*, whole cell pertussis (Pw) vaccines were developed using formalin-killed bacteria in 1930s. In the mid-1940s, administration of the pertussis vaccine to children was initiated in the United States. First vaccines were prepared from only *B. pertussis* cells, but in 1947, vaccines including diphtheria and tetanus toxoids (DTP) were available and recommended (Özcengiz, 2005). During the 1950s, other countries also initiated routine pertussis immunization. Since 1974 high coverage pertussis vaccination has been started with WHO Expanded Program of Immunization (EPI). Providing immunization against six diseases, namely, whooping cough, diptheria, tetanus, tuberculosis, polio and rubeola was the subject of the original program (Mattoo and Cherry, 2005; Crowcroft and Pebody, 2006). The Pw vaccination studies showed that neither Pw vaccination nor *B. pertussis* infection provides a protection against *B. parapertussis* infections (Neimark, 1961; Khelef et al., 1993).

In the 1970s, there were concerns about safety of Pw vaccines. This caused the acceleration of the development of acellular pertussis (Pa) vaccines. In Japan, the first Pa vaccine which contains known components with specific amounts was developed by Sato et al. (1984). After this, different DTaP vaccines were developed by different manufacturers in Japan and routine immunizations with DTaP vaccines has been started in 1981 for 23 years (Mattoo and Cherry, 2005; Özcengiz, 2005). The success of DTaP vaccine in Japan stimulated other countries to develop new vaccine candidates and in the late 1990s, instead of Pw vaccines Pa vaccines started to be used. It is decided that the Pa vaccine was safer and caused less adverse effects. In many industrialized countries, Pa vaccines consist of five antigens, in which PT, FHA, PRN and FIM2 and FIM3 are used currently (Table 1.1) (Mattoo and Cherry, 2005; Crowcroft and Pebody, 2006; Storsaeter *et al*., 2007; Friedrich, 2011). The CDC's Advisory Committee on Immunization Practices (ACIP) recommends that children receive a total of 5 doses of the diphtheria, tetanus, and acellular pertussis vaccine (DTaP): one at each 2, 4, and 6 months of age; a reinforcing dose between 15 and 18 months; and a booster between 4 and 6 years. Another booster dose is recommended between the ages of 11 and 12 years (Friedrich, 2011) (Table 1.2).

Recently, the CDCs Advisory Committee on Immunization Practices (ACIP) has recommended routine Tdap for adolescents from 11–18 years. Continuously, Tdap vaccines (Boostrix and ADACEL) are licensed for adolescents in the US (Bamberger and Srugo, 2008). Formulation of adolescent and adult Tdap vaccines (Infanrix and Daptacel) is prepared based on the company's pediatric DTaP formulation. For both vaccines, the antibody response to a single dose of Tdap was similar to that following three doses of DTaP in infants. It is proposed to that both vaccines have similar clinical efficacy as DTaP vaccine because similar antibody levels against vaccine components was achieved. Infanrix and Daptacel Tdap vaccines are approved by the Food and Drug Administration for a single booster dose for persons who have completed the recommended childhood

DTP/DTaP vaccination series (http://www.cdc.gov/vaccines/pubs/pinkbook/downloads/pert.pdf).

Australia introduced an adolescent booster in 2003. In Argentina, a DTaP vaccine for people older than 10 years of age was licensed in 2003 (Forsyth et al., 2005). In Finland, Hungary, India, Netherlands, Poland and Romania, Pw vaccines are produced which meet the requirements for WHO standards. Recently, in the USA, Australia, Canada, France, Germany and Switzerland DTaP vaccine has been licensed and recommended for adults and adolescents (Wood, 2008; Zamir, 2011). But still to confirm the efficacy of the adult immunization, surveillance is needed (Forsyth et al., 2007).

Nowadays also there is concern that the Pa may not work as well as the Pw vaccine and that its duration of protection may be shorter (Friedrich, 2011). The data obtained from global immunization campaigns revealed that the immunity induced by Pa is less persistent than that induced by Pw (Gustafsson et al., 1996) or immunity induced by natural infection (Esposito et al., 2001). Pa-induced immunity lasts for 10 to 15 year period. This also shows the importance of adolescent booster immunization to prevent adolescent reservoir of infection (Skerry and Mahon, 2011).

Table 1.1. Composition of acellular vaccines and combinations from major manufacturers of acellular vaccines (Storsaeter *et al*., 2007).

			Pertussis Antigens (μg per dose-0.5ml)			
Vaccine	Manufacturer	Trademark	PT	FHA	PRN	FIM
Pediatric Vaccines						
DTPa	Chiron	Acelluvax	5	2.5	2.5	
	GSK	Infanrix	25	25	8	
	SP	Tripedia	23.4	23.4		
	SP	Tripacel/Daptacel	10	5	3	5
DTPa-Hib	GSK	Infanrix/Hib	25	25	8	
	SP	Actacel	10	5	3	5
DTPa-HBV	GSK	Infanrix-HBV	25	25	8	
DTPa-IPV	GSK	Infanrix-IPV/Infanrix polio	25	25	8	
	SP	Tetravac	25	25		
	SP	Quadracel	20	20	3	5
DTPa-IPV-Hib	GSK	Cinquerix	25	25	8	
	SP	Pentacel/Pediacel	20	20	3	5
	SP	Pentavac	25	25		
DTPa-HPV-IPV	GSK	Infanrix penta/Pediarix	25	25	8	
DTPa-HBV-IPV-Hib	GSK	Infanrix hexa	25	25	8	
	SP	Hexavac	25	25		
Reduced Antigen content Vaccines						
dTpa	GSK	Boostrix	8	8	2.5	
	SP	Adacel/Covaxis	2.5	2.5	5	3
dTpa-IPV	GSK	Boostrix polio	8	8	2.5	
	SP	Repevax	2.5	2.5	5	3

GSK — GlaxoSmithKline Biologicals, SP — Sanofi Pasteur, HBV — hepatitis B vaccine, IPV — inactivated poliovirus vaccine, Hib —conjugated *Haemophilus influenzae* type b vaccine

1.7. Vaccine Manufactured Against *Bordetella pertussis* in Turkey

Pertussis considered as an important disease is the one of the important childhood infections in Turkey (Özkan et al., 2007). In the 1950s, Pw were introduced in many countries and the reduction in the incidence of pertussis cases was noticeable. The routine administration of whole cell DTP vaccines to children was started at 1968 in Turkey and these vaccines were manufactured between the years 1968 and 1996 in the same country. After 1996, the local production of human vaccines was quitted as a government policy and imported human vaccines started to be used. EPI of the WHO was initiated in 1981 in Turkey and was accelerated by the National Vaccination Campaign commenced in 1985. With this campaign DTP3 vaccination percentage increased from 46% in 1986 to 96% in 2007 (Figure 1.6) (Nar Otgun et al., 2011). Pa vaccine has been administered since 2007 (Kurugöl, 2009).

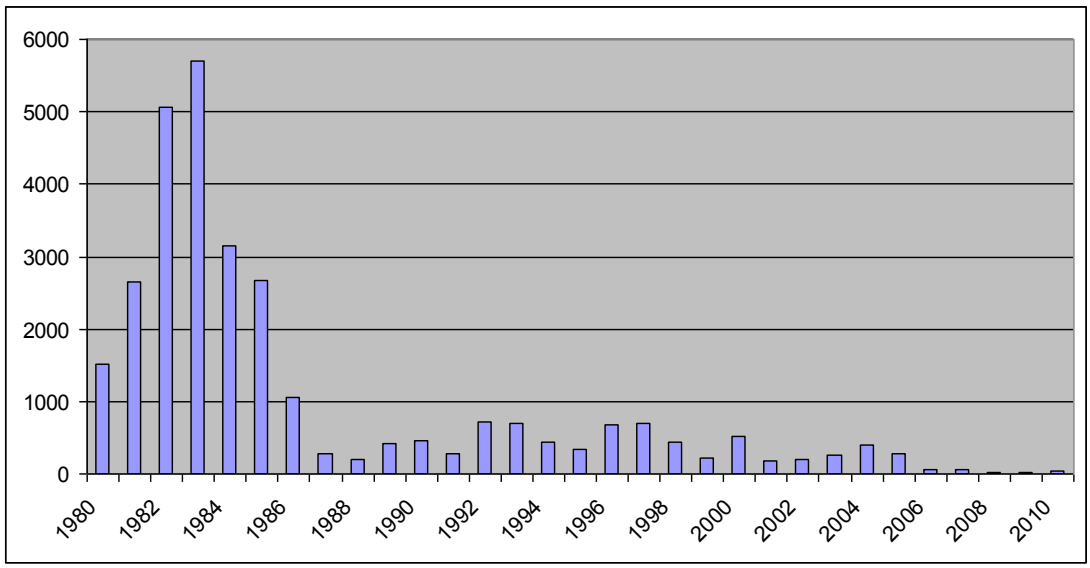

Figure 1.6. Pertussis incidence in Turkey between the years 1980 and 2010 (WHO).

In Turkey, children were vaccinated with Pw as a triple vaccine including Tetanus and Diphteria at the second, third, and fourth months and with a booster between the 16th and twenty fourth months. Children up to 6 years old can be vaccinated, if the booster is delayed (Nar Otgun et al., 2011). After switching of Pw into Pa vaccine, administration dates were changed as second, fourth and sixth months, and the period for booster dose administration was limited between 18th and 24th months (Table 1.3) (Özmert *et al*. 2008; Kurugöl, 2009).

In spite of decline in the disease incidence, the circulation of *B. pertussis* has not been eliminated. In Turkey, it was observed that unfortunately, neither immunity nor vaccination protects against the disease in the long-term and it has been reported that the number of pertussis cases in adolescents and adults has been increasing since the 1990s (Özkan, 2007). It is necessary to formulate vaccines with more powerful approaches on a country by country basis (Vatansever *et al*., 2005).

Table 1.2 Current childhood vaccination schedule in Turkey (http://www.euvac.net/graphics/euvac/vaccination/turkey.html).

	DTaP	OPV	IPV	Hib	HepB	MMR	PCV	BCG	dT
At birth					Yes				
1 month					Yes				
2 months	Yes		Yes	Yes			Yes	Yes	
4 months	Yes		Yes	Yes			Yes		
6 months	Yes	Yes	Yes	Yes	Yes		Yes		
12 months						Yes	Yes		
18-24 months	Yes	Yes	Yes	Yes					
6 years[1]	Yes		Yes			Yes			
13 years[2]									Yes

DTaP: Diphteria-Tetanus- acellular Pertussis vaccine, OPV: Live Oral Polio vaccine, IPV: Inactivated Polio vaccine, Hib: *Haemophilus influenza* type b vaccine, MMR: Measles, Mumps and Rubella vaccine, PCV: Pneumococcal conjugate vaccine, BCG: Bacillus Calmette-Guérin vaccine, Td; Diptheria-Tetanus for adults.
[1] Given at the first grade of primary school.
[2] Given at the eight grade of primary school.

1.8. Proteomics

A proteome is the entire PROTein complement expressed by a genOME, or by a cell or tissue type. As an extrapolation of the concept of the genome project, a proteome project is a research which seeks to identify and characterize the proteins present in a cell or tissue and define their patterns of expression (Wilkins, 1996). Proteomics is the study of protein properties (expression level, post-translational modification, interactions, etc.) on a large scale to obtain a global, integrated view of disease processes, cellular processes and networks at the

protein level (Blackstock, 1999). Proteome analysis provides invaluable information about changes in protein synthesis, degradation rates, post-translational modifications, and protein interactions (Han, 2008). The concepts of proteome have some differences from those of genome; while there is only one definitive genome of an organism, the proteome is an entity which can change under different conditions, and can be dissimilar in different tissues of a single organism. A proteome, nevertheless, remains a direct product of a genome (Wilkins, 1996).

A proteomic analysis starts with the isolation of soluble proteins, followed by the separation and visualization of the protein mixture generally by two-dimensional gel electrophoresis (2-DE) (Washburn, 2000).

There are various methods for proteomic studies, including 2-DE or liquid chromatography (LC) followed by tandem mass spectrometry (MS). 2-DE with IPGs and protein identification by MS are currently the grindstone for proteomics (Görg, 2004). Although recent advances in 2-DE have improved resolution and reproducibility, to automate the technique is still hard in a high-throughput setting. Because of this reason, for large-scale proteomic studies, multidimensional protein technologies can be used as alternative approaches. Researchers expecting that, these technologies combined with high resolution analysis methods such as Fourier transform mass spectrometry will increase the detection of proteins in an extract (Bertone, 2005).

1.9. Proteomic Strategies

Protein identification by MS is usually performed either as whole protein analysis (top-down proteomics) or enzymatically produced peptide analysis (bottom-up proteomics (Figure 1.5) (Han et al., 2008).

1.9.1. Bottom-up Proteomics

In "bottom up'' shotgun proteomics, in order to detect a protein, first peptide detection is needed. These bottom-up strategies are mostly used for the large-scale or high-throughput analysis of complex samples. "Sort-then break" approaches start with off-line protein fractionation and separation continues with digestion, peptide mass fingerprinting (PMF) and LC/MS/MS. In 'break-then-sort' approaches, proteins are digested directly without separated, and then peptides are separated by multidimentional chromatography followed by MS/MS (Figure 1.7) (Resing, 2005; Han, 2008).

Among these techniques, the multidimensional protein identification technology (MudPIT) is a non-gel technique which needs a 2D chromatography separation before MS/MS sequencing. Generally, separation of proteins and their enrichment is performed before digestion. In another type MudPIT approach, proteins are separated by SDS-PAGE, cut from the gel, digested enzymatically and analyzed by multidimensional LC/MS/MS (Resing, 2005).

1.9.2. Top-down Proteomics

The "top-down" approach starts with separation of proteins from mixtures into single proteins or less complex protein mixtures, continues with off-line or on-line mass spectrometry analysis. This approach focuses on characterization of intact proteins (Cui et al., 2011). It involves gas-phase ionization of intact proteins, high-resolution MS analysis of intact protein ions and subsequently protein's gas-phase fragmentation inside the mass spectrometer. The advantage of this approach is that it enables the analysis of entire protein sequence which is important for characterization of isoforms and post-translational modifications (PTM) (Han, 2008).

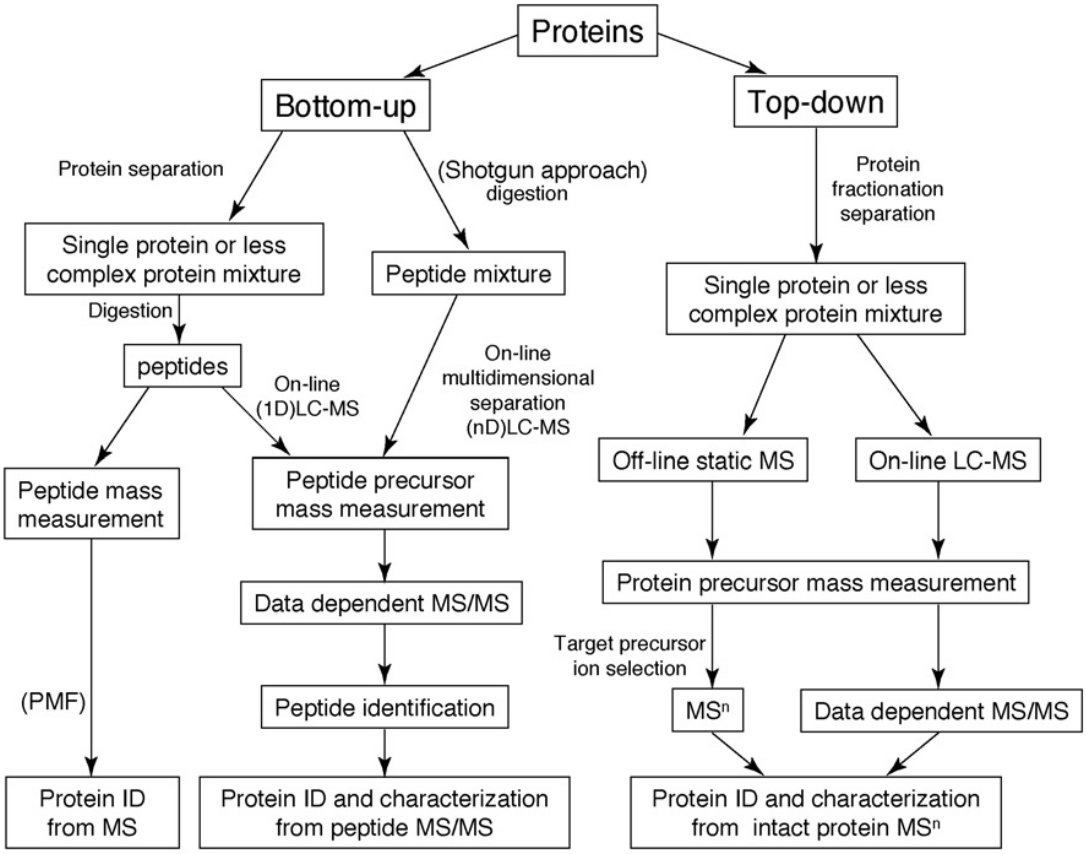

Figure 1.7. Steps in "bottom up" proteomics and "top down" proteomics strategies (Han, 2008).

1.10. Immunoproteomics

The availability of complete genome sequences provides identification of most protein products of an organism. However, because protein expression is time, location and condition dependent, the information obtained from just genome sequence could not be enough for determination of proteins. Nowadays, using various protein separation techniques followed by MS analysis facilitates protein identification even from very complex mixtures (Grandi, 2001).

Combination of proteomics with serological techniques, also known as serological proteome analysis (SERPA) or immunoproteomics is used for the identification of *in vivo* immunogens suitable as vaccine candidates (Serruto, 2006).

Immunoproteomics determines antigenic components of proteome such as tumor-specific antigens in cancer research or immunoreactive proteins of pathogens, generally in total proteome, outer membrane (surface) proteome and secreted protein fraction (secretome).

Although immunoproteomics is rather a new research tool, it has been quite effective to determine the virulence factors of various pathogenic microorganisms and hence, the development of new drugs and vaccines. Among the current proteomic techniques, generally 2 DE combined with Western blot is working tool in immunoproteomic applications (Falisse-Poirrier, 2006).

Much of information about immunogenic components can be derived from immunoproteomics which has been successfully applied for the discovery of antigens from various bacterial pathogens such as *Helicobacter pylori* (Nilsson, 2000; Backert, 2005), *Haemophilus influenzae* (Langen et al., 2000, Thoren et al., 2002), *Staphylococcus aureus* Vytvytska, (2002), *Francisella tularensis* Havlasová, (2002), *Bacillus anthracis* (Ariel, 2003), *Plasmodium falciparum* (Doolan et al., 2003), *Shigella flexneri* (Peng, 2004), *Anaplasma marginale* (Brown et al., 2005), *Brucella abortus* (Neubauer et al., 2006), *Corynebacterium diphtheria* (Hansmeier, 2006), *Streptococcus pyogenes* (Rodríguez-Ortega, 2006), *Neisseria meningitides* (Vipond et al., 2006; LinksHsu, 2008), *Mycoplasma hyopneumoniae* (Pinto, 2007) *Chlamydia pneumoniae* (Bunk, 2008), *Borrelia hermsii* (Lopez, 2009), *S. suis* serotype 9 (Wu, 2011).

1.11. Analysis of Hydrophobic Membrane Proteins

Outer membrane proteins (OMPs) of Gram-negative bacteria have various functions and they are important for cell to cell and cell to environment interactions such as signal transduction, cell adhesion, ion transport and endocytosis. In pathogenic microorganisms they also act as virulence factors and are involved in adhesion, invasion of host cells, and proliferation (Koebnik et al., 2000). For these reasons, understanding the features of OMPs will pave the way for discovering new antimicrobial drug and vaccine candidates (Santoni et al., 2000; Rodriguez-Ortega, 2006).

Despite their importance, membrane proteins especially OMPs are unfortunately underrepresented in 2D gels. The causes can be; (i) their lack of abundance compared with total proteins of the organism, (ii) their alkaline pI, (iii) their tendency to aggregate in aqueous media used for IEF. There are various extraction techniques to increase the solubilization and separation of membrane proteins in 2 DE like adding strong chaothrophic agents and new zwitterionic detergents in extraction solutions as well as in IEF media. However, it is considered that the best method for detection of OMP is 1D SDS-PAGE followed by LC-MS/MS. This method has been termed as geLC-MS/MS (Santoni et al., 2000; Görg, 2004).

1.12. Steps in Immunoproteomics

An ideal proteome screening methodology would combine high throughput capabilities with detection of as many protein products as possible in a sensitive, reproducible, and quantifiable manner. The wide-ranging biochemical heterogeneity of proteins makes it unlikely that any single separation and analysis method will be suitable for profiling the full proteome of any cell type, tissue, or biological fluid (Steel, 2005). The major steps of the 2 DE-MS workflow include: (i) sample preparation and protein solubilization; (ii) protein separation by 2-DE; (iii) protein detection and quantitation; (iv) computer-assisted analysis of 2-DE

patterns; (v) protein identification and characterization; (vi) 2-D protein database construction (Görg, 2004).

1.12.1. Sample Preparation and Protein Solubilization

In order to obtain a 2 D gel with high resolution, proteins of the sample have to be denatured, solubilized and stabilized. Complexicity, isoelectric point (pI) and molecular weight (Mr) values of proteins from various cell or tissue extracts are major problems for proteomic research. Unfortunately, there are thousands of different extraction methods for different samples for 2 DE. Since the starting sample is important for any research, preparing sample and finding suitable experimental technique is important. Sample preparation is important, especially in comparative proteomics (Bodzon-Kulakowska et al., 2007). Increasing reproducibility is one of the major factors in proteomic studies; therefore, sample preparations should be simple. The stability of samples can be obtained by adding protease inhibitors into the solutions. Because decomposition of urea causes carbamylation of the proteins, urea containing samples should not be heated. Proteins generally can be found as insoluble in their native form. For increasing their solubilization, denaturization by disrupting their intra- and intermolecular interactions without changing their charge properties is needed. Sample solubilization is carried out by using chaotrophes (e.g., urea and/or thiourea), nonionic/zwitterionic detergents (3-[(3-Cholamidopropyl)-dimethyl-ammonio]- 1-propane sulfonate (CHAPS) or Triton X-100), reducing agents dithiothreitol/dithioerythritol (DTT/DTE)), carrier ampholytes (CA), and if necessary protease inhibitors to prevent aggregation, precipitation and protein modifications (Görg, 2004).

1.12.2. Two Dimensional Gel Electrophoresis (2-DE)

Two-dimensional gel electrophoresis is a technique in which proteins are separated by two different physicochemical principles. Proteins are first separated

on the basis of their isoelectric points (pI) and further separated on the basis of their molecular masses in the presence of SDS. These both high-resolution techniques are carried out in polyacrylamide gels (Garfin, 2003).

IEF is an electrophoretic method which separates proteins according to their pI values (O' Farrell, 1975). In nature, protein net charges are determined by pH of their environment, which means they carry either negative, positive or zero net charge, depending on their surrounding pH level. Isoelectric point (pI) is the specific pH where the net charge of a protein is zero. Proteins show variations in pI values, but these values are generally between 3 to12.

Under an electrical field, a protein migrates always towards the electrode with the opposite charge in a linear pH gradient medium and it stops when its total charge is zero. The effect which concentrates proteins at their pI values and separates them according to their small charge differences is called “focusing” effect. After IEF step, proteins are separated according to their molecular masses by SDS-PAGE.

The first separation methods for 2D PAGE by IEF were done by capillary gels within pH gradients generated by CA. Because preparing capillary gels are difficult and their reproducibility is poor, immobilized pH gradients (IPGs) are preferred for IEF. The pH gradients of IPGs are generated by buffering compounds that are covalently bound into porous, polyacrylamide gels and the pH gradient is stable and resistant to high voltages for high-resolution separations. Nowadays, IPGs are widely used for 2D PAGE and they are prepared as mechanically stable strips for practical use (Garfin, 2003).

1.12.3. Protein Detection

Proteomics can detect and measure quantitative changes in expression levels. That is why the selection of the best protein detection method has great importance in

proteomic works. Among all of the different techniques, choosing the correct one with optimal signal to noise ratio is important (Westermeier and Marouga, 2005). The most important properties of protein visualization methods are low detection limit, high linear dynamic range (for quantitative accuracy), reproducibility, non-toxicity and mass-spectrometry compatibility. Protein detection methods can be categorized as staining with anionic dyes (*e.g.,* Coomassie Brilliant Blue (CBB)), negative staining with metal cations (*e.g.,* zinc imidazole), Silver-staining, fluorescence staining, and radioactive isotopes, using autoradiography, fluorography, or phosphor-imaging (Görg, 2004).

CBB staining methods are most frequently used ones for protein detection because of their low price and compatibility with MS. The most important limitation of this method is its insufficient sensitivity (detection limit is between 200–500 ng protein *per* spot). A modified method, Colloidal CBB staining (Neuhoff et al., 1988), which is highly reproducible and sensitive when repeatedly applied, can be used as a substitute (Görg, 2004). A wide used, relatively low-cost, highly sensitive (1 ng per spot) method for the detection of protein spots is silver staining (Merril et al., 1981). The drawback of this staining method is because it is a multistep procedure, it does not have a general endpoint, and the intensities of stained spots may vary. Fluorescence staining is the optimum method which is highly sensitive, reproducible and shows very wide linear dynamic ranges. There are various dyes with different sensitivities and properties such as the family of Sypro® dyes and Deep Purple™ dyes (Westermeier and Marouga, 2005).

1.12.4. Computerized Image Analysis

The complexity of proteomic works creates a need for image analysis. So the protein patterns are converted into images in digital format. For optimum analysis the software must be reliable and reproducible, human interference must be kept at very low levels to prevent the variation. Computerized image analysis starts

with spot detection, continues with image normalization (e.g. backgrounds subtraction), spot matching, if needed quantitative comparisons of volumes of spots and data presentation and interpretation. Modified document scanners, laser densitometers, charge coupled device cameras, and fluorescent imagers can be used for producing digital images. The statistic tools which are required for analysis of protein expression levels are generally provided by the image analysis software. If the protein spot contains more than one protein because of low gel resolution, the volume of the spot will not reflect the real situation. For such kind of analysis MS is needed (Görg, 2004; Westermeier and Marouga, 2005).

1.12.5. Immunoblotting

Blotting is the transfer of macromolecules into immobilizing membranes for immunoreaction (Towbin et al., 1979). The immunoblotting technique provides information about the presence, relative molecular weight, and/or quantity of an antigen by combining protein separation via gel electrophoresis with specific recognition of antigens by antibodies.

Blotting followed by immunodetection is called as 'Western Blotting. In this technique, after transferred to support membrane (nitrocellulose (NC), polyvinylidene fluoride (PVDF)) proteins are probed with monoclonal antibodies or with immunoglobulins. Free binding sites on the membrane are blocked with skim milk powder, casein, bovine serum albumin (BSA), non-fat dry milk (NFDM), or fish gelatin, which are not taking place in visualization reaction. Enzyme-, gold-, radioactive label-conjugated secondary antibodies are used for visualization. In the last step, the proper substrate is incubated with the membrane for the detection of conjugated enzymes (Westermeier and Marouga, 2005).

1.12.6. Mass Spectrometry Analysis

Mass spectrometry (MS) based studies have been started by J. J. Thomson (Thomson, 1913) and his student F. W. Aston (Aston 1933) in the beginning of 1900s. The mass spectrometer is an apparatus which measures the masses and relative concentrations of atoms and molecules. It produces ions from a sample, separates them according to their mass-to-charge ratio (*m*/*z*) and records the relative abundance of each of the ions to obtain a mass spectrum (Bonner et al., 2002). MS is based on the peptide mass fingerprints (PMF) which are obtained by digesting the protein of interest finding. By comparing the experimental PMF with the theroretical PMF generated *in silico,* the protein identification is completed (Görg, 2004). Advantages of mass spectrometers are their high accuracy (~0.01–0.001%) and sensitivity (detection of 10^{-9}–10^{-18} mol of sample required) in the determination of the Mr of compounds (Graham et al., 2007). Even there are a lot of techniques to identify a protein, MS based techniques are the most efficient ones because of their ability to solve the complexities in proteome studies (Han et al., 2008).

With the introduction of the "soft ionization" techniques (Fenn et al., 1989), the obstacle of observing large, polar and thermally label molecules without fragmentation has been removed. Two "soft ionization" techniques which are important for the characterization and quantification of proteins are electrospray ionization mass spectrometry (ESI-MS) (Yamashita and Fenn, 1984) and matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) (Karas and Hillenkamp, 1988; Tanaka et al., 1988). Because of importance of these techniques in life sciences, 2002 Nobel Prize for Chemistry was awarded to both John Fenn and Koichi Tanaka for their revolutionary work in ESI and MALDI, respectively.

The parts of a mass spectrometer can be categorized as the ion source which produces ions from the sample, one or more mass analyzer which is used for the

separation of proteins according to their *m*/*z* ratios and the detector which detects the reaching time and number of ions (Figure 1.8). An inlet device to introduce the analyte into the ion source (e.g., liquid chromatograph, direct probe) and a computer to processing the data to create a mass spectrum is also needed (Lane, 2005).

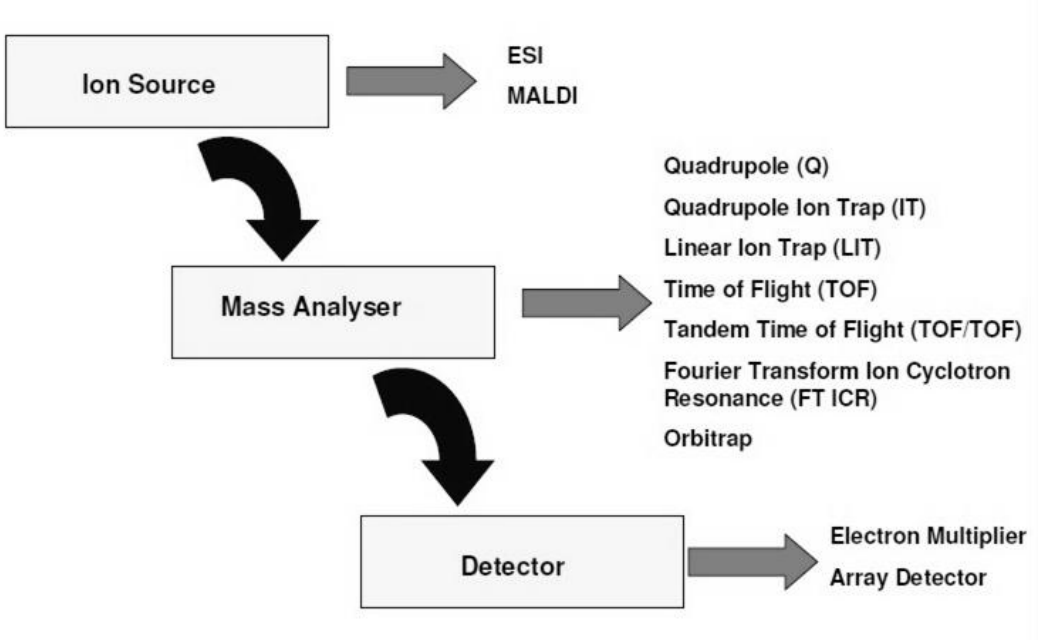

Figure 1.8. Three important components of a mass spectrometer (Graham et al., 2007).

1.12.6.1. Ion Source

The sample which will be analyzed must be vaporized and ionized. Two ionization techniques generally used for the analysis of proteins are ESI and MALDI.

Because of its high sensitivity and broad applicability, ESI is a mainstream method for biological researches. It produces gaseous ions from sample solution,

and can therefore be easily coupled to liquid-based separation techniques such as liquid chromatography (LC) for proteomic studies (Mann et al., 2001). ESI basically used for producing charged microdroplets which are composed of analyte ions. After dissolved, the sample is pumped through a thin capillary metal tube which is positioned in front of counter electrode. With the application of electricity, pumped liquid exits the capillary in the conical shape (Taylor cone) (Manisali et al., 2006) because a cylindrical shape can hold more charge than a sphere. The droplets at the tip of the cone are scattered from the capillary when the electrostatic repulsion of the charged molecules reaches the surface tension of the solution. These small charged droplets fly towards the counter electrode also called a collector. While flying to evaporate, they pass through a heated capillary or curtain of heated nitrogen. When the electrostatic repulsion is greater than the surface tension, the droplet scatters smaller droplets (Figure 1.9) (Lane, 2005).

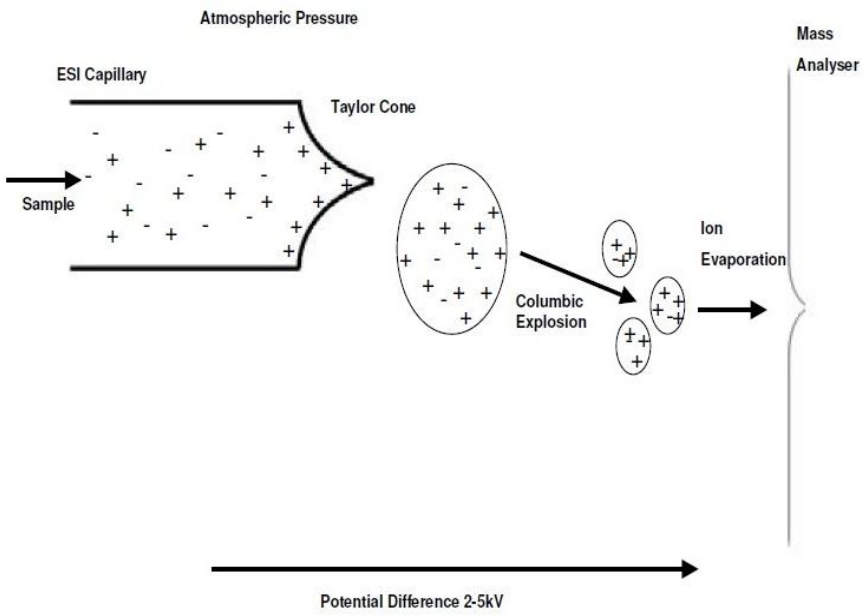

Figure 1.9. ESI process (Graham et al., 2007).

In MALDI analysis, the sample is first co-crystallized with a large molar matrix, usually a UV-absorbing weak organic acid that prevents the analyte from forming large aggregates. 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) and α-cyano-4-hydroxycinnamic acid (CHCA) are used as matrixes to analyze proteins and peptides, respectively. After mixing, analyte and matrix are both placed onto a metal plate. When laser bombardment is initiated in a vacuumed chamber, the irradiation of the matrix causes heating and sublimation of the matrix. Generally a nitrogen laser at 337 nm is used. While shifting into the gas phase, matrix also takes intact analyte molecules with it, which are ionized without fragmentation (Lane, 2005). The ionization depends on the matrix–analyte combination, but is not dependent on the number of acidic or basic groups of the analyte. The matrix serves as a proton donor and receptor, acting to ionize the analyte in both positive and negative ionization modes, respectively (Figure 1.10) (Graham et al., 2007).

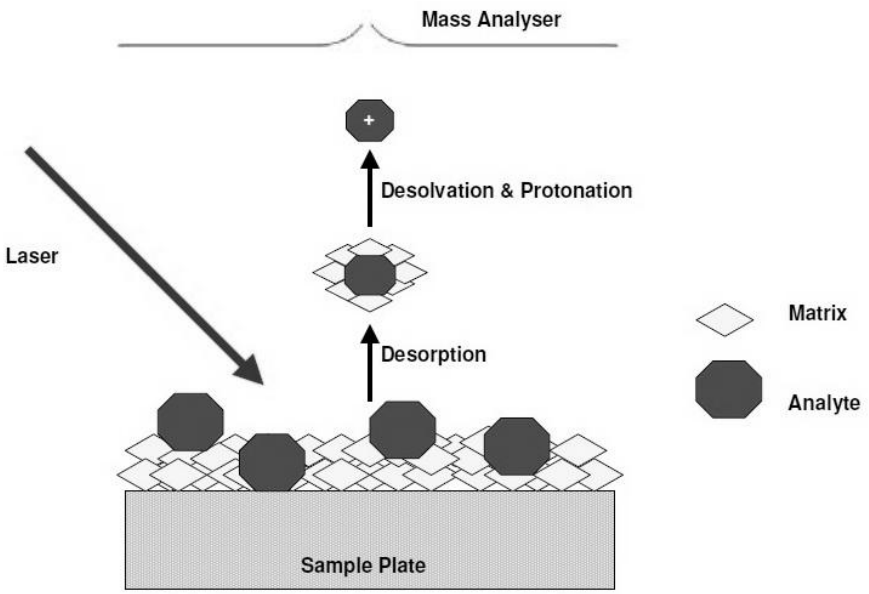

Figure 1.10. MALDI process (Graham et al., 2007).

The MALDI systems allow desorption and ionization of very high molecular mass analytes (greater than 100,000 Da) and it is generally used to analyze simple peptide mixtures, while ESI systems are favored for the analysis of more complex samples (Lane, 2005).

1.12.6.2. Mass Analyzers

For MS technology, mass analyzer stands in the middle. For proteomics research generally four types of mass analyzers are used: quadrupole (Q), ion trap (quadrupole ion trap, QIT; linear ion trap, LIT or LTQ), time-of-flight (TOF), and Fourier-transform ion cyclotron resonance (FTICR) mass analyzer. They may vary in their design and performance, and can be used either alone or tandem (Table 1.3) (Han et al., 2008; Yates et al., 2009).

TOF mass spectrometer is the most incomplex one among them. It composed of an ion-accelerating region, a flight tube, and a detector and generally used with MALDI to observe high kDa molecules. Theoretically, at the same position and at the same time, being accelerated by electrostatic current, their velocities will depend on their mass to charge ratio (*m/z*) and the time that they reach the detector will show their masses. Advantages of this technique are unlimited mass range, getting a complete mass spectrum for each ionization event, high transmission, no need for scanning the ion beam, obtaining spectra from extremely small sample amounts and low cost. One drawback of TOF technology is its insufficient mass resolution which is caused by the time variations of ions have same *m/z*.

The quadrupole mass analyzer contains four axial rods which are parallel to the flight paths of the ions with an electric field applied through a combination of direct current (DC) and radio frequency (RF) to the rods. It filters ions according to their *m/z* by changing the voltages in the rods: While passing along the middle of the quadrupoles, the motion of ions will depend on the electric fields. By adjusting DC and RF voltages, ions which have certain *m/z* can pass through the

detector while the others collide with the rods and discharged. These analyzers are generally used with gas chromatography (GC) or LC and also can be placed in tandem (generally as triple quadrupole (QQQ)). Q-TOF in which the third quadrupole is replaced with TOF analyzer is also used in biological studies (Graham et al., 2007).

The ion-trap (IT) mass spectrometer uses ring shaped electrodes; a rotating three-dimensional electrostatic field, to capture the ion of interest. By changing the voltage, trapped electrons are ejected from the trap according to their *m/z* ratio. In this technique, ions can be trapped and analyzed many times in tandem. Advantages of IT mass spectrometers are their high sensitivity with fast data acquisition (Graham et al., 2007).

A Fourier transform mass spectrometer (FTMS), also known as an ion-cyclotron resonance mass spectrometer (ICR-MS), is an ion trap containing a cubic cell inside a strong magnetic field. Inside the cubic cells, there are trapping, transmitter and receiver. If a beam of ions is pointed up to the trap, the ions in the cell start to move in cyclotron motion in a plane perpendicular to the magnetic field. An oscillating electric field is also applied to the trapping plate which is also perpendicular to magnetic field. While passing closer to the receiver, the cyclotron motion of the ions will be detected by the receiver (Lane, 2005).

Table 1.3. Characteristic of mass spectrometers which have been commonly used for proteomic studies (Han et al., 2008).

Instrument	Mass resolution	Mass accuracy (ppm)	Sensitivity	*m/z* range	Scan rate	Dynamic range	MS/MS capability	Ion source	Main applications
QIT	1000[a]	100–1000	Picomole	50–2000; 200–4000	Moderate	1E3	MS^{nd}	ESI	Protein identification of low complex samples; PTM identification
LTQ	2000[a]	100–500	Femtomole	50–2000; 200–4000	Fast	1E4	MS^{nd}	ESI	High throughput large scale protein identification from complex peptide mixtures by on-line LC–MS^n; PTM identification
Q-q-Q	1000	100–1000	Attomole to femtomole	10–4000	Moderate	6E6	MS/MS	ESI	Quantification in selective reaction monitoring (SRM) mode; PTM detection in precursor ion and neutral loss scanning modes
Q-q-LIT	2000[a]	100–500	Femtomole	5–2800	Fast	4E6	MS^{nd}	ESI	Quantification in SRM mode; PTM detection in precursor ion and neutral loss scanning modes
TOF	10 000–20 000	10–20[b]; <5[c]	Femtomole	No upper limit	Fast	1E4	n/a[e]	MALDI	Protein identification from in-gel digestion of gel separated protein band by peptide mass fingerprinting
TOF–TOF	10 000–20 000	10–20[b]; <5[c]	Femtomole	No upper limit	Fast	1E4	MS/MS	MALDI	Protein identification from in-gel digestion of gel separated protein band by peptide mass fingerprinting or sequence tagging via CID MS/MS
Q-q-TOF	10 000–20 000	10–20[b]; <5[c]	Femtomole	No upper limit	Moderate to fast	1E4	MS/MS	MALDI; ESI	Protein identification from complex peptide mixtures; intact protein analysis; PTM identification
FTICR	50 000–750 000	<2	Femtomole	50–2000; 200–4000	Slow	1E3	MS^{nd}	ESI; MALDI	Top-down proteomics; high mass accuracy PTM characterization
LTQ–Orbitrap	30 000–100 000	<5	Femtomole	50–2000; 200–4000	Moderate to fast	4E3	MS^{nd}	ESI; MALDI	Top-down proteomics; high mass accuracy PTM characterization; protein identification from complex peptide mixtures; quantification

[a] Mass resolution achieved at normal scan rate; higher resolution achievable at slower scan rate.
[b] With external calibration.
[c] With internal calibration.
[d] $n > 2$, up to 13.
[e] Fragmentation achievable by post-source-decay.

1.13. Aim of the Study

In the present study, we took the advantage of the availability of complete *B. pertussis* genome sequence and attempted to construct the first systematic immunoproteome map of *B. pertussis* with the aim of providing further insight into the biology and immunogenicity of this pathogen. Our approach involved the analysis of the total soluble cytosolic proteome, secretome and outer membrane subproteome, respectively. For the identification of the predominant immunogenic proteins 2-DE was coupled with MALDI-TOF MS analyses and geLC/MS was also performed when necessary.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

All the materials used in this study are listed in the Appendix B. All chemicals were of analytical grade and were obtained from the commercial sources at the highest grade of purity available.

2.2. Bacterial Strains

The strains of *B. pertussis* used in this study were Tohama I and Saadet, the latter being a local strain. All *B. pertussis* strains were kindly provided by Dr. Erkan Özcengiz (Berk Farma Res. Co.., Ankara).

2.3. Culture Media and Growth

B. pertussis strains were grown for 60 h in Cohen-Wheeler agar medium (Sato and Arai, 1972), then stored at 4˚ C and subcultured at 2 week intervals. For long term maintenance, the colonies were collected from the surface of the agar, and then resuspended in phosphate buffered saline (PBS) to an OD_{630} of 0.6 (corresponding to ca. 10^9 cells/ml). After covering with 30% glycerol, the cells were stored at – 80˚ C. When necessary, the strains were also grown on modified Morse and Bray liquid medium (Morse and Bray, 1969) for 60 h at 37˚ C, by shaking at 180 rpm (Appendix C).

2.4. Preparation of Cytoplasmic Proteomes

B. pertussis Tohama I and Saadet strains were grown on Cohen-Wheeler agar medium (Sato and Arai, 1972) for 48 h at 37^{o} C. For cytoplasmic protein extraction three different methods with slight modifications were used:

In the first method, cells were suspended in cold PBS, then centrifuged and resuspended in lysis buffer (137 mM NaCl, 10% glycerol, 1% *p*-octyl-b-D-glucopyranoside, 50 mM NaF, 1 mM Na_3VO_4. Then bacterial suspension freezed and thawed in liquid nitrogen for 10 times and undisrupted cells were removed by centrifugation at 16,000 g for 15 min (Eppendorf, Germany). Proteins were precipitated overnight in 20% TCA in acetone 0.2% DTT. Proteins were solubilized in IEF buffer (9 M urea,4%w/v CHAPS, 70mM DTT, and 5% v/v pH 3–10 carrier ampholytes) (Hubalek et al., 2004). In the second method, cells were harvested and washed in PBS containing EDTA and centrifuged at 16,000 g for 15 mins at 4 ^{o} C. Pellet was suspended in reswelling buffer containing 7 M urea, 2 M thiourea, 2% w/v CHAPS, 2% v/v pH 3–10 carrier ampholytes, and 1% w/v DTT (Bernardini et al., 2004). In the third method, for the preparation of whole cell lysates, the cells were collected and suspended in cold TE buffer and centrifuged at 4500 g for 10 min. The pellet was resuspended in 1 ml 0.04 M lysosyme in TE (0.2 mM EDTA, 2 mM Tris-HCl) buffer and incubated at 37^{o} C for 35 min, followed by a centrifugation at 16,000 g for 10 min. The pellet was solubilized in 8M urea, vortexed at 4^{o}C for 30 min and centrifuged at 16,000g for 10 min. The supernatant was stored at -20^{o} C. Protein samples were stored at -20˚C at most for 10 days till usage. Since the latest method resulted in better resolution in 2DE experiments, for the rest of the study this method was used.

2.5. Preparation of Outer Membrane Proteins

B. pertussis Tohama I and Saadet strains were grown in Morse-Bray medium (Morse and Bray, 1969) for 60 h at 37 ^{0}C and then collected by centrifugation at 7,000 g for 10 min. Two modified methods are used for isolation of outer

membrane proteins. In the first method cells were disrupted by sonication for four cycles. Extract was centrifuged at 16,000 g for 25 min at 4^0 C. Supernatant was diluted with ice cold 0.1 M Na_2CO_3, pH 11. The mix was incubated at 4^0 C. for 1 h then centrifuged at 26,000 g for 1 h. Pellets were washed in 2 mL of 50 mM Tris/HCl (pH 7.5) and centrifuged again. Extracted proteins were solubilized in rehydration buffer (7 M urea, 2 M thiourea, 0.5% v/v Triton X-100, 40 mM Tris-base, 30 mM DTT) (Yu et al., 2007). The second method for isolating OMPs was modified from Wright *et al*. (2005). Cells were collected by centrifugation at 7,000 g for 10 min at 4 ^{0}C. Then the pellet was treated with extraction buffer (3 M Urea, 28 mM DTT 0,5% w/v CHAPS) for 10 min and then centrifuged at 26,000 g for 15 min. The supernatant was recentrifuged at 4 ^{0}C at 26,000 g for 1 h and used as outer membrane protein source. Protein samples were stored at -20˚C at most for 10 days till usage. Since the method modified from Wright *et al*. (2005) gave most satisfactory results, for the rest of the study outer membrane protein extraction procedure was based on this method.

2.6. Preparation of Secreted Proteins

For secretome extraction, a slightly modified method reported by Damerval et al. (1986) was performed. In this method; *B. pertussis* strains were grown on Modified Morse-Bray medium (Morse and Bray, 1969) for 60 h at 37^{0}C. The cells were centrifuged at 16,000 g rpm for 15 min and pellet was discarded. 15% TCA+Acetone solution was added to the supernatant and incubated –20 ^{0}C for 45 min. After the incubation the solution was centrifuged and the pellet was washed two times with ice cold acetone containing 28 mM DTT at –20 ^{0}C for 2 h. After the centrifugation the pellet was vacuum dried. The powdered sample was stored at –20 ^{o}C.

2.7. Determination of Protein Concentration

To determine total protein concentration, the modified Bradford assay described by Ramagli and Rodrigez (1985) was used. 5X Bradford reagents (containing 500

mg Coomassie Brillant Blue G-250, 250 ml of 96% ethanol and 500 ml of 85% ortho-phosphoric acid; completed to a 1 L with dH_2O) was diluted 1:3 with dH_2O and filtered at least three times using Whatman No. 1 filter paper. For determination of the total protein concentration, bacterial cell extract was dissolved in 500 µl of rehydration buffer (containing 8 M urea, 2 M thiourea, 28 mM DTT, 2% (w/v) CHAPS, 0.5% (v/v) ampholyte pH 3-10). The suspension was mixed and incubated at room temperature for 1 h and then centrifuged at 12,000 xg for 5 min. To 20 µl of aliquots of the supernatant, 80 µl of 0.1 N HCl were added to protonate samples and mixed thoroughly. To this mixture, 3.5 mL of 1:3 diluted 5 X Bradford reagent was added, incubated at room temperature for 10 min and absorbance was measured at 595 nm. Bovine Serum Albumin (BSA) fraction number V was used as a standard for the construction of calibration curves.

2.8. 1D Gel Electrophoresis

For 1-D gel separation, an aliquot of 30 µg protein was solubilized with one volume of SDS-PAGE sample buffer and separated in 12 % acrylamide/bis-acrylamide gel with a Bio-Rad Cell system (Bio-Rad, USA), applying approximately 16 mA per gel. To visualize the separated proteins, gels were stained with colloidal Coomassie blue.

2.9. 2D Gel Electrophoresis

IPG strips were passively rehydrated by applying 400 µl of rehydration buffer (8 M urea, 2 M thiourea, 2% w/v CHAPS, 28 mM DTT and 0,5% v/v ampholyte 3-10) containing 350 µg protein sample for 14 h**.** IEF was performed with commercially available IPG strips (17 cm, pH 3–10, Bio-Rad) and the Protean IEF Cell (Bio-Rad USA). Rehydrated strips were taken to the IEF process. The following voltage profile was used for IEF: 1 h 100 V; 1 h 300 V; 1h 600 V; 1 h 1000 V; 2 h 3000 V; 2 h 5000 V followed by a linear increase to 8000 V. The final phase of 8000 V was terminated after 50,000 Vh. The IPG strips were

equilibrated for 15 min each in 5 mL of solution 1 (6 M urea, 50 mM Tris-HCl (pH 8.8), 30% v/v glycerin, 2% w/v SDS, 50 mg DTT) and then in 5 mL of solution 2 (6 M urea, 50 mM Tris-HCl (pH 8.8), 30% v/v glycerin, 2% w/v SDS, 225 mg iodacetamide) (Görg, 2004). The isolated proteins were separated in 12 % acrylamide/bis-acrylamide gels with a Bio-Rad Cell system (Bio-Rad, USA), applying approximately 25 mA per gel. To visualize the separated proteins, each gels were stained with colloidal Coomassie blue (Neuhoff et al., 1988). Coomassie stained gels were digitized by using a scanner (HP Scanjet 4070 Photosmart scanner, USA). Spot pattern analyses were accomplished by using the 2D image analysis software Delta2D version 3.3 (Decodon, Germany).

2.10. Preparation of Antisera against *B. pertussis*

B. pertussis Tohama and Saadet strains were grown on Cohen-Wheeler agar media for 48 h. The cells were suspended in 0.85 % saline solution to contain ca. $4x10^{10}$ bacteria/ml. For subcutaneous immunization, the suspension was inactivated at 56^{o} C for 30 min. Inactivated bacterial cells were used as the antigen and anti-*B. pertussis* polyclonal antibodies were raised by immunizing mice. For each strain, ten mice received two subcutaneous injections of 0.5 ml per animal at two week intervals between the first and second injections. Their sera were collected and pooled 14 days after the second injection.

In Western Blot analysis, the antisera Th (sc), Sa (sc) were used. The antisera were obtained from the mice which were subcutaneously immunized with inactivated whole cells of *B. pertussis* Tohama I and Saadet strains, respectively.

2.11. Western Blotting of 2-DE gels

Proteins from the identical, but non-stained gels were transferred to a NC membrane for 1 h at 400 mA in transfer buffer (25 mM Tris, 192 mM glycine, 2% w/v SDS and 20% v/v methanol) by using semi dry blotter (Cleaver Scientific Ltd.). After the transfer, the membrane was blocked for 2 h with 10% skim milk

in TBS solution (20 mM Tris and 5M NaCl) at 37^{o}C. After rinsing for 10 min with TBS-Tween 20, the membrane was incubated with primary antibody, mouse anti-*B. pertussis*, at a dilution of 1:300 in 0.05% Tween-20 in TBS (TTBS) containing 5% skim milk for 1 h at room temperature on a gentle shaker. The membrane was rinsed for 10 min and incubated with rabbit anti-mouse IgG-alkaline phosphatase, (Sigma) at a dilution of 1: 15,000 in TTBS containing 5% skim milk for 1 h. The membrane was then washed with TBS for 10 min and developed with substrate (AP Conjugate Substrate Kit, Bio-Rad) until optimum color was developed. The immunoreactivity of each spot that gave positive signal in Western blot analysis was verified through their excision from 2-D gels followed by dot-blotting.

2.12. Dot Blotting

In order to confirm the immunogenic reaction of the spots after the Western Blot analysis, the serum reactive protein spots were cut from the stained 2-DE gels and placed into a 96 well plate. Spots were destained with destaining solution (10 % Methanol, 20 % Acetic acid, 70 % dH_2O) until the dye is removed. Spots were first washed with dH_2O and secondly with 10% SDS, two times each for 10 min. The protein gel spots were placed onto the NC membrane for Western blotting. For Western analysis, the modified method of Towbin et al. (1979) was used. After the second Western blot, the proteins which gave positive reaction were considered as 100 % immunogenic.

2.13. MALDI-TOF-MS Analysis

MALDI-TOF-MS/MS was performed in Greifswald, Germany for the identification of 2D spots as described in Eymann et al.(2004). Protein spots were excised from stained 2D gels, destained and digested with trypsin (Promega, Madison, WI, USA). For the extraction of peptides, the gel pieces were covered with 60 µl of 0.1% trifluoroacetic acid in 50% CH_3CN and incubated for 30 min

at 40^{O}C. Peptide solutions were mixed with an equal volume of saturated α-cyano-3- hydroxycinnamic acid solution in 50% acetonitrile-0.1% trifluoroacetic acid (v/v) and applied to a sample plate for MALDI-TOF-MS. Mass analyses were carried out on the Proteome-Analyzer 4800 (Applied Biosystems). The three most abundant peptides in each MS spectrum were chosen for MS/MS experiment. The resulting sequence data were included in the database search to increase the reliability of protein identification. Mass accuracy was usually in the range between 10 and 30 ppm.

2.14. LC-MS Analysis

One lane of 1D-SDS gel was cut into 12 equidistant pieces. In-gel tryptic digestion as well as peptide elution for LC-MS/MS was performed for each of the gel pieces as described by Eymann *et al*. (2004). The nano-LC-MS/MS analysis of peptides derived from tryptic in-gel digestion was performed on a linear trap quadrupole (LTQ) Oribtrap (Thermo Fisher Scientific, Waltham, MA) equipped with a nanoACQUITY UPLC (Waters, Milford, MA). Peptides were loaded onto a trapping column (nanoAcquity Symmetry UPLC column, C_{18}, 5 µm, 180 µm by 20 mm; Waters) at a flow rate of 10 µl/min and washed for 3 min with 99% buffer A. Peptides were then eluted and separated *via* an analytical column (nanoAcquity BEH130 UPLC column, C_{18}, 1.7 µm, 100 µm by 100 mm; Waters) with a 80 min gradient (from buffer A (0.1% acetic acid) to buffer B (0.1% acetic acid, acetonitrile). The mass spectrometric analysis started with a full survey scan in the Orbitrap (*m/z* 300 to 2,000, resolution of 60,000) followed by collision-induced dissociation and acquisition of MS/MS spectra of the five most abundant precursor ions in the LTQ. Precursors were dynamically excluded for 30 s, and unassigned charge states as well as singly charged ions were rejected. Proteins were identified via an automated database search using the SEQUEST software (Bioworks v.3.2, Thermo Electron). The search results were imported to Scaffold 2.02.01 (Proteome Software) used to validate MS/MS-based peptide and protein identifications. Peptide and protein identifications were accepted if they could be

established at greater than 99.9 % probablility and contained at least two identified peptides.

2.15. Protein Identification and Database Searches

Amino acid sequences for *B. pertussis* proteins were obtained from Sanger Institute organism's genome project web site (http://www.sanger.ac.uk/Projects/B_pertussis/). The peak lists of each protein spot were analyzed with the aid of "PMF" and "MS/MS Ion Search" engines of MASCOT software (http://www.matrixscience.com/). The searches considered oxidation of methionine and modification of cysteine by carbamidomethylation as well as partial cleavage leaving one internal cleavage site. Of the results given by the MASCOT software, those having a probability score value higher than 53 were considered for successful protein identification. To find out putative functions, protein accession numbers of the identified spots were searched in the website for *B. pertussis*. To predict epitopes of the identified immunogenic proteins of *B. pertussis*, the artificial network based B-cell epitope prediction server ABCpred (Saha and Raghava, 2006) was used. The PSORTb version 2.0.4 (Gardly et al., 2005) was used for prediction of subcellular localization of the cytoplasmic immunogenic proteins. The CELLO version 2.5 (Yu et al., 2006) was used for prediction of subcellular localization of the secretome and outer membrane proteoms. The SignalP webserver (http://www.cbs.dtu.dk/services/signalp/) was employed for signal peptide prediction. Functional categories of the identified proteins were determined using KEGG BRITE database (http://www.genome.jp/kegg/brite.html).

2.16. Relative abundance of surface proteins

Relative spectral counts (R_{SC}) were calculated for quantitation of abundance differences of the proteins identified by LC-MS/MS from two strains (Beissbarth et al., 2004; Old et al., 2005). For each protein, $\log_2$ ratio of abundance between

Sample 1 and Sample 2 constituted an R_{SC} value (Equation 1). n_1 and n_2 designate spectral counts for the protein in Sample 1 and Sample 2, t_1 and t_2 are total spectral count (sampling depth) for Sample 1 and 2; and f is the correction factor set to 1.25 instead of 0.5, as proposed by Old et al. (2005).

$$R_{SC} = \log_2[(n_2 + f)/(n_1 + f)] + \log_2[(t_1 - n_1 + f)/(t_2 - n_2 + f)]$$

The changes in R_{SC} values greater than 2 fold were accepted as significant.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Theoretical Proteome Map of *B. pertussis*

For proteomic studies, it is important to cover as many proteins as possible by keeping the gel number limited. The theoretical gel of *B. pertussis* was obtained from gelbank database (http://gelbank.anl.gov/2dgels/index.asp, Figure 3.1). Based on this theoretical proteome map gel, pH range 3-10 was chosen for the rest of the experiments to cover most of the proteins of the organism.

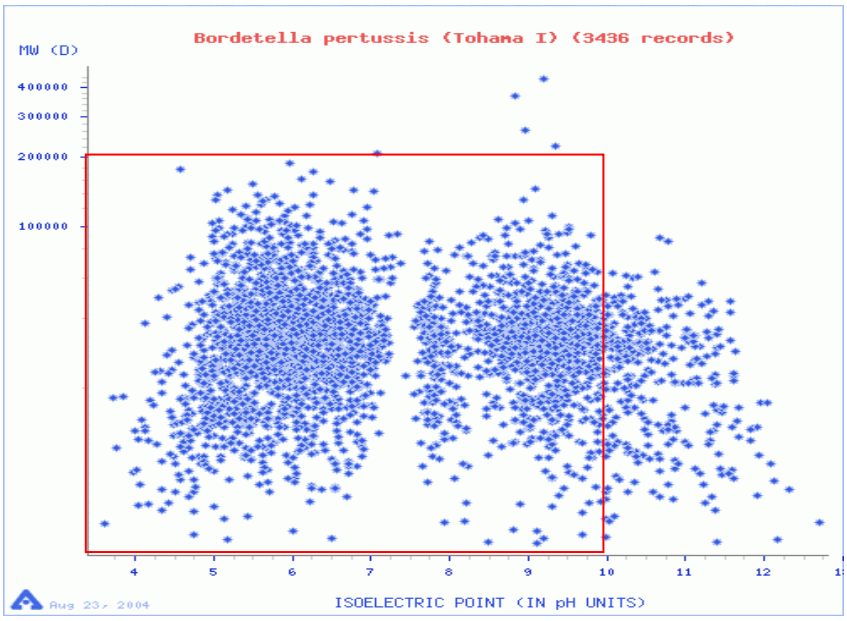

Figure 3.1. Theoretical proteome gel of *B. pertussis*. pH 3-10 range was indicated by a rectangle.

3.2. Master Gels of *B. pertussis* Strains

Since running all 2D gels identically is difficult in proteomic experiments, replicate analysis of all gels for a sample must be done. Master gel, generally composed of three gel replicates, can be defined as a reference expression image. With the advantage of master gels, additional analysis such as comparing differences between biological variations can also be performed.

In this study, master gels for cytoplasmic proteome were prepared by proteome samples of *B. pertussis* at exponential phase to visualize wide range of proteins (Figure 3.2 and 3.3). Subproteome master gels were prepared from the cultures at stationary phase to observe virulence associated secreted and outer membrane proteins of the organism (Figure 3.4, 3.5, 3.6 and 3.7). Soluble cytoplasmic protein samples were collected at 48^{th} our and subproteome samples at 72^{nd} hours. By using pH 3-10 range gels, the proteins of *B. pertussis* Tohama I and Saadet strains were observed. Three technical replicate of 2-DE gels for each of the three independent biological samples were run for each strain. The master gels obtained were used as the templates for the rest of the study.

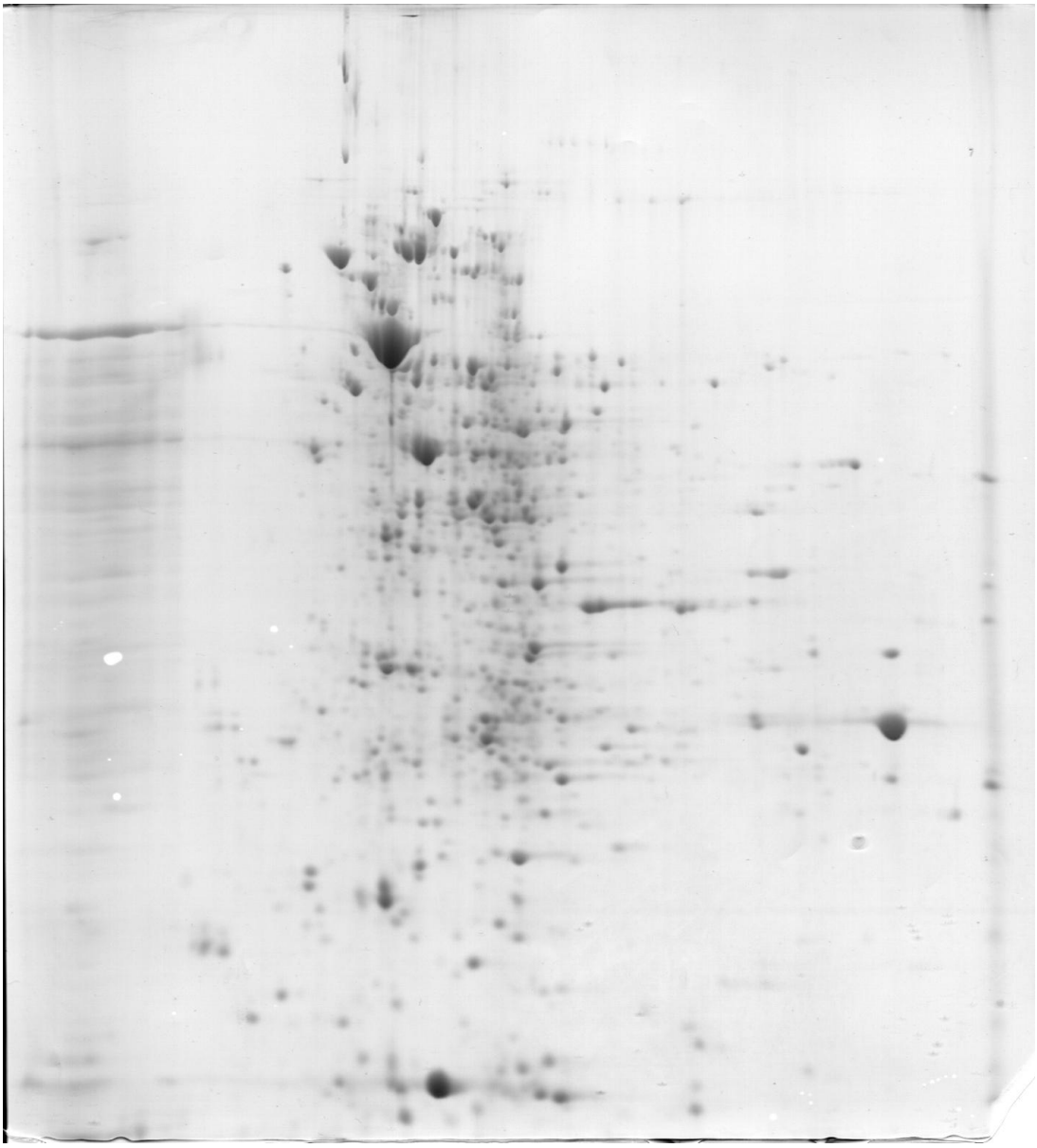

Figure 3.2. 2-D master gel of the cytoplasmic proteome of *B. pertussis* Tohama I.

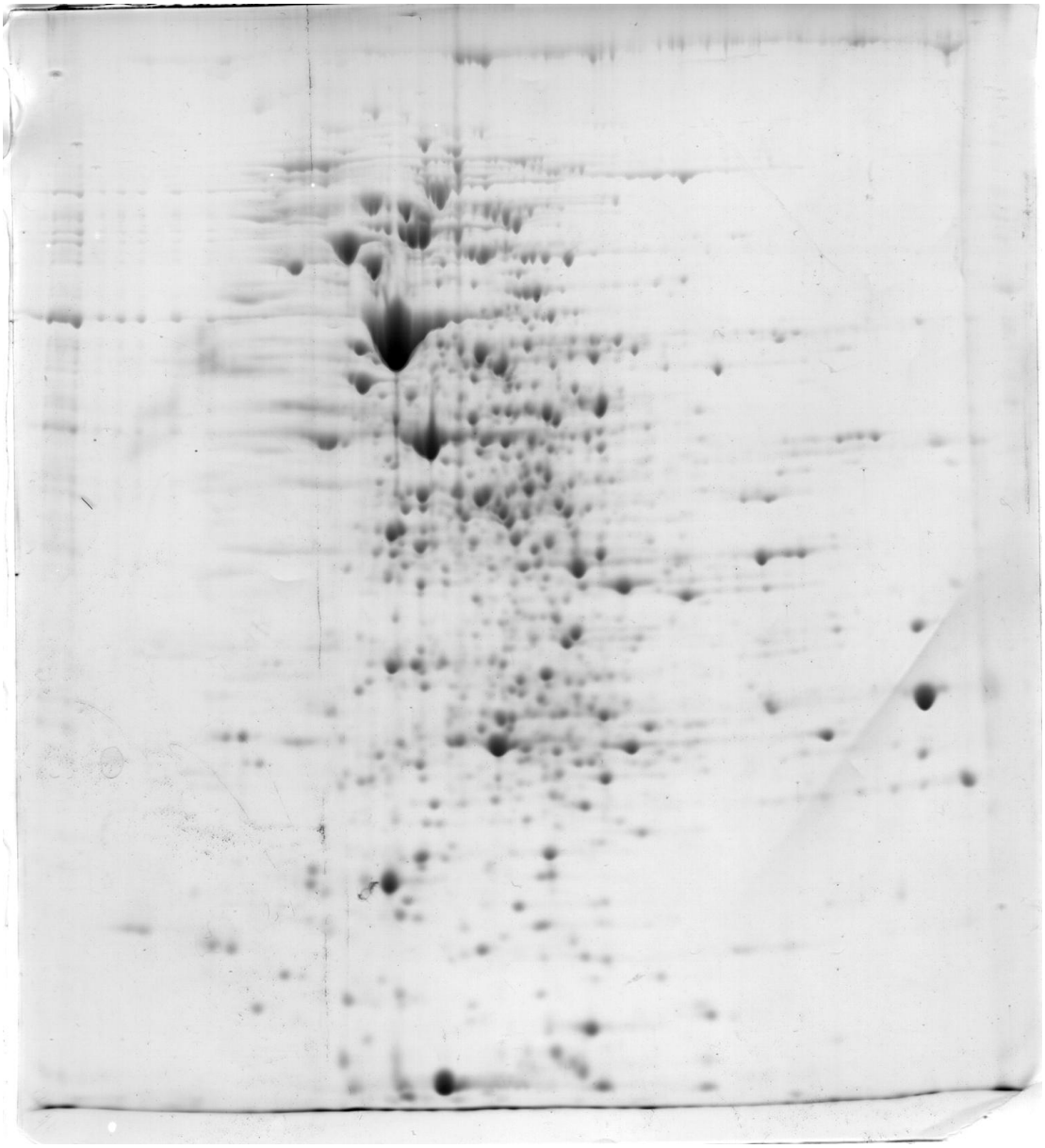

Figure 3.3. 2-D master gel of the cytoplasmic proteome of *B. pertussis* Saadet.

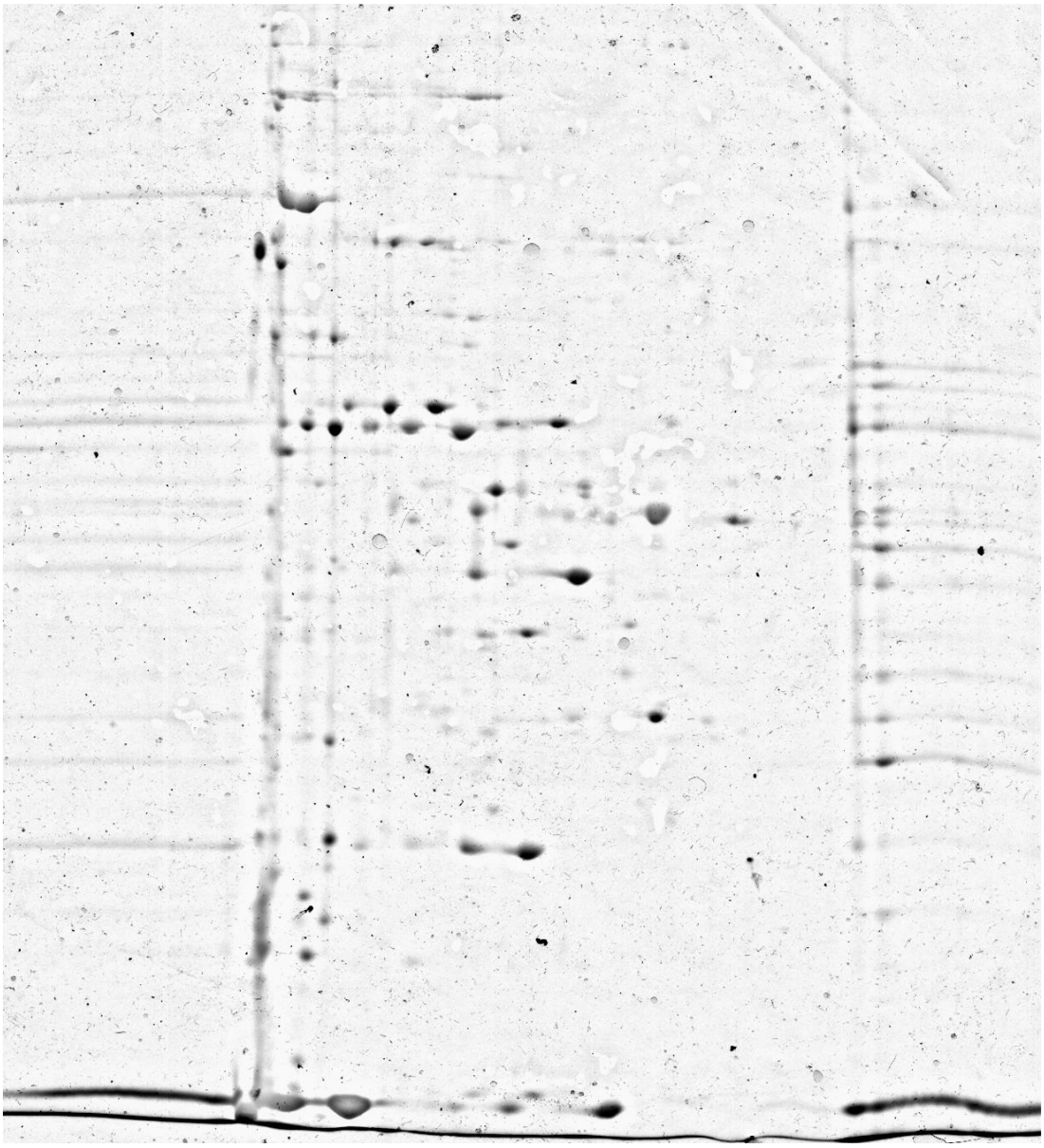

Figure 3.4. 2-D master gel of the surface proteins of *B. pertussis* Tohama I.

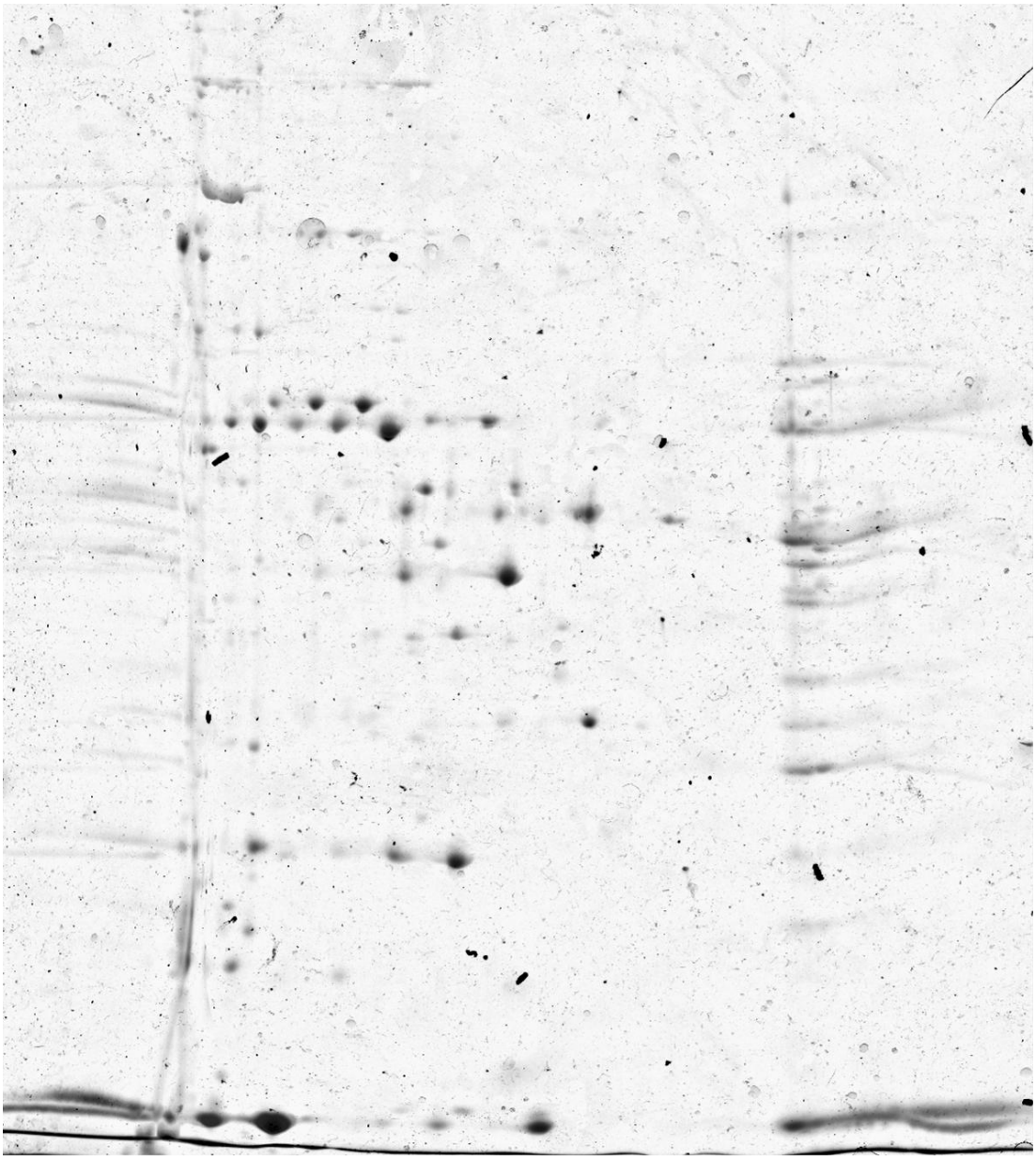

Figure 3.5. 2-D master gel of the surface proteins of *B. pertussis* Saadet.

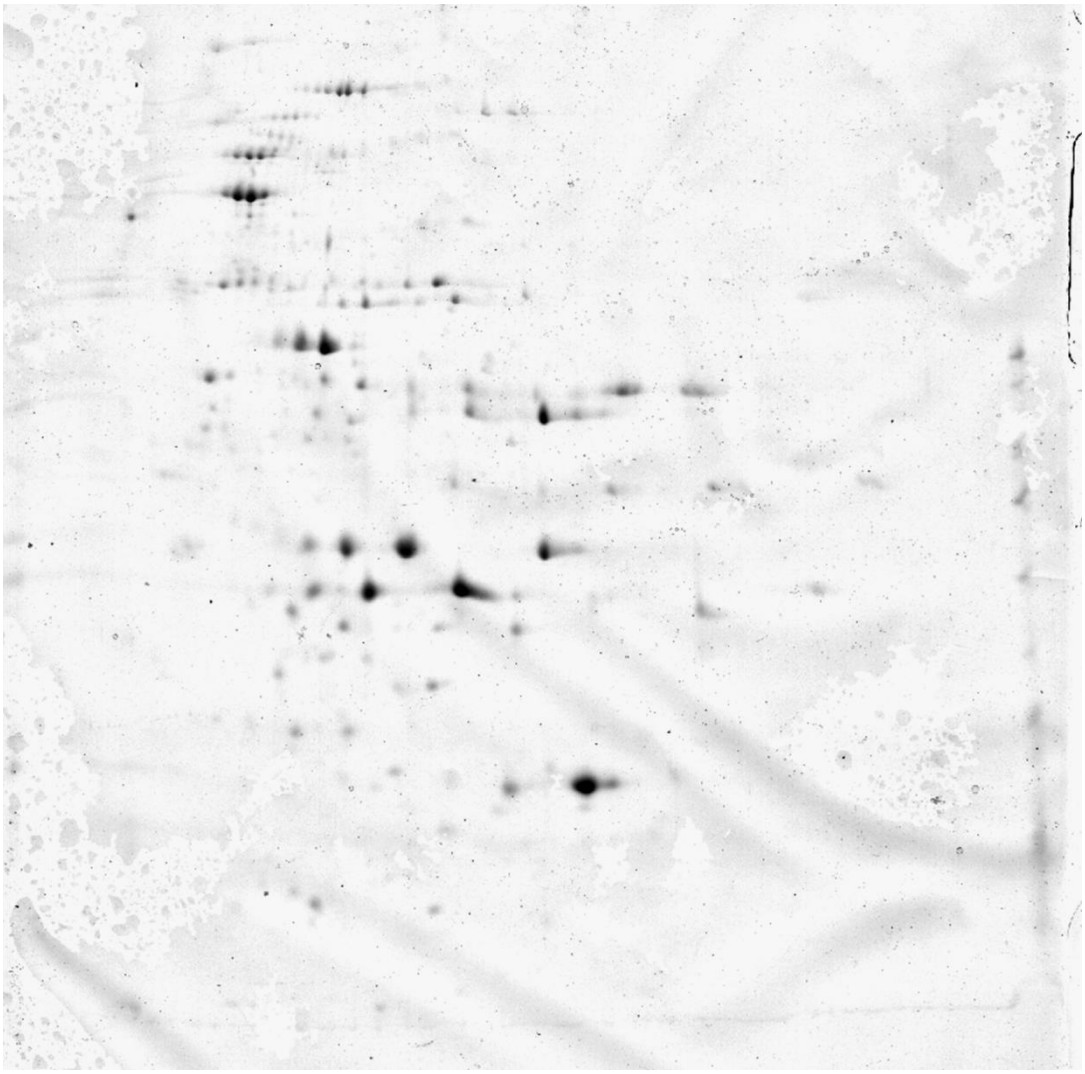

Figure 3.6. 2-D master gel of the secretome of *B. pertussis* Tohama I.

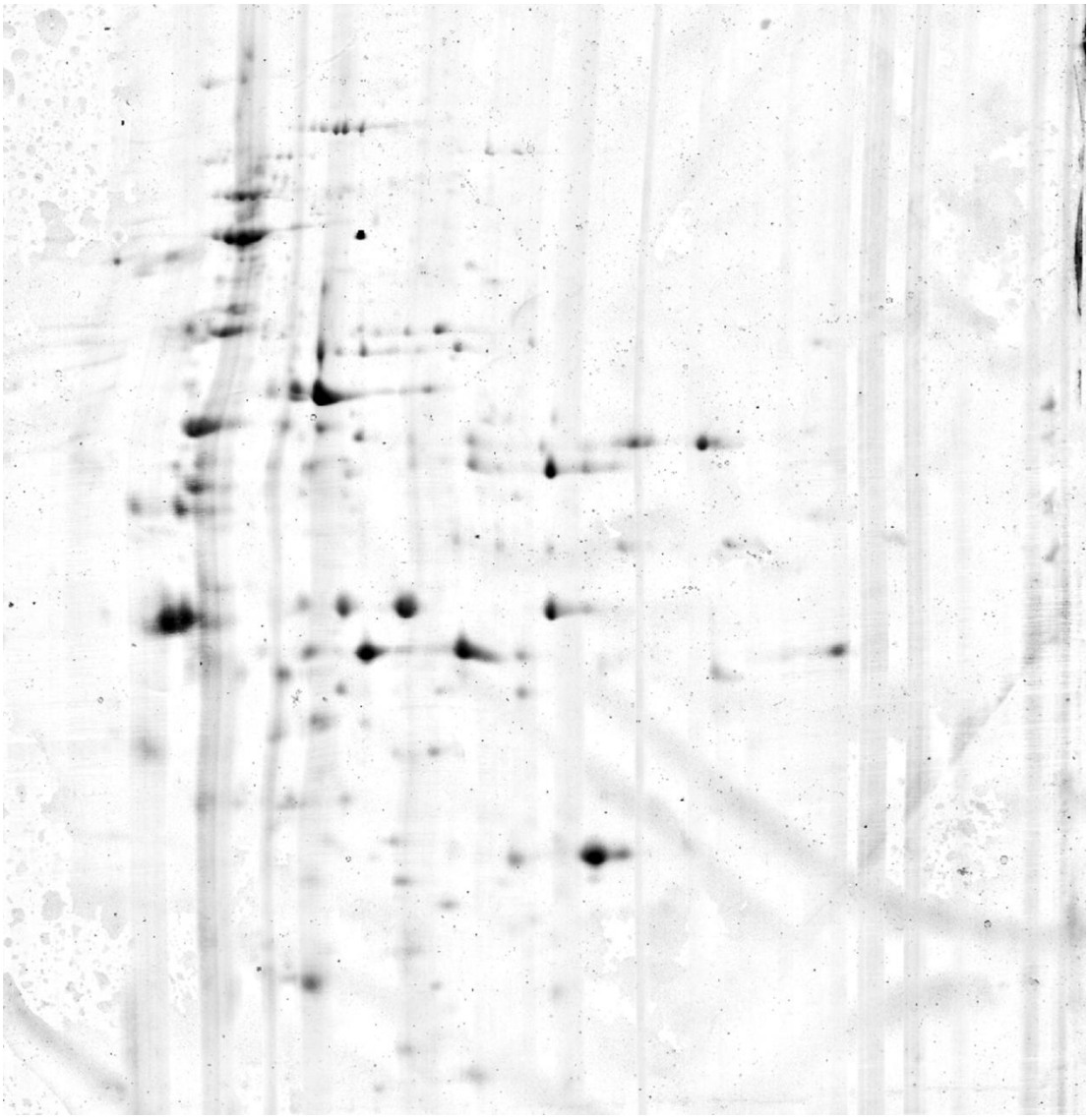

Figure 3.7. 2-D master gel of the secretome of *B. pertussis* Saadet.

3.3. Cytoplasmic Proteomes of *B. pertussis* Tohama and Saadet Strains

Cytoplasmic proteomes of *B. pertussis* Tahoma I and Saadet strains were separated by 2DE. 350 µg of protein samples were applied to the IPG strips and protein spots on 2D gels were visualized by colloidal CBB G250 staining method. Within the pH range 3-10, over 600 spots could be detected on the gels (Figure 3.2 and 3.3). For dual channel 2D image analysis, master gels of *B. pertussis* Tohama I (green) and Saadet (red) were used (Figure 3.8).

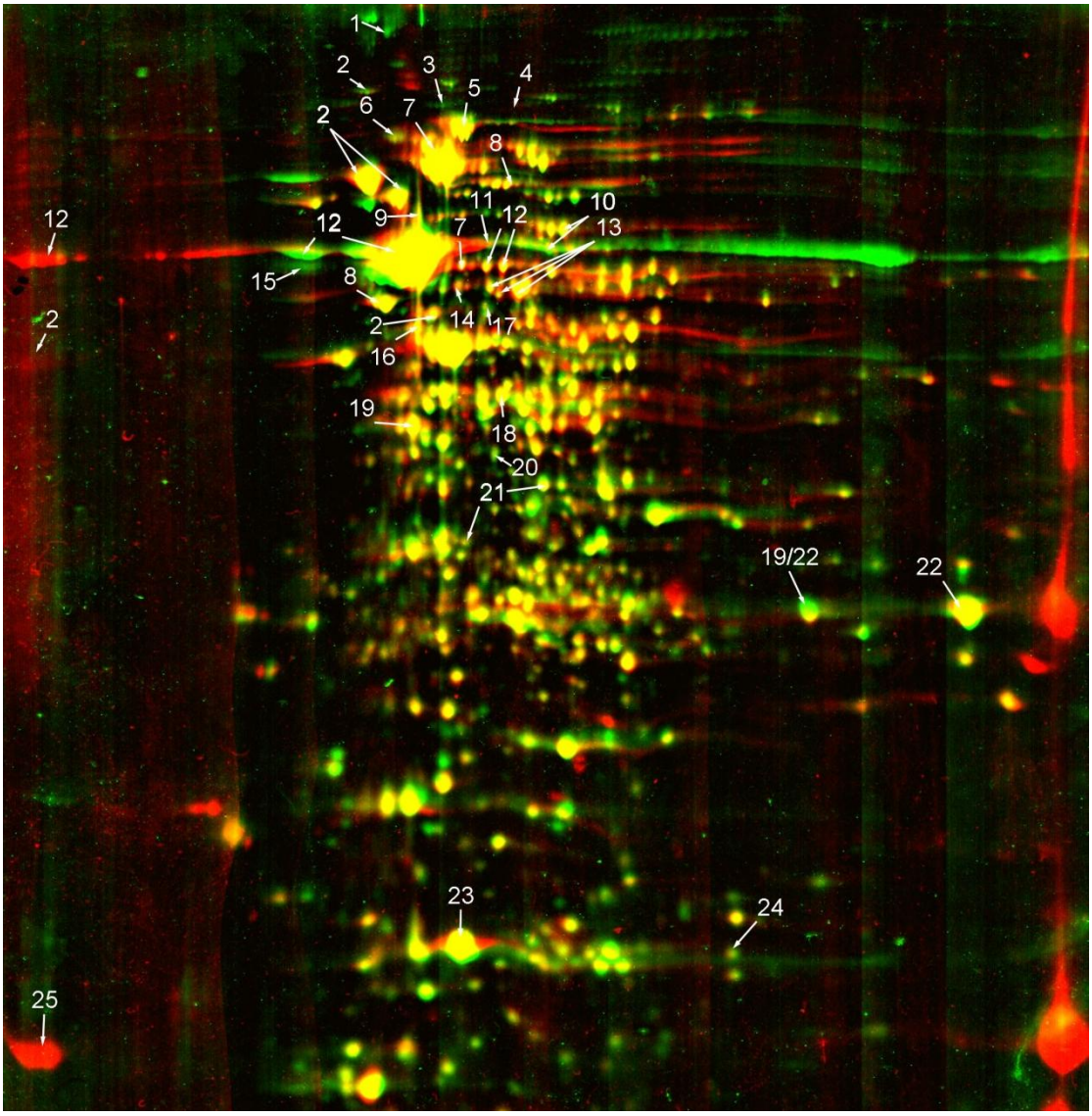

Figure 3.8. Dual channel 2-D imaging of *B. pertussis* strains Tohama I (green) and Saadet (red) (Altındiş et al., 2009).

3.3.1. Identification of Cytoplasmic Immunoreactive Proteins

Cytoplasmic immunogenic proteins of two strains were detected by Western blotting of the 2D gels using antisera: Th (sc) and Sa (sc). A total of 45 immunorective proteins were observed from Western blots. These spots matched well with the spots seen on the Coomassie-stained gels. For protein characterization, the corresponding spots were excised from gels, digested and analyzed by using MALDI-TOF/MS. Forty out of 45 spots could be identified which corresponded to 25 different gene products (Fig. 3.9 and 3.10; Table 3.1) (Altındiş et al., 2009).

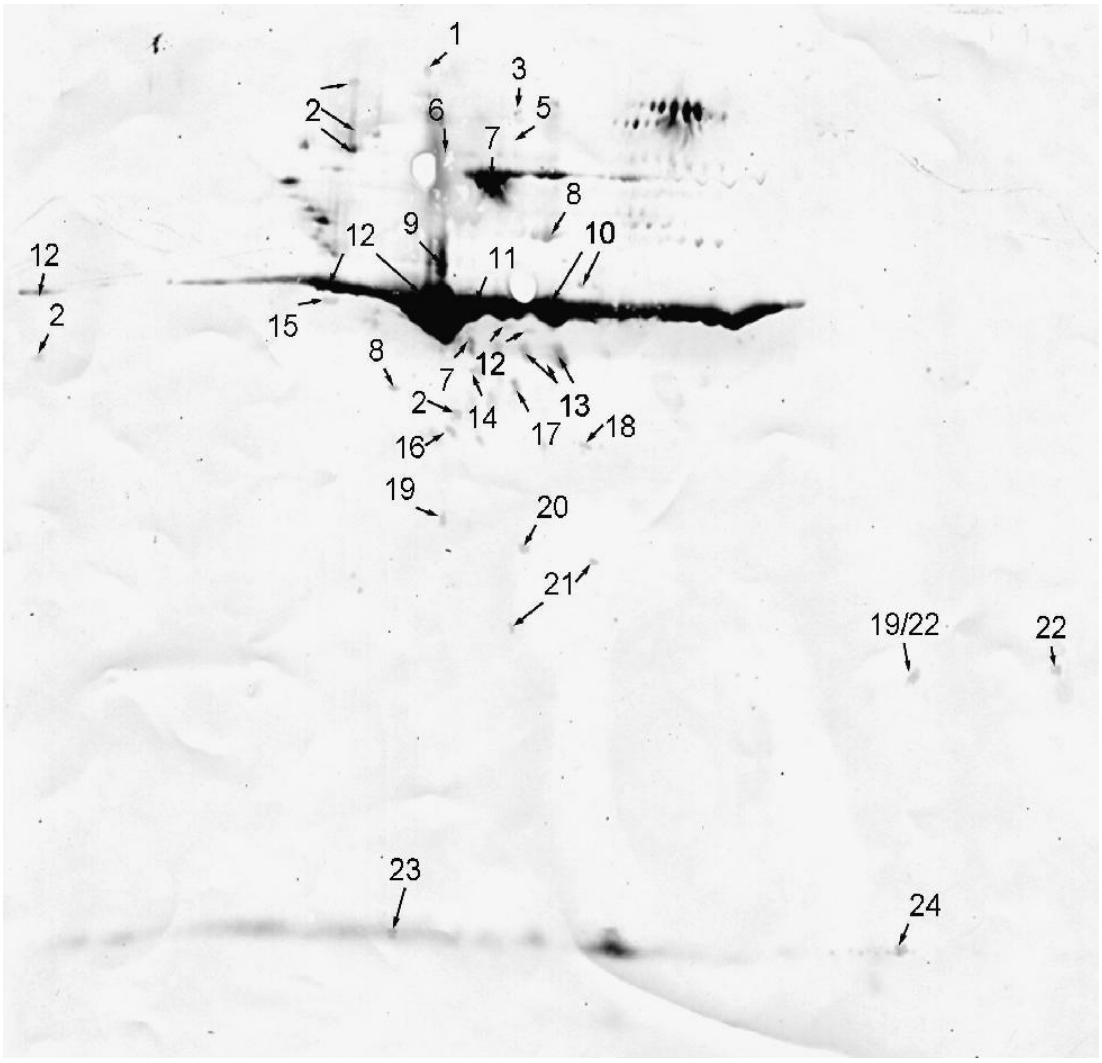

Figure 3.9. 2-D Western blot analysis of the total soluble proteome of *B. pertussis* strain Tohama I against antiserum Th (sc) (Altındiş et al., 2009).

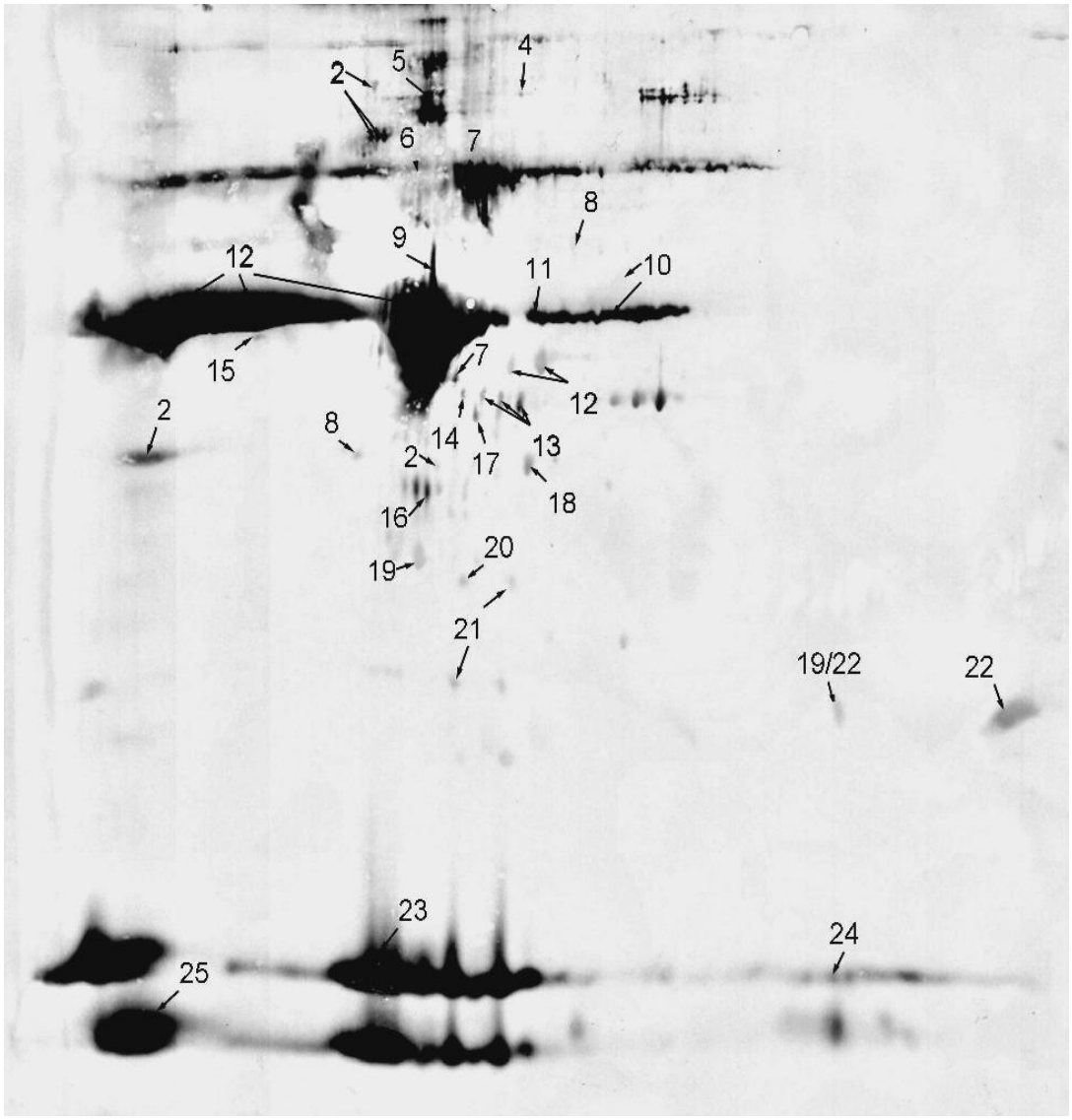

Figure 3. 10. 2-D Western blot analysis of the total soluble proteome of *B. pertussis* strain Saadet against antiserum Sa (sc) (Altındiş et al., 2009).

3.3.2. Epitope Prediction, Subcellular Localization and Signal Peptides

An epitope is the part of a macromolecule that is recognized by immune system, specifically by antibodies, B cells or cytotoxic T cells. Peptide-based vaccines in which epitopes are used to provoke an immune reaction have attracted

considerable attention recently as a potential means both of treating infectious diseases and promoting the destruction of cancerous cells by a patient's own immune system. The server ABCpred was used for B-cell epitope prediction. In our study, the accuracy scores of the predicted epitopes of all identified immunogenic proteins were within a meaningful range. PSORTb analysis predicted that 13 of the 25 immunogenic proteins are localized in cytoplasm, 4 proteins on outer membrane and 1 protein in the periplasm of the organism. The localization of 7 proteins could not be determined. According to SignalP analysis, 5 of the 25 immunogenic proteins were predicted to have a signal peptide sequence (Altındiş et al., 2009) (Table 3.1).

Table 3.1. Cytoplasmic immunogenic proteins detected in total soluble proteome of *B. pertussis* Tohama I and Saadet strains (Altındiş et al., 2009).

Order[a]	Protein name/function	Gene locus	Mass (Da)	pI	Number of isoforms [b]	Epitope predicton	Subcellular localization[c]	Signal peptide	Straiin[d]	Antiserum[e]
1	Putative chromosome partition protein	BP3558	130596,01	5,02	-	RELAEMPDEWRKASWL	C	-	Th	Th (sc)
2	Heat shock protein 70	BP2499	71110,52	5,04	3 (C-M)	KVKEFFGREPRKDVNP	C	-	Th, Sa	Th (sc), Sa (sc),
3	Preprotein translocase secA subunit	BP3014	103237,4	5,47	-	TRSIETAQRKVEGRNF	C	-	Th	Th (sc)
4	Carbamoyl-phosphate synthase large chain	BP1453	118066,52	5,25	-	VEKIIEREKPDALLPT	ND	-	Sa	Sa (sc)
5	ATP-dependent protease, ATPase subunit	BP1198	96274,57	5,37	-	RMEIDSKPEVMDRLDR	C	-	Th, Sa	Th (sc), Sa (sc)
6	Phosphoenolpyruvate sytnhase	BP1436	86182,65	5.02	-	GGSRYEPEEENPMLGL	C	-	Th, Sa	T h(sc), Sa (sc)
7	Serum resistance protein	BP3494	103314,7	6.62	-	YSLAEDPKTHVWSLQR	OM	+	Th, Sa	Th (sc), Sa (sc),
8	ATP synthase subunit B	BP3284	50498,8	4.94	-	SMIEQARREAEAERAR	ND	-	Th, Sa	Th (sc), Sa (sc),
9	30S ribosomal protein S1	BP0950	62979	5,1	-	KQLGEDPWVGLARRYP	C	-	Th, Sa	Th (sc), Sa (sc),
10	Pertactin	BP1054	68644,38	6,92	2 (M)	TLTGGADAQGDIVATE	OM	+	Th, Sa	Th (sc), Sa (sc),)
11	Putative substrate-CoA ligase	BP0624	54538,05	5,62	-	CVALLAPPTPDAMVCL	C	-	Th, Sa	Th (sc), Sa (sc)
12	Heat shock protein 60	BP3495	53383,87	5,09	5 (C)	TGLKGDTADQNAGIKL	C	-	Th, Sa	Th (sc), Sa (sc),
13	Serine protease	BP2434	5210,42	7.79	3 (C)	PLTIGDPKTLKKGQWV	P	+	Th, Sa	Th (sc), Sa (sc)

Table 3.1. Continued

14	Glutamyl-tRNA amidotransferase subunit A	BP0372	53383,87	5.37	-	KMLAGYASPFDATVVE	ND	-	Th, Sa	Th (sc), Sa (sc)
15	Lysyl-tRNA synthetase	BP1102	56593,81	5,27	-	IGDIIAIEGPVFKTNK	C	-	Th, Sa	Th (sc), Sa (sc)
16	S-adenosylmethionine synthetase	BP3071	41976,16	5,12	-	TSESVSEGHPDKVADQ	C	-	Th, Sa	Th (sc), Sa (sc)
17	Fumaryl acetoacetase	BP3135	47214,71	5.49	-	AQLVAHHTVNGCNLQP	ND	-	Th, Sa	Th (sc), Sa (sc)
18	RNA polymerase alpha subunit	BP3642	36136,08	5,6	-	NELLKTPNLGRKSLNE	C	-	Th, Sa	Th (sc), Sa (sc)
19	Elongation factor Tu	BP3611	42889,12	5,34	-	PGSINPHTDFTAEVYI	C	-	Th, Sa	Th (sc), Sa (sc)
20	Ketol-acid reductoisomerase	BP0791	36211,39	5,76	-	QGHAHALNLHDSGVKV	ND	-	Th, Sa	Th (sc), Sa (sc)
21	Putative peptidyl-prolyl cis-trans isomerase	BPP3352	28946.21	8.93	-	PITQKSLDEFVKLVVS	OM	+	Th, Sa	Th (sc), Sa (sc)
22	Aspartate-semialdehyde dehydrogenase	BPP1945	40340.78	5.08	2 (C)	AGTQWAKVVPNTKEDT	ND	-	Th, Sa	Th (sc), Sa (sc)
23	Putative DNA-binding protein	BB2935	18512.33	6.19	-	MFMAQYTEEWNALDSI	ND	-	Th, Sa	Th (sc), Sa (sc)
24	Heat schock protein 10	BPP0869	10265.62	5.39	-	AVGPGKKTEDGKILPV	C	-	Th, Sa	Th (sc), Sa (sc)
25	Putative outer membrane protein	BB2614	20920.96	9.69	-	RKRREFQEDFNRRRNE	OM	+	Sa	Sa (sc)

[a] Protein spot corresponding to position on gel (Fig. 1).
[b] C, charge modification; M, mass modification; C-M, both charge and mass modification
[c] Predicted location of proteins by PSORTb version 2.0.4. C: Cytoplasm, OM: Outer membrane, P: Periplasm and ND: Not determined
[d] Strains used in this study. Th: Tohama I and Sa : Saadet
[e] Th (sc) and Sa (sc) were obtained from mice subcutaneously immunized with inactivated whole cells of Tohama and Saadet, respectively , Th (ip) and Sa (ip) were obtained from mice intraperitoneally challenged with live whole cells of Tohama and Saadet, respectively.

3.3.3. Evaluation of Cytoplasmic Immunogenic Proteins

The immunoproteomic approach in this study led to the identification of a total of 25 immunogenic proteins of *B. pertussis* which can be divided into three main groups. The first group contains 3 well-known antigens of *B. pertussis*: pertactin, serum resistance protein and Hsp60. The second group includes 10 proteins that have already been shown antigenic in certain pathogenic bacteria, but not in *B. pertussis* before and the third group comprised 12 proteins which have not been shown to be immunogenic in any pathogen till the present study (Altındiş et al., 2009).

3.3.3.1. Putative Chromosome Partition Protein

In a previous study, it was found that putative chromosome partition protein Spo0J of *B. subtilis* plays an important role in chromosome partition during sporulation. The authors proposed that Spo0J protein assembles on a centromere-like site which results in the partitioning of the nucleoids to daughter cells (Lin et al., 1997). It is also known that in Gram-negative bacteria, plasmid partition system P1*par* family members assist the active segregation of a variety of plasmids and plasmid prophages (Sergueev et al., 2005). A Walker type ATPase which is crucial for plasmid movement is encoded by *parA.* Before partition, when ParS protein, encoded by *parB* gene, binds to the partition site, the plasmid is captured at the center of the cell. It is known that some other pathogens such as *S. flexneri* and *Y. pestis* posses these genes (Youngren et al., 2000). Our study constituted the first report on the presence of putative chromosome partition protein in *B. pertussis* as well as its immunogenic function (Altındiş et al., 2009).

3.3.3.2. Heat Shock Protein 70 (Hsp70)

Hsp70 has various functions such as folding of newly synthesized proteins, refolding of misfolded/aggregated proteins, membrane translocation of proteins,

and activity of proteins which have regulatory functions (Mayer and Bukau, 2005). The antigenic activity of *Mycobacterium tuberculosis*-derived Hsp70 was shown earlier (Kaufmann et al. 1987; Young et al., 1988; Kaufmann, 1990) and its function as an adjuvant in stimulating host immune responses has been characterized (Lussow et al., 1991; Suzue and Yang, 1996). The immunogenic activity of Hsp 70 has also been shown in *Mycobacterium leprae* (Adams et al., 1993), *Cryptococcus neoformans* (Kakeya et al., 1999) and *Francisella tularensis* (Havlasová et al., 2002). It was found that the DnaK protein of *B. anthracis* showed high immunogenic activity and also stimulated humoral and cell-mediated immunity. Although immunization with DnaK conferred no protection to mice against *B. anthracis* infection (Sinha and Bhatnagar, 2010). It is also known that whole cell *B. pertussis* vaccine induced immune response against Hsp70 (Del Giudice et al., 1993).

3.3.3.3. Preprotein Translocase SecA Subunit

SecA is a component of a prokaryotic translocase which can be found as a structural component of the preprotein channel and second, as an ATP-dependent membrane cycling factor which is responsible for the translocation of proteins across the cytoplasmic membrane by binding to their signal sequences (Schmidt and Kiser, 1999). In *B. pertussis*, the transportation of important virulence factors like BrkA, PRN FHA and PT is Sec-dependent (Rambow-Larsen and Weiss, 2004). The immunogenic activity of this protein has been demonstrated by our group for the first time (Altındiş et al., 2009).

3.3.3.4. Carbamoyl-phosphate Synthase Large Chain

It is a 160 kDa enzyme composed of one large and one small subunit. Large subunit is responsible for the catalysis of carbomoyl phosphate synthesis only when the nitrogen source is ammonium. This subunit also serves as the site for the binding of allosteric ligands and divalent and monovalent cations (Schofield,

1993; Thoden et al., 1997). Our study constituted the first report about the immunogenicity of this protein (Altındiş et al., 2009).

3.3.3.5. ATP-dependent Protease, ATPase Subunit

Molecular chaperones and energy-dependent proteases are crucial for the maintenance of cell homeostasis. Energy-dependent proteases are responsible for targeted degradation of regulatory proteins, misfolded, denatured or aggregated polypeptides. Well-studied Clp proteases in *Escherichia coli* are composed of two subunits: a regulatory ATPase/chaperone subunit (ClpA, ClpX, ClpY) and a proteolytic subunit (ClpP, ClpQ). After recognized by chaperone subunit, damaged proteins are unfolded there and then transferred to proteolytic subunit (Singh et al., 2000). Inside this subunit, proteins are degraded to peptide fragments. These proteases sharing a conserved structure are found in eubacteria, plants and mammals; however, the type and number of ATPase/chaperone subunit shows difference among organisms (Schelin et al., 2002).

Lon is an ATP-dependent protease which is an important contributor to proteolysis in *E. coli*. The mutant of *S. enterica* serovar Typhimurium with a disruption of Lon was able to efficiently invade cultured epithelial cells and showed increased production and secretion of three identified *Salmonella* pathogenicity island 1 proteins, SipA, SipC, and SipD (Takaya et al., 2002). It was also shown that *S. enterica* with a disrupted ATP-dependent protease can be useful in developing live vaccine strains (Matsui et al., 2003). In our study, ATP-dependent protease showed an immunogenic activity during *in vivo* infection.

3.3.3.6. Phosphoenolpyruvate Synthase (PEP Synthase)

PEP synthase is involved in the glucose metabolism by catalyzing the phosphorylation of pyruvate to PEP with the hydrolysis of ATP to AMP. This reaction is required for the synthesis of carbohydrates and citric acid cycle

intermediates that are essential for cell growth (Smyer and Jeter, 1989). The immunogenic activity of this protein was shown by our laboratory for the first time (Altındiş et al., 2009).

3.3.3.7. Serum Resistance Protein (BrkA)

Serum resistance protein (BrkA) is a well-known virulence factor with autotransporter function of *B. pertussis*. It confers serum resistance and mediates adherence and invasion (Del Giudice et al., 1993; Fernandez and Weiss, 1994). In a recent study, it was shown that BrkA enhances the efficacy of the two-component DTPa vaccine containing pertussis toxin (PT) and filamentous hemagglutinin (FHA). The protection level of this vaccine was similar to that of the commercial *Infanrix*™ vaccine, containing pertussis toxin (PT), FHA and PRN (Marr et al., 2008).

3.3.3.8. ATP Synthase Subunit B

ATP synthase is a ubiquitous membrane enzyme that playing a key role in biological energy metabolism. It consists of two portions F_1 and $F_{0.}$ The F_0 region is a proton pore and contains three main subunits A, B and C. The B subunit includes a membrane anchor, one transmembrane α-helix in *E. coli*, that interacts with the intramembrane A subunit. B subunit interacts with F_1 and holds back the ring of A and B subunits, keeping it from rotating along with the central stalk (Deckers-Hebestreit and Altendorf, 1996). Our study constituted the first report about the immunogenicity of this protein (Altındiş et al., 2009)

3.3.3.9. 30S Ribosomal Protein S1

S1 protein is needed for binding mRNA; thus facilitating recognition of initiation point and translation of mRNA with purine-rich sequences. It is an acidic protein and considered as fractional which is found less then one copy per ribosome. S1

to ribosome ratio is 0.1 to 0.3 in monosomes and 1.0 in polysomes. The protein is elongated, contains two domains, and appears to function as a relatively nonspecific mRNA binding protein that brings the mRNA into the vicinity of the ribosomal region at which translation is initiated (Hermoso and Szer, 1974). The immunogenic activity of this protein was reported by Altındiş et al. (2009) for the first time.

3.3.3.10. Pertactin

Pertactin is an outer membrane autotransporter protein which is responsible for attachment and invasion of eukaryotic cells. It is also used as a component of acellular vaccines (Everest et al., 1996). Natural infection with *B. pertussis* induces an anti-pertactin humoral response and a cell mediated immune response. It was shown to protect mice against aerosol challenge; furthermore, FHA and PRN combination also protects mice against intracerebral challenge with *B. pertussis* (Poolman and Hallander, 2007; El-kady et al., 2010).

3.3.3.11. Putative Substrate-CoA Ligase

The short-chain fatty acid acetate is found abundant in nature and used for metabolic processes in prokaryotes and eukaryotes. The activation of endogenous and exogenous fatty acids to their corresponding acyl-CoA esters is achieved by the action of acyl-CoA ligases (AMP forming) which are also known as acyl-CoA synthetases (Knights and Drogemuller, 2000; Deborah et al., 2004). This protein is partially membrane bound and known to moves between cytosol and plasma membrane to aid in the esterification of exogenous fatty acids (Overath et al., 1969; Black and DiRusso, 2003). The immunogenic activity of this protein was first demonstrated by Altındiş et al. (2009).

3.3.3.12. Heat Shock Protein 60 (Hsp60)

Molecular chaperones are conserved proteins which facilitate very important intracellular functions and also the major antigenic proteins of many pathogens (Yushan et al., 2010). A GroEL-like protein from *B. pertussis* was purified and it was found that immunization of neonatal mice with this protein provided a very low level protection against aerosol challenge with the same organism (Burns et al., 1991). The immunogenicity of GroEL family members was shown in *Mycobacterium tuberculosis* and *M. leprae* (Thole et al., 1988; Young et al., 1988), as well as *Chlamydia trachomatis* (Morrison et al., 1989). In another study, the antigenic and the immunogenic activity of Hsp60 were shown in *Yersinia enterocolitica* which induced pathogen specific CD4 and Th1 and Th2 T cells (Mertz et al., 1998). In 2007, Gebara et al. purified Hsp60 of *B. pertussis* and evaluated this protein in terms of its protective and adjuvant activities. While Hsp60 alone provided no protection against *B. pertussis* infection, it showed a very good performance as an adjuvant combined with DTaP vaccine as compared to the traditional one (Gebara et al., 2007).

3.3.3.13. Serine Protease

Serine proteases which contain a Ser residue at their active site constitute almost one-third of all known proteolytic enzymes. These enzymes include both exo- and endo-peptidases. Serine peptidases in prokaryotic organisms function in cellular processes such as heat shock response, growth, host invasion, signal transduction, cell signaling and removal of misfolded proteins. It is also known that these proteins are needed for the virulence in many pathogenic bacteria (Triphati and Sowdhamini, 2008). In 2002, Turner et al. identified a novel immunogenic serine protease namely serine protease A (AspA), which is surface-exposed, secreted and meningococcal autotransporter protein. The serine protease autotransporters of *Enterobacteriaceae* (SPATEs) are released by pathogenic *E. coli* and *Shigella* spp. via autotransporter pathway. A class 2 SPATE may have various effects on

the immune response like activation, migration and leukocyte apoptosis (Ruiz-Perez et al., 2011). It was found that serine protease protein SphB1 is involved in the maturation of filamentous haemagglutinin (FHA) in *B. pertussis* (Dé et al., 2008).

3.3.3.14. Glutamyl-tRNA Amidotransferase Subunit A

Immunogenicity of cell wall non-lectin protein glutamyl-tRNA amidotransferase subunit A was first reported for *S. pneumoniae* (Portnoi et al., 2006). The researchers have demonstrated differential capacity of *S. pneumoniae* cell wall lectin (CW-L) and cell wall non-lectin (CW-NL) proteins in eliciting protective immunity. They found that CW-L proteins provided a significantly better protection against intranasal inoculation and CW-NL proteins provided a significantly beter protection from intraperitoneal inoculation. The claimed that proteins identified by sera from mice immunized with the cell-wall derived fractions may constitute candidates for future development of anti *S. pneumoniae* vaccines.

3.3.3.15. Lysyl-tRNA Synthetase

In addition to their function in protein synthesi, aminoacyl tRNA synthetases are also known to play role in RNA splicing, RNA trafficing, apoptosis, translational and transcriptional regulations and tRNA processing (Lee and Razin, 2005). The lysyl-tRNA synthetase (LysRS) is found as class I LysRS in many bacteria. The causative agent of Lyme borreliosis, *Borrelia burgdorferi* also contains a functional class I lysyl-tRNA synthetase, whereas its mammalian host contain class II type. This major difference makes LysRS an important target candidate for developing antibiotics against the pathogen (Dieter et al., 1997; Mejlhede et al., 2002). *poxA* encodes lysRS in *Salmonella enterica* and its existence was also shown in other pathogens like *S. flexneri*, *Y. enterocolitica*, *K. pneumoniae*, and *P. multocida*. A mutation in *poxA* gene causes formation of an attenuated strain

which may be important for designing live vaccines against *Salmonella* and various pathogens (Kaniga et. al., 1998). Our study has been the first report about the immunogenicity of this protein (Altındiş et al., 2009).

3.3.3.16. S-Adenosylmethionine Synthetase

To regulate virulence, many pathogens use quorum sensing. LuxS is a protein produced from S-adenosylmethionine (SAM) synthetase by three enzymatic reactions. It was shown that the LuxS protein behaves as an AI-2 synthase which is an autoinducer in qourum sensing mechanism. The biosynthetic pathway and biochemical intermediates in AI-2 biosynthesis are identical in *E. coli, S. typhimurium, Vibrio harveyi, V. cholerae,* and *Escherichia faecalis*. The immunogenicity of SAM synthetase was previously shown in a *H. pylori* immunoproteomics study (Lin et al., 2006).

3.3.3.17. Fumaryl acetoacetase

Fumaryl acetoacetase is a protein which takes place in the tyrosine and phenylalanine catabolic pathway by cleaving carbon-carbon bond in fumarylactoacetate (Bateman et al., 2001). The immunogenicity of this protein was shown by Altındiş et al. (2009) for the first time.

3.3.3.18. RNA polymerase α subunit

DNA-dependent RNA polymerase (RNAP) of *E. coli* consists of two α subunits (each 36.5 kDa), one β subunit (150.6 kDa) and one β' subunit (155.2 kDa). It is known that α subunit acts as an initiator for RNAP assembly and binds to regulatory factors (Igarashi and Ishihama, 1991). The C-terminal domain of this subunit recognizes the UP elements and Class-I transcriptional activators, and N-terminal contains the determinants for the interaction of α with the rest of RNAP domain (Chen et al., 2003). It is reported that a mutation in the *rpoA* gene,

encoding RNAP α subunit, causes overexpression of RNAP α subunit while reducing the expression of PT and AC virulence factors (Carbonetti et al., 1994). In another study, Carbonetti et al. (2000) concluded that this effect of RNAP α subunit was mediated by its C-terminal domain, most likely by reducing the level of the response regulator BvgA in cells. Boucher et al. (2003) has found that the BvgA and RNAP α subunit C-terminal domain (CTD) interact synchronously with the same linear domain of the *fha* promoter but with two different faces of th DNA helix, clarifying the interaction between RNAP α subunit CTD and BvgA. The immunogenicity of RNA polymerase β subunit of *Klebsiella pneumoniae* and DNA-directed RNAP α subunit of *H. pylori* were also shown by immunoproteomics (Bumann et al., 2002; Kurupati et al., 2006; Lin et al., 2006).

3.3.3.19. Elongation factor Tu (EF-Tu)

One of the most abundant proteins in prokaryotic organisms is elongation factor Tu (EF-Tu), which constitutes 5% of the total cellular proteins of *E. coli* (Jacobson and Rosenbusch, 1976). It is known that EF-Tu catalyzes the interaction between aminoacyl-tRNAs and ribosomes during translation (Parmeggiani and Swart, 1985) and also interacts with ribosomal proteins, various molecules and guanine nucleotides (Weber et al., 1995). EF-Tu is related with Gram-positive bacterial membrane and Gram-negative outer membrane (Kolberg et al., 2008). The immunogenic activity of this protein was shown in *S. epidermidis* (Sellman et al. 2005), *A. marginale* (Lopez et al., 2005), *H. pylori* (Park et al., 2006), *L. garvieae* (Shin et al., 2007), *Leptospira* spp. (Sakolvaree et al., 2007) and *C. pneumoniae* (Bunk et al., 2008). Recently Nieves et al. (2010) reported immunogenicity of EF-Tu of *Burkholderia pseudomallei* and its ability to elicit cellular immune response in mice model.

3.3.3.20. Ketol-acid Reductoisomerase

One of the enzyme in this pathway the biosynthetic cascade for the three branched chain amino acids is ketol-acid reductoisomerase KARI, which catalyzes two-step reaction from S-2-acetolactate to 2,3-dihydroxy-isovale- rate (Chunduru et al., 1989). In this reaction alkyl migration and a ketone reduction occur at a single active site on the enzyme (Rane and Calvo, 1997). Stübs et al. (2005) showed that KARI also acts as a cold inducible factor in *B. bronchiseptica*. Cold inducible factors which are also heat shock proteins can be related with virulence. The immunogenic activity of KARI of *Candida albicans* was demonstrated by Fernández-Arenas et al. (2004). In our study we showed the immunogenicity of this protein for the first time (Altındiş et al., 2009).

3.3.3.21. Putative Peptidyl-prolyl Cis-trans Isomerase

The prokaryotic peptidyl-prolyl cis-trans isomerases (PPIases), also known as rotamases, catalyze cis/trans isomerization of peptide bonds followed by proline residues. They play various roles in the cell such as facilitating the initial folding of immature proteins, protein repair by reconversion of randomly occurring cis linkages after protein synthesis, reactivation of denatured proteins, signal transduction, developmental regulation, cell cycle control and host pathogen interaction (Visick and Clarke, 1995; Kromina et al., 2008). It is also known that these proteins are members of large chaperone complexes (Pratt, 1998) and some membrane channels (Nicolli et al., 1996). It has been shown that PPIases increase the protein folding rate in many microorganisms (Schmid et al., 1993). They can be classified as cyclophilins, FK506-binding proteins, parvulins and chimerical proteins with FK506- and cyclosporine-binding parts (Kromina et al., 2008). In Gram negative microorganisms, PPIases are also responsible for folding of periplasmic proteins and OMPs. It was shown that PPIases, especially SurA, are involved in the pili assembly in *E. coli* (Justice et al., 2005) Mip protein in *Legionella pneumophila* is involved in entry of host cells and intracellular

replication (Hacker and Fischer., 1993). A trigger factor which is also a PPIase is needed for secretion of different virulence factors in *S. pyogenes* (Lyon et al., 1998)**.** Our study was the first report about the immunogenicity of this protein (Altındiş et al., 2009).

3.3.3.22. Aspartate-semialdehyde Dehydrogenase

Aspartate β-semialdehyde dehydrogenase (Asd) is a conserved enzyme which is encoded by *asd* gene. This protein is important for the biosynthesis of essential amino acids; lysine, threonine, methionine, and isoleucine. It also plays role in the synthesis of diaminopimelic acid (DAP) which is both a cell wall component and lysine precursor (Datta, 1969). The immunogenic activity of this protein was demonstrated for the first time by Altındiş et al. (2009).

3.3.3.23. Putative DNA-binding Protein

Bacterial DNA binding proteins, also known as histone-like proteins are basic, small proteins. Small dimeric HU protein in *E. coli* is one of the well-known DNA binding proteins, which contains conserved sequences among bacterial species and is capable of wrapping DNA. These proteins provide resistance to DNA in harsh environmental conditions (Drlica and Rouviere-Yaniv, 1987; Pettijohn, 1988). Our study is the first report about the immunogenicity of this protein (Altındiş et al., 2009)

3.3.3.24. Heat Shock Protein 10 (Hsp10)

Normally, Hsps constitute up to 5% of the total cytoplasmic protein mass; however, under stress conditions Hsp amount in the cell can be increasedto 15% in prokaryotic cells (Qamra et al., 2005). The function of Hsp60 is dependent upon a different heat shock protein, Hspl0 which also known as GroES or

chaperonin l0. By binding to the Hsp60, Hsp10 regulates the ATPase activity and substrate relations of Hsp60 (Langer et al., 1992).

It is known that Hsp 60 and Hsp 10 are most potent stimulators of the immune system (Qamra et al., 2005). The immunogenic activity of Hsp10 or its homologs was shown in *M. tuberculosis* (Minden et al. 1986; Barnes and Hirshfield, 1989; Ragno et al., 1996), *M. leprae* (Mehra et al., 1992) and *Campylobacter jejuni* (Wu, 1994).

3.3.3.25. Putative Outer Membrane Protein

In bacteria nearly half of the mass of the outer membrane is protein (Osborn and Wu, 1980). The bacterial outer membrane proteins (OMPs) have been an object of intensive research during the last decade. It is well known that OMPs of bacteria play an important role during infection and induction of host immune response. OMPs have crucial role in many cellular and physiological processes, which are particularly used for development of vaccine candidates and diagnostic kits (Qian et al., 2007).

B. pertussis Saadet, isolated in 1948 in Turkey, had been the primary strain to manufacture cellular pertussis vaccine for many years in the country. In this study, the immunogenic proteins identified were common to both Tohama I and Saadet strains, except for a putative chromosome partition protein and preprotein translocase secA subunit found only in Tohama I and carbamoyl-phosphate synthase large chain and a putative outer membrane protein found only in Saadet. The latter protein was of particular interest with its location, very strong reactivity and specificity to Saadet strain (Altındiş et al., 2009).

3.4. Surface Proteome

Surface proteins of *B. pertussis* Tahoma I and Saadet strains were resolved by 2DE to obtain an overview of protein distribution. After staining with colloidal CBB G250, nearly 170 spots could be detected on the gels by the 2D image analysis software Delta2D version 3.4 (Decodon, Germany). There was no detectable difference between the protein patterns obtained from two strains (Figure 3.11). A total of 141 spots were cut from each replicate for each strain and analyzed by MALDI-TOF-MS analysis. A total of 125 spots which corresponded to 45 different gene products could be identified for each strain (Table 3.2). Of these gene products, 19 occurred in multiple spots (Tefon et al., 2011).

1D-SDS PAGE followed by LC-MS/MS was next used to identify surface proteins of *B. pertussis* strains. Surface proteins were separated by 1DE and proteins were visualized by CBB G250 staining. One gel lane was cut into 12 equidistant pieces. After in-gel tryptic digestion as well as peptide elution, peptides were analysed by LC-MS/MS, resulting in identification of 226 proteins in total (Table 3.2), covering also those already identified via 2DE MALDI-TOF-MS analysis. (Tefon et al., 2011). The proteins differentially expressed in Tohama I and Saadet are shown with bold fonts.

Of 226 proteins, 16 were differentially expressed in *B. pertussis* Saadet and Tohama I strains. 5 proteins were expressed only in Saadet (adhesin, chaperone protein DnaJ, fimbrial protein FimX, putative secreted protein Bsp22 and putative universal stress protein), and 2 (ABC transporter substrate-binding protein and a putative binding protein-dependent transport periplasmic protein) only in Tohama I. Also, 6 proteins, OmpQ, PT subunit S1 and S2, putative outer protein D-BopD, putative uncharacterized protein Bcr4 and serotype 3 fimbrial subunits were more abundant in Saadet while 3 proteins, a probable extracellular solute binding protein and two putative exported proteins were expressed at a higher level in Tohama I (Tefon et al., 2011).

3.4.1. Identification of Immunoreactive Proteins

Western blotting of the 2D gels using antisera designated as Th (sc) and Sa (sc) as primary antibody and anti-mouse IgG as secondary antibody revealed a total of 27 immunoreactive protein spots (Figure 3.12). The surface immunoproteomes of Tohama I and Saadet strains were nearly identical. The corresponding spots were excised, digested and analyzed using MALDI-TOF-MS. These spots corresponded to 11 different gene products (Figure 3.11, Table 3.2). Of these, 6 proteins [60 kDa chaperonin (Hsp 60), serum resistance protein (BrkA), pertactin (PRN), Hsp 10, putative peptidyl cis-trans isomerase and ATP synthase subunit beta] have already been shown in total soluble immunoproteome of *B. pertussis* (Altındiş et al., 2009) while 5 proteins, namely glutamine-binding periplasmic protein, leu/ile/val-binding protein, one putative exported protein, serotype 2 fimbrial subunit (FIM2) and iron-superoxide dismutase (Fe-SOD) were detected by immunoproteomics for the first time in this pathogen. The immunogenic ones appeared to undergo charge modification included PRN, BrkA, Fe-SOD, Hsp10 and leu/ile/val-binding protein while FIM2 appeared as a mass variant antigen. The accuracy scores of the epitopes of these immunogenic proteins predicted by the server ABCpred (Table 3.3) were within a meaningful range (Tefon et al., 2011).

3.4.2. Functional Classes, Protein Localization and Signal Peptides

When functional classes of a total of 226 identified proteins were annotated, they fell into 6 categories: (i) General metabolism and enzyme families (27%), (ii) membrane transport, secretion system, signal transduction, and cell motility (29%), (iii) folding, sorting and degradation (6%), (iv) bacterial toxins (2%), (v) replication, repair and translation (3%), and (vi) yet unknown ones (33%) (Figure 3.13). Localization prediction analysis revealed that ca. 60% of these proteins were located at periplasm and/or outer membrane, the detection of which was also supported by signal peptide analysis (Table 3.2) (Tefon et al., 2011).

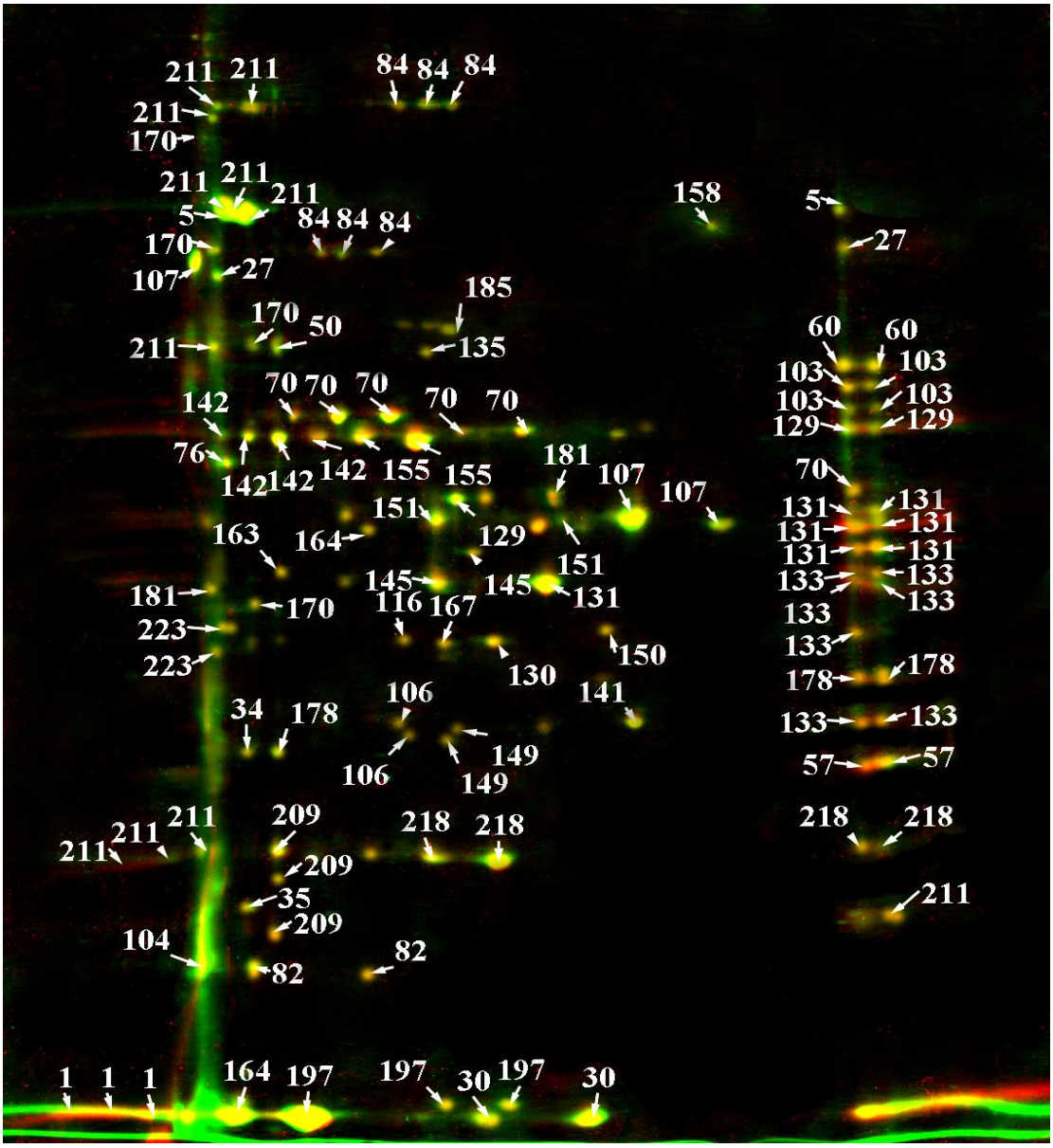

Figure 3. 11. Dual channel 2D imaging of *B. pertussis* strains Tohama I (green) and Saadet (red) (Tefon et al., 2011).

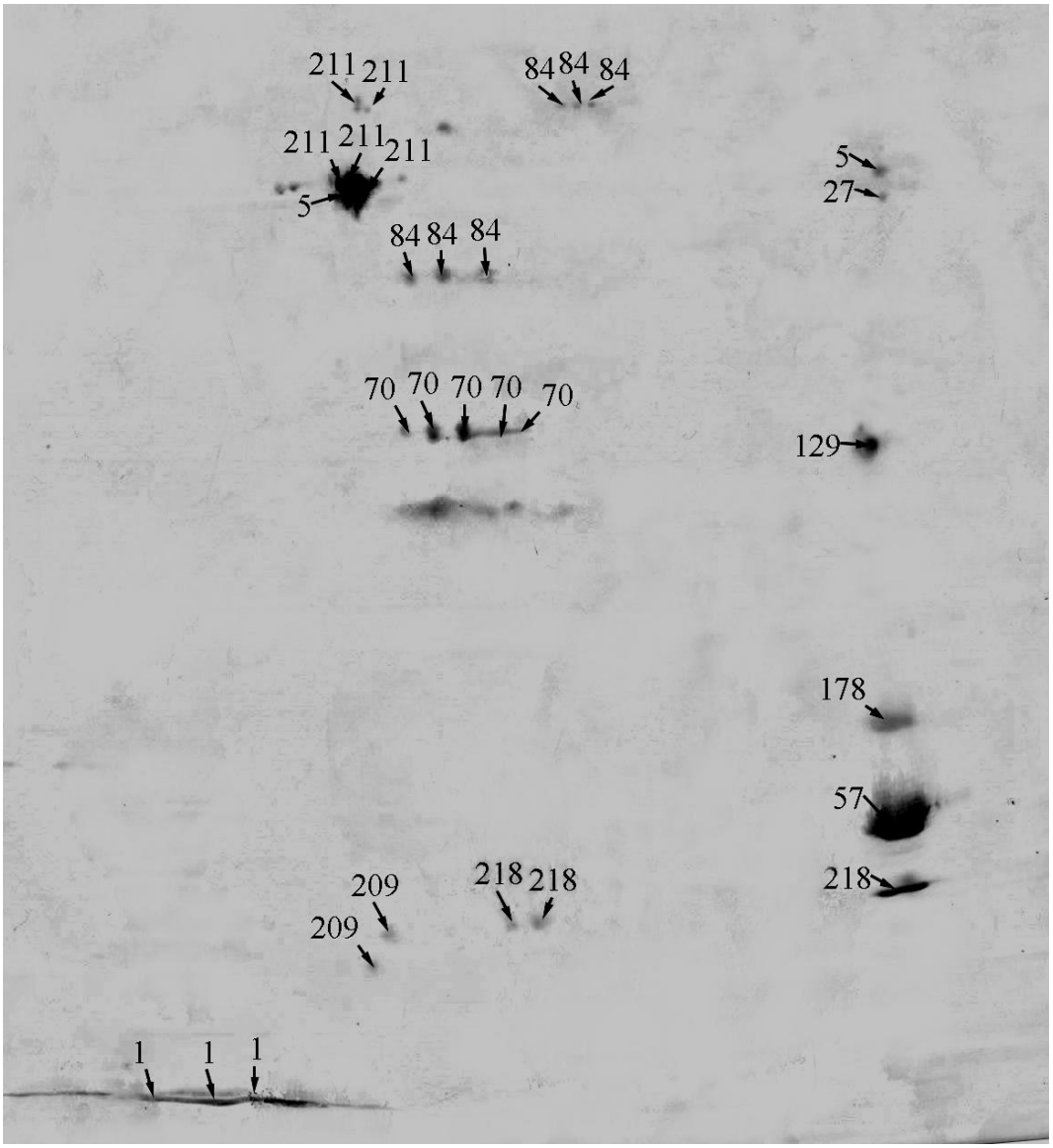

Figure 3. 12. Fused 2D Western blot analysis of the surface proteins of *B. pertussis* strains Tohama and Saadet (Tefon et al., 2011).

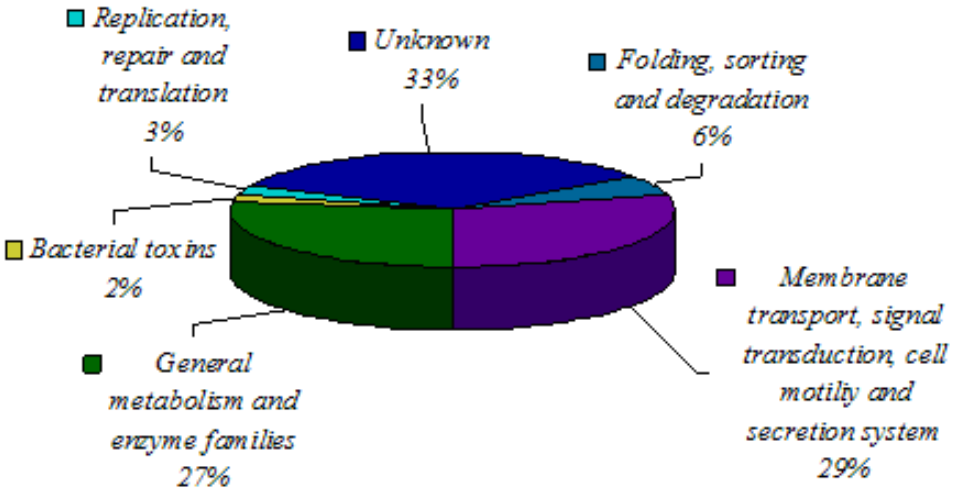

Figure 3.13. Functional classes of the surface proteins identified from *B. pertussis* (Tefon et al., 2011).

Table 3.2. Surface proteins *of B. pertussis* Tohama I and Saadet strains identified by geLC-MS/MS (Tefon et al., 2011).

Protein no.[a]	Protein name	Gene locus	MW (kDa)	Number of isoforms[b]	Subcellular localization[c]	Signal peptide	Function	Spectral counts[d] Th	Sa	*Rsc*[e]
1	10 kDa chaperonin	BPP0869	10,25	3(C)	C	-	Folding, sorting and degradation	16	12	-0,2948
2	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase	BB2183	29,24		C	-	Amino acid metabolism	15	14	-0,0052
3	3-oxoacyl-[acyl-carrier-protein] synthase II	BP2439	43,33		C	-	Lipid metabolism	10	13	0,4282
4	50S ribosomal protein L9	BB1917	16,34		C	-	Translation	4	5	0,3383
5	60 kDa chaperonin	BP3495	57,46		C	-	Folding, sorting and degradation	131	104	-0,2470
6	ABC transport protein, periplasmic component	BP2616	35,01		P	+	Unknown	8	8	0,0866
7	**ABC transporter substrate-binding protein**	**BAV1159**	**57,24**		**P**	**+**	**Membrane transport**	**14**	**0**	**-3,5250***
8	ABC transporter substrate-binding protein	BAV1080	40,47		P	+	Membrane transport	16	32	1,0367
9	ABC transporter substrate-binding protein	BAV3033	41,86		P	+	Membrane transport	20	30	0,6451
10	ABC transporter substrate-binding protein	BAV1088	43,38		P	+	Membrane transport	7	13	0,8763
11	ABC transport protein, solute-binding component	BP2692	55,69		P	+	Membrane transport	7	5	-0,3144
12	Acetylornithine aminotransferase 2	BB4951	42,78		C	-	Amino acid metabolism	20	20	0,0867
13	Adenosylhomocysteinase	BB0198	51,51		C	-	Amino acid metabolism	23	28	0,3582
14	Adenylosuccinate synthetase	BB3165	46,82		C	-	Nucleotide metabolism	15	8	-0,7277
15	**Adhesin**	**BP2667**	**263,68**		**OM**	**+**	**Unknown**	**0**	**23**	**4,3692**
16	Alkyl hydroperoxide reductase	BP3552	20,15		C	-	Enzyme	12	16	0,4680
17	Amino acid-binding periplasmic protein	BP0558	36,122		P, OM	+	Membrane transport	20	21	0,1532
18	Aminomethyltransferase	BP0195	39,33		C	-	Energy metabolism	13	9	-0,3895
19	Antioxidant protein	BP0965	23,75		C, P	-	Unknown	12	18	0,6267
20	Arginine biosynthesis bifunctional protein argJ	BB4426	42,66		C	-	Amino acid metabolism	34	28	-0,1836

Table 3.2. Continued

21	Argininosuccinate synthase	BB1986	49,35		C	-	Amino acid metabolism	3	7	1,0442
22	Aromatic-amino-acid aminotransferase	BP1795	43,07		C	-	Amino acid metabolism	9	12	0,4576
23	Aspartate-semialdehyde dehydrogenase	BP1484	40,31		C	-	Amino acid metabolism	16	10	-0,5312
24	Aspartokinase	BP1913	45,28		C	-	Amino acid metabolism	12	13	0,1918
25	ATP synthase gamma chain	BB4606	33,32		C	-	Energy metabolism	11	6	-0,6712
26	ATP synthase subunit alpha	BB4607	55,44		C	-	Energy metabolism	55	26	-0,9644
27	ATP synthase subunit beta	BP3288	50,48		C	-	Energy metabolism	32	21	-0,4950
28	ATP-dependent Clp protease proteolytic subunit	BB2254	23,75		C	-	Enzyme families	8	6	-0,2653
29	Autotransporter subtilisin-like protease (SphB1)	BP0216	99,63		P, OM, E	-	Membrane transport, secretion system	21	77	1,9125
30	Azurin	BB3856	15,95	2 (C)	P	+	Membrane transport	857	316	-1,4584
31	Bifunctional hemolysin-adenylate cyclase	BB0324	177,03		E	-	Bacterial toxinss	0	3	1,8526
32	Bifunctional protein glmU	BB4817	48,36		C	-	Carbohydrate metabolism	8	14	0,8091
33	Branched-chain amino acid-binding protein	BP1948	44,34		P	+	Membrane transport	16	16	0,0867
34	Carbonic anhydrase	BP3425	23,64		C	-	Energy metabolism	10	10	0,0866
35	Chaperone protein clpB	BP1198	96,31		C	-	Folding, sorting and degradation	7	6	-0,1001
36	**Chaperone protein dnaJ**	**BB3933**	**40,23**		**C**	**-**	**Folding, sorting and degradation**	**0**	**9**	**3,1239**
37	Chaperone protein dnaK	BB3934	69,66		C	-	Folding, sorting and degradation	127	116	-0,0437
38	Chaperone protein fimB/fhaD	BP1881	26,41		P	+	Folding, sorting and degradation	26	21	-0,2067
39	Chaperone protein htpG	BP0074	71,13		C	-	Folding, sorting and degradation	12	14	0,2898
40	Chaperone surA	BB4101	56,8		P	+	Folding, sorting and degradation	66	38	-0,6954
41	Cytochrome c oxidase polypeptide II	BP3744	42,86		CM, P	+	Energy metabolism	14	18	0,4235
42	D-methionine ABC transporter, substrate-binding protein	BAV2852	28,54		P	+	Membrane transport	13	15	0,2765
43	Dihydrodipicolinate synthase	BP1570	31,07		C	-	Amino acid metabolism	6	6	0,0865
44	Dihydrolipoyl dehydrogenase	BAV1205	49,97		C	-	Carbohydrate metabolism	10	5	-0,7624
45	DNA polymerase iii, beta chain	BP0490	41,23		C	-	Nucleotide metabolism	6	7	0,2732

Table 3.2. Continued

46	DNA-directed RNA polymerase alpha chain	BAV0060	36,27	C	-	Nucleotide metabolism	10	13	0,4282
47	Electron transfer flavoprotein alpha-subunit	BP0962	31	C	-	Unknown	11	7	-0,4845
48	Electron transfer flavoprotein beta-subunit	BP0961	26,8	C, P	-	Unknown	17	26	0,6669
49	Elongation factor Ts	BB2606	30,88	C	-	Translation	34	43	0,4168
50	Elongation factor Tu	BPP0007	42,89	C	-	Translation	62	41	-0,4992
51	Enolase	BAV1166	45,95	C	-	Carbohydrate metabolism	36	24	-0,4765
52	Filamentous hemagglutinin	BP1879	367,49	OM	+	Unknown	576	354	-0,6550
53	**Fimbrial protein fimX**	**BP2674**	**21,44**	**P**	**+**	**Unknown**	**0**	**6**	**2,6237**
54	Fructose-bisphosphate aldolase	BP1519	38,39	C	-	Carbohydrate metabolism	21	16	-0,2815
55	Glutamate dehydrogenase	BP1857	46,3	P	-	Energy metabolism	6	8	0,4384
56	Glutamine ABC transporter, glutamine-binding protein	BAV1960	26,86	P	+	Membrane transport	40	30	-0,3157
57	Glutamine-binding periplasmic protein	BP1852	27,13	P	+	Membrane transport	43	49	0,2717
58	Glutathione reductase	BP2120	49,26	C	-	Amino acid metabolism	7	11	0,6577
59	Glyceraldehyde-3-phosphate dehydrogenase	BP1000	36,26	C	-	Carbohydrate metabolism	5	11	1,0586
60	Glycerol-3-phosphate-binding periplasmic protein	BP1281	47,68	P	+	Membrane transport	89	77	-0,1208
61	High-affinity branched-chain amino acid ABC transporter,	BAV1895	39,26	P	+	Membrane transport	25	26	0,1409
62	Histidinol dehydrogenase	BB4854	46,86	C	-	Amino acid metabolism	3	7	1,0442
63	Imidazole glycerol phosphate synthase subunit hisF	BB4859	28,3	C	-	Amino acid metabolism	5	15	1,4671
64	Indole-3-glycerol phosphate synthase	BP3261	28,54	C	-	Amino acid metabolism	12	15	0,3817
65	Inorganic pyrophosphatase	BP2533	20,02	C	-	Energy metabolism	20	16	-0,215
66	Inosine-5'-monophosphate dehydrogenase	BP2625	51,74	C	-	Nucleotide metabolism	8	7	-0,0787
67	Isocitrate dehydrogenase [NADP]	BP2488	45,77	C	-	Carbohydrate metabolism	24	19	-0,2326
68	Ketol-acid reductoisomerase	BAV2671	36,34	C	-	Amino acid metabolism	5	9	0,8010
69	L-amino acid ABC transporter, substrate-binding protein	BAV3058	36,82	P	+	Membrane transport	139	140	0,0986

Table 3.2. Continued

70	Leu/ile/val-binding protein	BP1285	39,59	5 (C)	P	+	Membrane transport	166	94	-0,7387
71	Leu/ile/val-binding protein	BP1277	39,38		P	+	Membrane transport	26	26	0,0868
72	Malate synthase G	BP3680	78,55		C	+	Carbohydrate metabolism	44	41	-0,0126
73	Molybdate-binding periplasmic protein	BP3095	26,43		P	+	Membrane transport	33	13	-1,1824
74	**OmpQ**	**BP3405**	**39,14**		**OM**	**+**	**Unknown**	**1**	**11**	**2,5333**
75	Orotate phosphoribosyltransferase	BB4533	23,4		C	+	Nucleotide metabolism	10	7	-0,3615
76	Outer membrane porin protein BP0840	BP0840	41,02		OM	+	Unknown	91	109	0,3484
77	Outer membrane protein	BAV1743	20,53		C, P	+	Unknown	12	14	0,2898
78	Outer membrane protein A	BP0943	20,96		P	+	Unknown	1	5	1,5612
79	Penicillin-binding protein	BAV0135	45,17		P	+	Glycan biosynthesis and metabolism	2	5	1,0305
80	Peptide deformylase 1	BB0247	19,31		C	-	Unknown	9	9	0,0866
81	Peptidoglycan-associated lipoprotein	BAV2916	17,79		P, OM	+	Unknown	10	9	-0,0479
82	Peptidyl-prolyl cis-trans isomerase B	BP1906	18,54	2 (C)	C, P	-	Folding, sorting and degradation	28	34	0,3572
83	Periplasmic solute-binding protein	BP3674	34,41		P	+	Membrane transport	13	3	-1,6609
84	Pertactin	BP1054	93,43	3 (C)	OM, E	+	Membrane transport, secretion system	6	12	0,9577
85	Pertussis toxin subunit 1	BP3783	29,95		OM, E	+	Bacterial toxiins	0	14	3,6981
86	**Pertussis toxin subunit 1 homolog**	**BB4890**	**29,97**		**OM, E**	**+**	**Bacterial toxiins**	**4**	**44**	**3,2022**
87	**Pertussis toxin subunit 2**	**BP3784**	**24,78**		**OM, E**	**+**	**Bacterial toxiins**	**1**	**10**	**2,4102**
88	Pertussis toxin subunit 3	BP3787	24,97		CM, P, E	+	Bacterial toxiins	4	13	1,5289
89	Phosphate ABC transporter, phosphate-binding periplasmic protein	BAV0939	36,43		P	+	Membrane transport, signal transduction	116	60	-0,8605
90	Phosphate-binding periplasmic protein	BP1071	36,57		P	+	Membrane transport, signal transduction	97	49	-0,8896
91	Phospho-2-dehydro-3-deoxyheptonate aldolase, Phe-sensitive	BP2908	38,78		C	-	Amino acid metabolism	40	22	-0,7439
92	Phosphoglycerate kinase	BB1382	40,96		C	-	Carbohydrate metabolism	12	10	-0,1499

Table 3.2. Continued

93	Polyribonucleotide nucleotidyltransferase	BP0795	77,32		C	-	Nucleotide metabolism	20	15	-0,3013
94	Porphobilinogen deaminase	BB2085	33,19		C	-	Metabolism of cofactors and vitamins	5	12	1,1720
95	Probable class IV aminotransferase	BP0103	32,27		C	-	Amino acid metabolism	14	6	-0,9878
96	**Probable extracellular solute-binding protein**	**BP0121**	**47,13**		**P**	**+**	**Membrane transport**	**107**	**10**	**-3,1986**
97	Probable periplasmic solute-binding protein	BP0128	36,44		P	+	Membrane transport	7	2	-1,2584
98	Probable short-chain dehydrogenase	BP2770	26,09		C, CM	-	Unknown	8	13	0,7110
99	Probable surface antigen	BP1427	86,39		OM	+	Unknown	18	7	-1,138
100	Probable tonB-dependent receptor bfrD	BP0856	81,56		OM	+	Unknown	55	38	-0,4355
101	Probable zinc-binding dehydrogenase	BP0800	33,67		C	-	Unknown	5	6	0,3009
102	Protein grpE	BB3936	19,6		C, P	-	Folding, sorting and degradation	13	5	-1,1041
103	Protein tolB	BB4237	47,43	2 (M)	OM	+	Unknown	47	69	0,6335
104	Protein-export protein secB	BPP0292	19,06		C	-	Folding, sorting and degradation, membrane transport, secretion system	5	10	0,9355
105	Putative ABC transport protein, substrate-binding component	BP2418	42,91		P	+	Membrane transport	6	6	0,0865
106	Putative ABC transport solute-binding protein	BP2747	40,65	2 (C-M)	P	+	Membrane transport	282	160	-0,7486
107	Putative ABC transporter periplasmic amino acid-binding protein	BP3831	36,66	2 (C)	P, OM	+	Membrane transport	94	173	0,9754
108	Putative ABC transporter substrate binding protein	BP0301	41,55		P	+	Membrane transport	36	37	0,1253
109	Putative amino acid-binding periplasmic protein	BP1532	28,37		P	+	Membrane transport	7	16	1,1525
110	Putative amino-acid ABC transporter, periplasmic a.a.-binding protein	BP1364	28,26		P	+	Membrane transport	70	37	-0,8169
111	Putative antioxidant protein	BP3551	18,36		P	-	Unknown	7	10	0,5346
112	Putative bacterioferritin comigratory protein	BP1307	19,35		P	+	Unknown	52	12	-1,9279
113	**Put binding-prot-dependent trans. pperip.protein**	**BP2396**	**57,58**		**P**	**+**	**Membrane transport**	**10**	**0**	**-3,0854**

Table 3.2. Continued

114	Putative binding-protein-dependent transport protein	BP3237	58,87		P	+	Membrane transport	4	15	1,7188
115	Putative carboxy-terminal processing protease	BP0609	51,15		P	+	Enzyme families	11	10	-0,0365
116	Putative cell surface protein	BP2219	35,43		P	+	Unknown	9	20	1,1407
117	Putative cyclase	BP3130	35,68		C	-	Unknown	27	23	-0,1343
118	Putative DNA-binding protein	BP1616	18,5		C, P	-	Replication and repair	27	22	-0,1953
119	Putative exported protein	BAV3166	28,62		P	+	Membrane transport	5	3	-0,4703
120	Putative exported protein	BAV2471	33,42		P	+	Membrane transport	26	21	-0,2067
121	Putative exported protein	BAV0735	34,37		P	+	Unknown	17	14	-0,173
122	Putative exported protein	BAV2755	34,52		P	+	Unknown	16	25	0,6942
123	Putative exported protein	BAV1140	42,48		P	+	Membrane transport	7	13	0,8763
124	**Putative exported protein**	**BP0250**	**34,51**		**P**	**+**	**Unknown**	**29**	**4**	**-2,4449**
125	**Putative exported protein**	**BP0205**	**19,35**		**P**	**+**	**Unknown**	**24**	**4**	**-2,1832**
126	Putative exported protein	BP2936	37,24		C	-	Unknown	44	16	-1,3101
127	Putative exported protein	BP0698	22,16		P	+	Unknown	60	32	-0,7999
128	Putative exported protein	BP1480	29,01		P	+	Membrane transport	36	20	-0,7262
129	Putative exported protein	BP3827	35,39		P	+	Unknown	55	32	-0,6761
130	Putative exported protein	BP2818	28,72		P	+	Membrane transport	53	31	-0,6678
131	Putative exported protein	BP0454	33,43	4 (M)	P	+	Unknown	16	9	-0,6658
132	Putative exported protein	BP3867	35,63		P	+	Signal transduction	66	39	-0,6589
133	Putative exported protein	BP2068	33,69	4 (M)	P	+	Membrane transport	14	9	-0,4876
134	Putative exported protein	BP0334	33,83		C, CM, P	+	Unknown	18	13	-0,3482
135	Putative exported protein	BP3568	40,35		P	+	Membrane transport	5	4	-0,1652
136	Putative exported protein	BP0561	33,63		P	+	Unknown	6	5	-0,1278
137	Putative exported protein	BP1292	29,59		C, CM, P	+	Unknown	155	138	-0,0814
138	Putative exported protein	BP1506	40,77		C, CM, P	+	Membrane transport	8	7	-0,0787

Table 3.2. Continued

139	Putative exported protein	BP2802	34,18		P	+	Unknown	55	50	-0,0482
140	Putative exported protein	BP1900	34,56		P	+	Unknown	9	9	0,0866
141	Putative exported protein	BP0385	28,5		P	+	Membrane transport	11	12	0,200
142	Putative exported protein	BP3575	43,16	4 (C)	P	+	Membrane transport	38	43	0,2609
143	Putative exported protein	BP1887	35,62		P	+	Membrane transport	44	50	0,2679
144	Putative exported protein	BPP3617	34,53		P	+	Unknown	6	7	0,2732
145	Putative exported protein	BP0461	37,92	2 (C-M)	P	+	Membrane transport	18	21	0,2962
146	Putative exported protein	BP1838	20,22		P	+	Unknown	59	86	0,6269
147	Putative exported protein	BP0664	35,84		CM	+	Unknown	12	19	0,7000
148	Putative exported protein	BP0562	17,16		P	+	Unknown	27	44	0,7699
149	Putative exported protein	BPP1941	29	2 (C-M)	P	+	Unknown	45	74	0,7952
150	Putative exported protein	BPP4198	33,85		C, CM, P	+	Unknown	38	89	1,2987
151	Putative exported protein	BPP3542	34,48		P	+	Unknown	1	4	1,3095
152	Putative exported protein	BP3732	21,68		P	+	Unknown	1	5	1,5612
153	Putative exported protein	BP0782	39,82		P	+	Membrane transport	15	44	1,5701
154	Putative exported protein	BP3481	37,17		P	+	Membrane transport	5	17	1,6349
155	Putative exported solute binding protein	BP2963	40,29	2 (C)	P	+	Membrane transport	142	110	-0,2832
156	Putative extracellular solute-binding protein	BP3159	57,03		P	+	Membrane transport	23	42	0,9254
157	Putative extracellular solute-binding protein	BP1529	36,54		C, P	+	Membrane transport	12	31	1,3738
158	Putative extracellular solute-binding protein	BP3862	57,35		P	+	Membrane transport	5	17	1,6349
159	Putative glutamine-binding periplasmic protein	BP1852	27,13		P	+	Membrane transport	61	40	-0,5108
160	Putative glutathione S-transferase	BP3659	24,31		C, CM, P	-	Amino acid metabolism	3	3	0,0865
161	Putative glutathione transferase	BP1300	26,41		C	-	Amino acid metabolism	5	7	0,4875
162	Putative hemin binding protein	BP0345	29,48		C, CM, P	+	Membrane transport	5	3	-0,4703
163	Putative iron binding protein	BP1605	37,38		P	+	Membrane transport	31	67	1,1758

Table 3.2. Continued

164	Putative lipoprotein	BP1569	41,47	OM	+	Unknown	5	6	0,3009
165	Putative lipoprotein	BP2992	16,51	E	+	Unknown	10	14	0,5263
166	Putative lipoprotein	BP2072	21,5	P, OM	+	Unknown	1	5	1,5612
167	Putative membrane protein	BP3012	37,33	P	+	Unknown	56	15	-1,7381
168	Putative membrane protein	BP1056	25,53	P	+	Unknown	36	23	-0,535
169	Putative orotidine 5'-phosphate decarboxylase	BP3490	29,34	C	-	Nucleotide metablosim	7	14	0,9743
170	Putative outer membrane ligand binding protein-BipA	BP1112	137,17	OM	-	Unknown	107	48	-1,0606
171	Putative outer membrane protein	BP3755	23,01	OM	+	Unknown	17	8	-0,8955
172	**Putative outer protein D (BopD)**	**BPP2223**	**31,95**	**OM**	**-**	**Unknown**	**1**	**25**	**3,6357**
173	Putative oxidoreductase	BP2454	26,54	C	+	Carbohydrate metablosim	7	8	0,2518
174	Putative penicillin-binding protein	BP0102	44,86	P	+	Glycan biosynthesis and metabolism	8	10	0,3694
175	Putative peptidase	BP0906	71,05	P	+	Unknown	41	16	-1,2105
176	Putative peptidase	BP1721	30,03	P	+	Unknown	12	17	0,5495
177	Putative peptidoglycan-associated lipoprotein	BP3342	17,87	OM	+	Unknown	8	6	-0,2653
178	Putative peptidyl-prolyl cis-trans isomerase	BP3561	28,94	P	+	Folding, sorting and degradation	168	77	-1,0434
179	Putative periplasmic protein	BP3341	24,98	P	+	Unknown	6	9	0,5867
180	Putative periplasmic solute binding protein	BP3080	33,39	C, P	+	Membrane transport	5	3	-0,4703
181	Putative periplasmic solute-binding protein	BP1487	40	P	+	Unknown	111	153	0,5551
182	Putative periplasmic substrate-binding protein	BP2055	36,89	P	+	Membrane transport	32	8	-1,7639
183	Putative periplasmic substrate-binding transport protein	BP2352	34,68	P	+	Membrane transport	7	8	0,2518
184	Putative polyamine transport protein	BP2348	40,89	P	+	Membrane transport	11	13	0,3052
185	Putative regulatory lipoprotein	BP2271	47,41	P	+	Membrane transport	6	17	1,4206
186	**Putative secreted protein (Bsp22)**	**BP2256**	**22,22**	**P,E**	**-**	**Unknown**	**0**	**10**	**3,2584**
187	Putative sigma factor regulatory protein	BP2435	39,19	C, CM, P	+	Unknown	15	7	-0,8930
188	Putative solute-binding periplasmic protein	BP3572	36,38	P	+	Membrane transport	16	22	0,5185

Table 3.2. Continued

189	Putative TonB-dependent receptor	BP2922	79,35		OM	+	Unknown	11	8	-0,3193
190	Putative uncharacterized protein	BP3819	26,19		P	+	Unknown	20	9	-0,9674
191	Putative uncharacterized protein	BP3128	68,57		C, OM	+	Unknown	25	18	-0,3621
192	Putative uncharacterized protein	BP2909	51,39		C	-	Enzyme families	5	4	-0,1652
193	Putative uncharacterized protein	BP2532	24,87		C	-	Unknown	8	10	0,3694
194	Putative uncharacterized protein	BP2953	23,86		P, E	-	Unknown	7	9	0,4001
195	Putative uncharacterized protein	BP0499	13,9		C, CM	-	Secretion system	4	7	0,7392
196	Putative uncharacterized protein	BP0479	35,64		P	+	Unknown	11	18	0,7401
197	Putative uncharacterized protein	BP1843	15,96	3 (C)	OM	-	Unknown	8	15	0,9009
198	Putative uncharacterized protein	BP3013	26,43		C	-	Unknown	3	7	1,0442
199	Putative uncharacterized protein	BP2213	16,59		C, P	-	Translation	7	15	1,0662
200	Putative uncharacterized protein	BP1320	29,64		C, OM	-	Unknown	0	2	1,4654
201	**Putative uncharacterized protein bcr4**	**BPP2226**	**18,12**		**C**	**-**	**Unknown**	**1**	**10**	**2,4102**
202	**Putative universal stress protein**	**BP0410**	**16,32**		**C**	**+**	**Folding, sorting and degradation**	**0**	**8**	**2,9756**
203	Putative UTP--glucose-1-phosphate uridylyltransferase	BP3403	32,52		C	-	Carbohydrate metabolism	1	4	1,3095
204	Putative zinc protease	BP2497	101,09		P, OM	+	Enzyme families	73	43	-0,6656
205	Ribosome recycling factor	BB2608	20,69		C	-	Translation	7	10	0,5346
206	S-adenosylmethionine synthetase	BB0195	41,98		C	-	Amino acid metabolism	7	10	0,5346
207	Serine hydroxymethyltransferase 2	BB4348	44,79		C	-	Energy metabolism	7	5	-0,3144
208	Serine protease	BP2434	52,11		P	+	Unknown	14	15	0,1785
209	Serotype 2 fimbrial subunit	BP1119	21,93	3 (M)	E	+	Membrane transport, secretion system, cell motility	48	128	1,4956
210	**Serotype 3 fimbrial subunit**	**BP1568**	**21,78**		**E**	**+**	**Unknown**	**1**	**76**	**5,2034**
211	Serum resistance protein	BP3494	103,31	3 (C-M)	OM, E	+	Membrane transport, secretion system	246	741	1,7842
212	Stringent starvation protein	BAV3332	23,77		C	-	Unknown	7	17	1,234
213	Succinate dehydrogenase flavoprotein subunit (Fragme)	BAV1184	64,86		C	-	Carbohydrate metabolism	6	18	1,4978

Table 3.2. Continued

214	Succinate dehydrogenase iron-sulfur protein	BAV1185	27,23		C	-	Carbohydrate metabolism	1	6	1,7756
215	Succinyl-CoA ligase [ADP-forming] subunit alpha	BP2540	30,66		C	-	Carbohydrate metabolism	7	15	1,0662
216	Succinyl-CoA synthetase, beta chain	BP2541	40,92		C	-	Carbohydrate metabolism	29	19	-0,4943
217	Superoxide dismutase [Cu-Zn]	BP2067	17,4		P	+	Carbohydrate metabolism	10	4	-1,0142
218	Superoxide dismutase [Fe]	BP2761	21,26	2 (C)	E	-	Unknown	27	42	0,7043
219	Thiol:disulfide interchange protein dsbA	BB4940	22,79		P	+	Folding, sorting and degradation	72	17	-1,9289
220	Tracheal colonization factor	BP1201	66,27		P, OM, E	+	Membrane transport, secretion system	51	100	1,0516
221	Transcription antitermination protein nusG	BAV0008	20,15		C	-	Unknown	8	15	0,9009
222	Trigger factor	BB2253	47,57		C	-	Unknown	19	41	1,1522
223	Triosephosphate isomerase	BPP3426	25,79	2 (C-M)	C	-	Carbohydrate metabolism	17	37	1,1583
224	Tryptophan synthase alpha chain	BB3773	29,82		C, CM	-	Amino acid metabolism	11	11	0,0866
225	Vag8	BP2315	94,89		OM, E	-	Unknown	72	147	1,12
226	Virulence factors transcription regulator bvgA	BB2994	22,93		C	-	Signal transduction	7	6	-0,1001

[a] Protein numbers also correspond to the numbers shown on the gel (Fig. 1A).
[b] C, charge modification; M, mass modification; C–M, both charge and mass modification.
[c] Predicted location of proteins by CELLO 2.5. C: Cytoplasm, OM: Outer membrane, CM: Cell membrane, P: Periplasm and E: Extracellular.
[d] Spectral counts of proteins of strains. Th: Tohama I and Sa: Saadet.
[e] R_{SC} values greater than 2 fold were accepted as significant which are shown in bold characters

Table 3.3. Immunogenic proteins detected in surface proteome of *B. pertussis* Tohama I and Saadet strains (Tefon et al., 2011).

Protein no.	Protein name/function	Gene locus	Mass (kDa)	p*I*	Epitope prediction	Accuracy
1	10 kDa chaperonin	BPP0869	10,26	5,39	AVGPGKKTEDGKILPV	0,87
5	60 kDa chaperonin	BP3495	57,44	5,09	TGLKGDTADQNAGIKL	0,90
27	ATP synthase subunit beta	BP3288	50,49	4,94	VVDIQFPRDNMPKIYE	0,94
57	Glutamine-binding periplasmic protein	BPP3010	26,84	8,77	YSLAEDPKTHVWSLQR	0,93
70	Leu/ile/val-binding protein	BP1285	39,38	6,67	YSLAEDPKTHVWSLQR	0,95
84	Pertactin	BP1054	93,81	9,23	TLTGGADAQGDIVATE	0,95
129	Putative exported protein	BP3827	35,38	8,61	ANLPYDPVKDFAPVTI	0,92
178	Putative peptidyl-prolyl cis-trans isomerase	BP3561	28,94	8,93	PITQKSLDEFVKLVVS	0,93
209	Serotype 2 fimbrial subunit	BP1119	21,93	8,39	EASAITTYVGFSVVYP	0,90
211	Serum resistance protein	BP3494	103,31	6,62	YSLAEDPKTHVWSLQR	0,95
218	Superoxide dismutase	BP2761	21,28	6,05	AYYIDYRNARPKYLEN	0,92

3.4.3. Evaluation of Surface Proteins

Besides protecting bacteria against harsh environments, outer membrane proteins play crucial roles such as signal transduction, solute and protein efflux/influx and bacterial pathogenesis (Koebnik et al., 2000; Lin et al., 2002). In this study we identified 45 proteins by 2DE and 226 proteins by geLC/MS. When 2D gels were subjected to Western blot analysis for their reactivity, a total of 27 immunogenic spots which corresponded to 11 different gene products were determined. Among these proteins, glutamine-binding periplasmic protein, leu/ile/val-binding protein, one putative exported protein, and iron-superoxide dismutase (Fe-SOD) were found as immunogenic for the first time in *Bordetella*. Of a total of 226 proteins identified by LC/MS, 16 were differentially expressed in *B. pertussis* Saadet and Tohama I strains. Five proteins were expressed only in Saadet (adhesin, chaperone protein DnaJ, fimbrial protein FimX, putative secreted protein Bsp22 and putative universal stress protein), and two (ABC transporter substrate-binding protein and a putative binding protein-dependent transport periplasmic protein) only in Tohama I (Tefon et al., 2011).

3.4.3.1. Well-known Virulence Factors of *B. pertussis*

Well known autotransporters of *B. pertussis* are PRN, tracheal colonization factor (TcfA), BrkA, subtilisin like serine protease (SphB1), and Vag8. Pertussis toxin (PT) is a primary component of DTaP and is responsible for pertussis associated lymphocytosis. Adenylate cyclase (CyaA) with adenylate cyclase/hemolysin activiy and dermonecrotic toxin (DNT) as a typical A-B toxin is also one of the important toxins of this microorganism (Locht, 1999). It is known that fimbrial proteins are needed for the adhesion of pathogen to the host cell. The immunogenicity of this protein is also shown. In the present study, we were able to identify all these well-known virulence factors of *B. pertussis* in both strains except for DNT which remains in cytoplasm. PT subunits S1 and S2 were among the proteins more abundant in Saadet than in Tohama I (Tefon et al., 2011).

3.4.3.2. Adhesin

The attachment of *B. pertussis* to the ciliated epithelial tracheal cells are mediated by filamentous hemagglutinin (FHA) and the autotransporters PRN, FIM2 and/or FIM3. [Locht, 1999; Mattoo and Cherry; 2005). FhaS adhesion which is a high molecular weight protein (236 kDa) was identified in outer membrane of Saadet, but not in Tohama I (Tefon et al., 2011). The BLAST search for this protein revealed the most significant database match to *B. parapertussis* adhesin, FhaS (88 % identity). FhaS protein was characterized only in *B. bronchiseptica* (Julio and Cotter, 2005). Comparison of the predicted FhaS proteins showed that *fhaS* gene of *B. bronchiseptica*, but not those of *B. pertussis* or *B. parapertussis*$_{hu}$ codes for a protein that is nearly identical to FHA. However, FhaS could neither mediate adherence of *B. bronchiseptica* to epithelial cell lines *in vitro*, nor was required for colonization in vivo. The allele distribution suggested that FHA and FhaS perform distinct functions during the *Bordetella* pathogenesis *B. parapertussis*$_{hu}$ most likely acquired its *fhaS* allele from *B. pertussis* horizontally, and *fhaS* may contribute to host-species specificity (Julio and Cotter, 2005).

3.4.3.3. FimX

In *B. pertussis*, *fim2* and *fim3* encode major fimbrial subunits FIM2 and FIM3, respectively (Locht, 1999). Expression of these two genes is regulated by mutations in their promoter regions, which results in serotype switching (Willems et al., 1990). *B. pertussis* genome also harbors a *fimX* gene, the product of which has not been identified yet (Mooi et al., 1987). This study constitutes the first report on identification of FimX protein in surfaceome of *B. pertussis*. To note, this protein was found in Saadet strain, but not in commonly used Tohama I (Tefon et al., 2011). The expression of 22 kDa fimbrial subunit FIM3, on the other hand, was negligible in Tahoma I, as determined by LC-MS/MS. These finding accords well with the results of an earlier study in which FIM3 could not be detected among the purified fimbria proteins both in SDS-PAGE and Western blot

analysis using anti-FIM3 monoclonal antibodies (Atakan-Ablay and Özcengiz, 2007). In another study, transcript abundance determined by significance analysis of microarrays between the ancestral and 12-times passaged strains of a clinical isolate revealed significant changes in transcript levels including that of *fim3* while the ancestral and passaged descendant (224 passages on plates) of Tohama I, as a laboratory strain, showed very few differences in transcript abundance (Brinig et al., 2006).

3.4.3.4. Type III Secretion System (T3SS) Proteins

Another virulence factor of *Bordetella* which is under two-component regulatory system BvgAS is type III secretion system (T3SS) required for long-term colonization during infection (Yuk et al., 1998). Molecules secreted via T3SS can be grouped as effectors which are exotoxins, exerting their functions when they are translocated into the host cells and translocons that take place in pore formation on the host membrane for the effectors to pass through (Abe et al., 2008). In *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*, the T3SS gene cluster has been found and designated the *bsc* locus comprising 30 ORFs that encode the T3SS machinery, BscN ATPase, type III-secreted proteins, and putative chaperones (Fauconnier et al., 2001). A highly immunogenic putative secreted protein Bsp22 is the most abundant T3SS effector protein exported from *B. bronchiseptica* (Yuk et al., 2000). It is a cell-surface exposed component of the T3SS system, required for T3SS-mediated cytotoxicity against host cells and for the function of the translocation pore complex (Medhekar et al., 2009). Putative outer protein D (BopD) is one of the T3SS translocator proteins which make a complex with BopB to form pores on the host plasma membrane (Nogawa et al., 2004). The putative uncharacterized protein Bcr4 is also encoded by an ORF located in T3SS locus (Brinig et al., 2006). In our work, as known T3SS components, Bsp22 and BopD were demonstrated well in the surfaceome of Saadet strain. Tohama I did not express Bsp22 while having BopD and Bcr4 only in negligible amounts (Tefon et al., 2011).

3.4.3.5. Outer Membrane Protein Q (OmpQ)

Another protein with a higher expression level in Saadet strain was OmpQ, a porin-like protein known to play an important role in allowing access to an essential nutrient. Although the lack of OmpQ did not affect the *B. pertussis*'s survival *in vitro* or *in vivo*, its role during infection, perhaps in the colonization of the human host or in the establishment of a carrier state can not be ruled out since it is a Bvg-regulated protein (Finn et al., 1995). The immunogenic activity of this protein was shown by Zhu et al. (2011) by using human sera against the total membrane enriched proteins of the Chinese WCV strain.

3.4.3.6. Superoxide Dismutase (SOD)

The immunogenic component Fe-SOD was predicted as an extracellular protein in both strains. SODs are crucial enzymes for pathogenic bacteria for detoxification of endogenous and exogenous reactive oxygen species produced during the infection process. A Fe-SOD-deficient mutant of *B. pertussis* had a reduced ability to express CyaA and PRN proteins of pathogenesis; moreover, it also had decreased abilities to colonize and persist in the murine respiratory infection model (Khelef et al., 1996). A cytosolic SOD belonging to the Fe-Mn SOD family was found to be immunogenic in *Mycobacterium immunogenum* (Gupta et al., 2009). The immunogenic activity of Fe-SOD which is excreted into the growth medium by the epimastigote form of the protozoan parasite *Trypanosoma cruzi* and promastigote form of *Phytomonas* is noteworthy (Marin et al., 2004; Villagran et al., 2005).

3.4.3.7. ATP Synthase Subunit beta

The well studied ATP synthase of *Escherichia coli* contains 8 different subunits, five of which belong to F1, subunits α_3, β_3, γ , δ, and ε, and three to F0, subunits *a*, b_2, and *c* (Deckers-Hebestreit and Altendorf, 1996). α and β subunits are

conserved among different species and are homologous to each other (Weber and Senior, 1997). The immunogenic activity of this protein has been showed in our study (Tefon et al., 2011). Moreover, ATP synthase subunit β in *M. pneumoniae* showed high immunogenic reactivity with serum samples from patients; however, this protein showed no reactivity when sera from healthy blood donors were used (Nuyttens et al., 2010). In another immunoproteomic study, the immunogenic activity of ATP synthase subunit β was shown in *Brucella abortus* (Ko et al., 2011).

3.4.3.8. Periplasmic Binding Proteins

Periplasmic binding proteins (PBPs) are a family of proteins which are important for chemotaxis and uptake of nutrient and other small molecules from the extracellular space (Ames, 1986). There are a variety of PBP ligands, including amino acids, peptides, simple and complex sugars, inorganic ions and metals (Dwyer and Hellinga, 2004; Crochet et al., 2010). Glutamine binding periplasmic protein and leucine/isoleucine/valine binding protein are the immunogenic PBPs found in this study (Tefon et al., 2011). The immunogenic activity of leucine/isoleucine/valine binding protein of *B. pertussis* Chinese WCV strain was also shown by Zhu et al. (2010).

As mentioned earlier, *B. pertussis* Saadet had been isolated as a Phase I strain from a case of whooping cough in 1948 in Turkey and extensively used both in research and cellular pertussis vaccine manufacture for many years in the country as a highly immunogenic strain. Saadet was given preference over other strains including Tohama I as a more protective vaccine strain, most likely owing to the stable expression of important virulence factors such as adhesin, DnaJ, FimX, Bsp22 and a putative universal stress protein as well as overexpression of OmpQ, PT subunits S1 and S2, BopD, Bcr4, FIM2 and FIM3, as the present study revealed. On the other hand, this strain constitutes an example for the lack of one-to-one relationship between virulence gene expression and long-term laboratory

maintenance since it is also a high-passage and laboratory-adapted strain like Tahoma I (Tefon et al., 2011).

Label-free geLC-MS/MS involves the comparison of multiple unprocessed LC-MS datasets based on the relative peptide peak intensities and simultaneously increases throughput, facilitating biomarker discovery (Nesatyy and Suter, 2007). It combines the robust nature of SDS-PAGE and the resolving power of LC–MS/MS. More proteins are derived from gel bands (1DE) than from gel spots (2DE) and this leads to a better separation and relative higher qualities of tandem mass spectra in a single LC–MS/MS run (Vidakovics et al., 2007). In addition, it can resolve low abundance proteins and large and hydrophobic ones typically not accessible via 2DE. As expected, GeLC-MS/MS detected more surface proteins than 2DE-MS/MS. 226 proteins identified by geLC-MS/MS included each of the 45 proteins identified by 2DE-MS/MS in this work, 21 out of 54 surfaceome components identified by 2DE-MS/MS from 3 vaccine strains and 1 clinical isolate of *B. pertussis* as reported by Bottero *et al*. (2007), 24 out of 49 identified proteins from biofilm-grown Tohama I analysed by Serra *et al*. (2009) by combining 2-DE and Fourier transform infrared (FT-IR) spectroscopy and 23 out of 40 total outer membrane enriched secreted proteins from Chinese WCV strain (Zhu et al., 2010).

3.5. Secretome

Secreted proteins of *B. pertussis* Tahoma I and Saadet strains were resolved by 2DE to obtain a view of secreted protein distribution. After gels were stained with colloidal CBB G250, nearly 160 spots could be detected on the gels by the 2D image analysis software Delta2D version 3.4 (Decodon, Germany). There was no detectable difference between the protein patterns obtained from two strains (Figure 3.14). A total of 135 spots were cut from each replicate for each strain and analyzed by MALDI-TOF-MS analysis. A total of 128 spots which corresponded

to 40 different gene products could be identified for each strain (Figure 3.14; Table 3.4). Of these gene products, 17 occurred in multiple spots.

GeLC-MS/MS was used to identify secreted proteins of *B. pertussi*s strains. These proteins were separated by 1DE and proteins were visualized by CBB G250 staining. After in-gel tryptic digestion as well as peptide elution, peptides were analyzed by LC-MS/MS, resulting in identification of 357 proteins in total (Table 3.4), covering also those already identified via 2DE MALDI-TOF-MS analysis.

3.5.1. Identification of Immunoreactive Proteins

Western blotting of the 2D gels using antisera designated as Th (sc) and Sa (sc) as primary antibody and anti-mouse IgG as secondary antibody revealed a total of 56 immunoreactive protein spots (Figure 3.15). The immune reactive proteins in secretome of Tohama I and Saadet strains were nearly identical. The spots were excised, digested and analyzed using MALDI-TOF-MS. These spots corresponded to 12 different gene products (Figure 3.15, Table 3.5). Of these, 11 proteins [serum resistance protein (BrkA), pertactin (PRN), Hsp 10, elongation factor Tu, glutamine binding periplasmic protein, pertussis toxin (PT), serotype 2 fimbrial subunit, Vag8, outer membrane porin protein precursor BP0840, putative exported protein (BP3575) and superoxide dismutase] have already been shown in total soluble immunoproteome (Altındiş et al., 2009) and in surface immunoproteome of *B. pertussis* (Zhu et al., 2010; Tefon et al., 2011). The immunogenic activity of a putative secreted protein (BP1047) was detected by immunoproteomics for the first time in this pathogen. The accuracy scores of the epitopes of these immunogenic proteins predicted by the server ABCpred (Table 3.5) were within a meaningful range.

3.5.2. Functional classes, Protein Localization and Signal Peptides

When functional classes of a total of 357 identified proteins were annotated, they fell into 6 categories: (i) General metabolism and enzyme families (33%), (ii) membrane transport, secretion system, signal transduction, and cell motility (17%), (iii) folding, sorting and degradation (5%), (iv) bacterial toxins and infection related proteins (3%), (v) replication, repair and translation (8%), and (vi) yet unknown ones (34%) (Figure 3.16). Localization prediction analysis revealed that ca. 50% of these proteins are located at periplasm, membrane, outer membrane or secreted which was also supported by signal peptide analysis (Table 3.4).

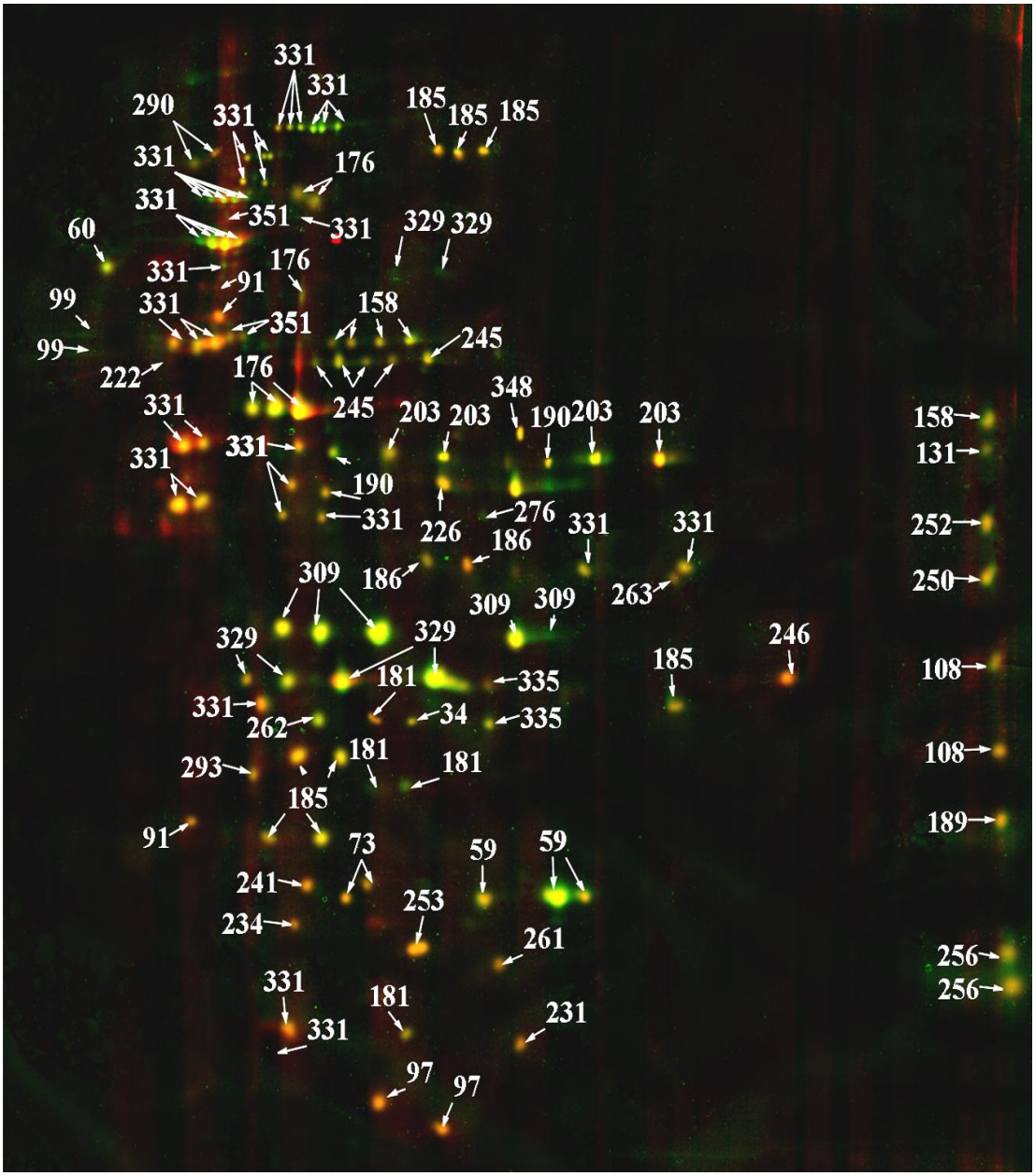

Figure 3.14. Dual channel 2D secretome imaging of *B. pertussis* strains Tohama I (green) and Saadet (red) for secretome.

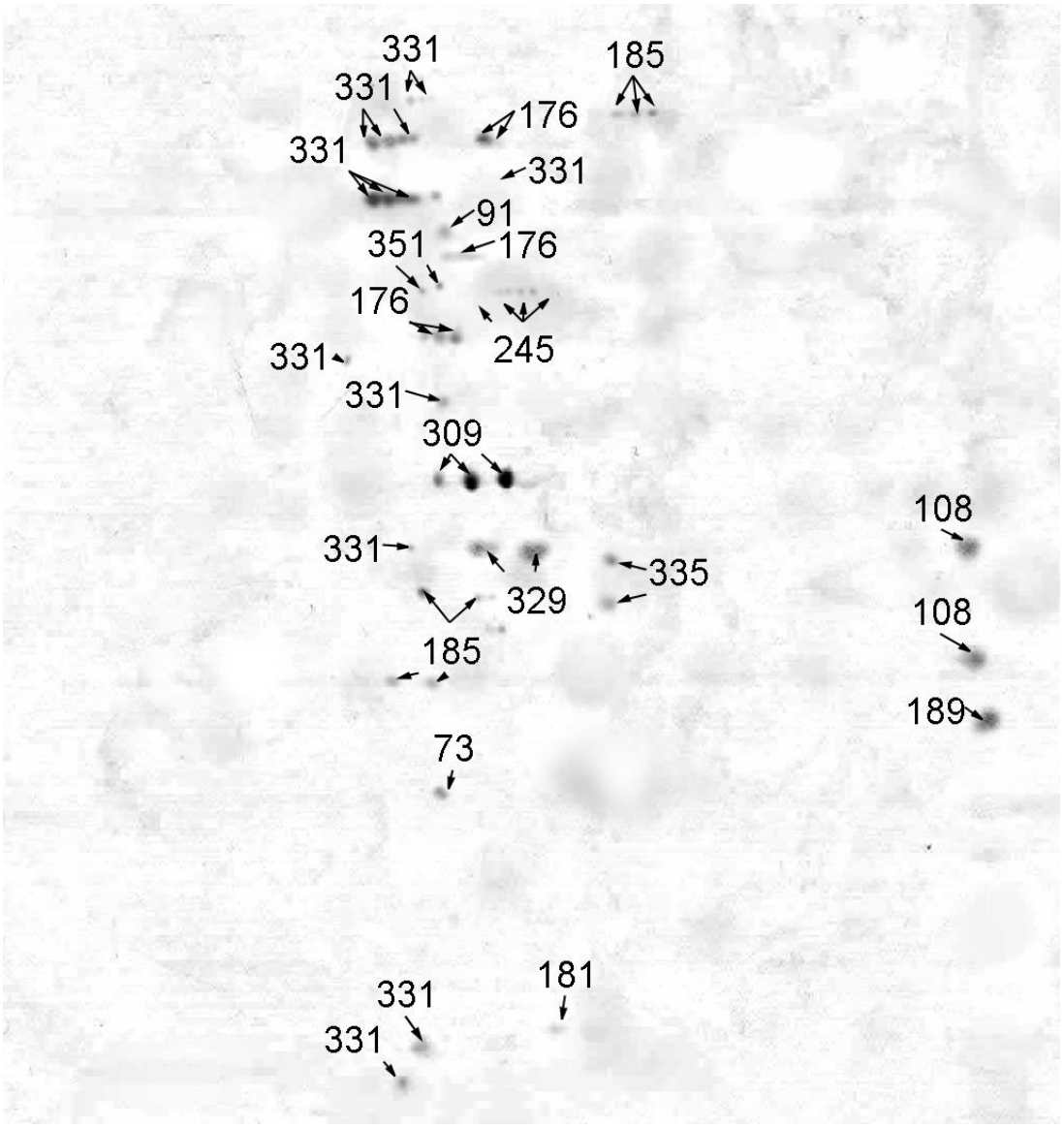

Figure 3.15. Fused 2-D Western blot analysis of the secreted proteins of *B. pertussis* strains Tohama and Saadet.

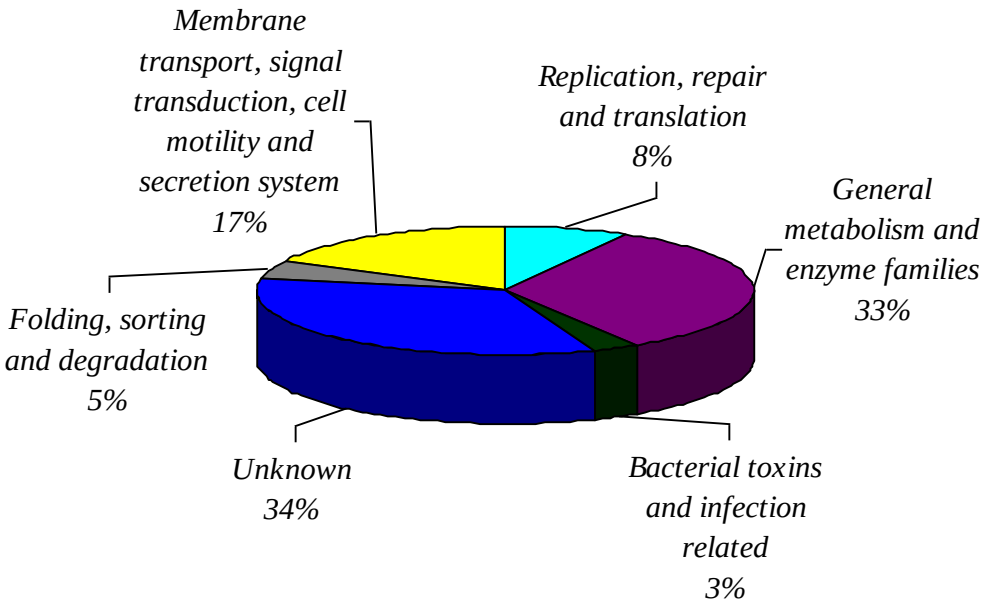

Figure 3.16. Functional classes of the secreted proteins identified from *B. pertussis*.

Table 3. 4. Secretome proteins *of B. pertussis* Tohama I and Saadet strains identified by geLC-MS/MS.

Protein no.[a]	Protein name	Gene locus	MW (kDa)	Number of isoforms[b]	Subcellular localization[c]	Signal peptide	Function	Spectral counts[d]		*Rsc*[e]
								Th	Sa	
1	2-oxoglutarate dehydrogenase complex, E3 component	BP1126	50 kDa		C	-	Carbohydrate metabolism, amino acid met	12	14	-0,0125
2	2-oxoglutarate dehydrogenase E1 component	BP1124	106 kDa		C	-	Carbohydrate metabolism, amino acid met	13	17	0,1424
3	30S ribosomal protein S1	BP0950	62 kDa		C	-	Translation	10	10	-0,2161
4	30S ribosomal protein S3	BP3619	29 kDa		C	-	Translation	6	4	-0,6824
5	30S ribosomal protein S5	BP3633	18 kDa		C	-	Translation	2	3	0,1719
6	3-isopropylmalate dehydrogenase	BP1483	39 kDa		C	-	Amino acid metabolism	4	9	0,7518
7	3-ketoacyl-(acyl-carrier-protein) reductase	BP1128	26 kDa		C	-	Lipid metabolism	3	5	0,3417
8	3-oxoacyl-(acyl carrier protein) synthase II	BP2439	43 kDa		C	-	Lipid metabolism	3	4	0,0897
9	4-aminobutyrate aminotransferase	BP1977	45 kDa		C	-	Carbohydrate metabolism,amino acid met	6	8	0,1365
10	50S ribosomal protein L1	BP0011	24 kDa		C	-	Translation	4	5	0,0363
11	50S ribosomal protein L10	BP0013	18 kDa		C, P	-	Translation	5	3	-0,7730
12	50S ribosomal protein L14	BP3626	13 kDa		C	-	Translation	4	3	-0,5210
13	50S ribosomal protein L15	BP3635	15 kDa		C	-	Translation	2	5	0,7292
14	50S ribosomal protein L16	BP3620	16 kDa		C	-	Translation	4	4	-0,2157
15	50S ribosomal protein L19	BP0975	14 kDa		C	-	Translation	5	3	-0,7730
16	50S ribosomal protein L24	BP3627	11 kDa		C	-	Translation	3	5	0,3417
17	50S ribosomal protein L3	BP3613	24 kDa		C	-	Translation	2	5	0,7292
18	50S ribosomal protein L5	BP3628	20 kDa		C	-	Translation	3	7	0,7431
19	50S ribosomal protein L6	BP3631	19 kDa		C	-	Translation	3	5	0,3417
20	50S ribosomal protein L9	BP2793	16 kDa		C	-	Translation	3	5	0,3417
21	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	BP2543	84 kDa		C	-	Amino acid metabolism	8	11	0,1906
22	ABC transport protein, periplasmic component	BP2616	35 kDa		P	+	Unknown	4	5	0,0363
23	ABC transporter	BP0697	28 kDa		C	-	Membrane transport	0	2	1,1640

Table 3. 4. Continued

24	ABC-transport protein, solute-binding component	BP2692	56 kDa	P	+	Membrane transport	10	7	-0,6649
25	Acetolactate synthase 2 catalytic subunit	BP1538	61 kDa	C	-	Carbohydrate metabolism, amino acid metabolism, metabolism of mofactors andvitamins	11	3	-1,7470
26	Acetolactate synthase 3 regulatory subunit	BP0790	18 kDa	C	-	Carbohydrate metabolism	2	3	0,1719
27	Acetyl-coa carboxylase carboxyltransferase subunit alpha	BP1910	36 kDa	C	-	Carbohydrate metabolism, lipid metabolism, metabolism of terpenoids and polyketides	3	4	0,0897
28	Acetylglutamate kinase	BP0383	32 kDa	C	-	Amino acid metabolism	4	7	0,4377
29	Acyl-coa dehydrogenase	BP0964	65 kDa	C	-	Metabolism of terpenoids and polyketides	5	2	-1,1605
30	Acyl-coa dehydrogenase	BP1445	42 kDa	C	-	Metabolism of terpenoids and polyketides	2	5	0,7292
31	Acyl-coa synthetase	BP0624	55 kDa	C	-	Carbohydrate metabolism	5	3	-0,7730
32	Adenylosuccinate lyase	BP2890	50 kDa	C	-	Amino acid metabolism, nucleotide metabolism	8	9	-0,0674
33	Adenylosuccinate synthetase	BP2188	47 kDa	C	-	Amino acid metabolism, nucleotide metabolism	7	10	0,2329
34	Adhesin	BP2667	264 kDa	OM	+	Unknown	13	12	-0,3217
35	Alanine racemase, catabolic	BP1221	40 kDa	C	-	Amino acid metabolism	2	5	0,7292
36	Aldehyde dehydrogenase	BP0465	51 kDa	C	-	Carbohydrate metabolism	5	5	-0,2157
37	Alkyl hydroperoxide reductase	BP3552	20 kDa	C	-	Enzyme families	7	8	-0,0504
38	Amidophosphoribosyltransferase	BP1415	56 kDa	C	-	Amino acid metabolism; nucleotide metabolism	5	4	-0,4677
39	Amino acid-binding periplasmic protein	BP0558	36 kDa	P, OM	+	Membrane transport, secretion system	7	10	0,2329
40	Amino-acid ABC transporter binding protein precursor	BP0057	32 kDa	P	+	Membrane transport	2	2	-0,2155
41	Aminopeptidase N	BP0869	100 kDa	C	-	Amino acid metabolism	12	14	-0,0125
42	Aminotransferase	BP3571	31 kDa	C	-	Amino acid metabolism, metabolism of cofactors and vitamins	5	4	-0,4677
43	Anthranilate synthase component I	BP3264	55 kDa	C	-	Amino acid metabolism	7	5	-0,6173
44	Antioxidant protein	BP0965	24 kDa	C, P	-	Unknown	5	7	0,1857
45	Argininosuccinate synthase	BP3537	49 kDa	C	-	Amino acid metabolism	11	8	-0,6228
46	Aromatic amino acid aminotransferase	BP2858	43 kDa	C	-	Amino acid metabolism	2	8	1,2961
47	Aspartate carbamoyltransferase	BP2562	48 kDa	C	-	Nucleotide metabolism, amino acid metabolism	8	4	-1,0349

Table 3. 4. Continued

48	Aspartate kinase	BP1913	45 kDa		C	-	Amino acid metabolism	10	10	-0,2161
49	Aspartate-semialdehyde dehydrogenase	BP1484	40 kDa		C	-	Amino acid metabolism	4	9	0,7518
50	Aspartyl/glutamyl-trna amidotransferase subunit A	BP0372	53 kDa		OM	-	Translation	12	6	-1,0888
51	Aspartyl/glutamyl-trna amidotransferase subunit B	BP0371	52 kDa		C	-	Translation	17	10	-0,9177
52	Aspartyl-trna synthetase	BP0709	67 kDa		C	-	Translation	9	16	0,5381
53	ATP phosphoribosyltransferase regulatory subunit	BP2189	42 kDa		C	-	Amino acid metabolism	6	3	-0,9877
54	ATP-binding protein	BP2307	57 kDa		C	-	Unknown	10	9	-0,3508
55	ATP-dependent Clp protease proteolytic subunit	BP1775	24 kDa		C	-	Enzyme families	0	4	1,8568
56	ATP-dependent protease ATP-binding subunit	BP3087	50 kDa		C	-	Folding, sorting and degradation	5	4	-0,4677
57	Autotransporter	BP0529	223 kDa		E	-	Unknown	31	17	-1,0453
58	Autotransporter subtilisin-like protease	BP0216	100 kDa		P, OM, E	-	Membrane transport, secretion system	20	24	0,0338
59	Azurin	BP0824	16 kDa	3 (C)	P	+	Membrane transport	4	5	0,0363
60	Bifunctional hemolysin-adenylate cyclase precursor	BP0760	178 kDa		E	+	Nucleotide metabolism	11	12	-0,1025
61	Bifunctional ornithine acetyltransferase/N-acetylglutamate synthase protein	BP3807	43 kDa		C	-	Amino acid metabolism	7	13	0,5753
62	Bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	BP3416	56 kDa		C	-	Nucleotide metabolism, metabolism of cofactors and vitamins	8	6	-0,5683
63	Branched-chain amino acid-binding protein	BP1948	44 kDa		P	+	Membrane transport	8	8	-0,2160
64	**Capsular polysaccharide biosynthesis protein**	**BP1629**	**47 kDa**		**C**	**-**	**Carbohydrate metabolism**	**0**	**8**	**2,6757**
65	**Capsular polysaccharide biosynthesis protein**	**BP1630**	**37 kDa**		**C, P**	**-**	**Carbohydrate metabolism**	**0**	**5**	**2,1088**
66	Carbamoyl phosphate synthase large subunit	BP1453	118 kDa		C	-	Lipid metabolism, amino acid metabolism, metabolism of terpenoids and polyketides	30	24	-0,5278
67	Cell division protein ftsa	BP3019	44 kDa		C	-	Unknown	4	6	0,2509
68	Cell division protein mraz	BP3031	16 kDa		C	-	Unknown	3	4	0,0897
69	Chaperone protein fhad	BP1881	26 kDa		P	+	Folding, sorting and degradation	6	7	-0,0290
70	Chaperone protein htpgheat shock protein 90	BP0074	71 kDa		C	-	Folding, sorting and degradation	25	28	-0,0597
71	Chaperonin GroEL (60-kDa chaperonin)	BP3495	57 kDa		C	-	Folding, sorting and degradation	59	51	-0,4288
72	Clpb; ATP-dependent protease, atpase subunit	BP1198;	96 kDa		C	-	Folding, sorting and degradation	20	23	-0,0250
73	Chaperonin GroES (10-kDa chaperonin)	BP3496	10 kDa	2 (C-M)	C	-	Folding, sorting and degradation	3	3	-0,2156
74	Cold shock-like protein	BP1770	7 kDa		C	-	Transcription	2	2	-0,2155
75	CTP synthetase	BP2389	61 kDa		C	-	Nucleotide metabolism	3	3	-0,2156

Table 3. 4. Continued

76	Cys/Met metabolism PLP-dependent enzyme	BP3528	45 kDa		C	-	Unknown	5	4	-0,4677
77	D-aminopeptidase	BP2393	40 kDa		C	-	Enzyme families	4	3	-0,5210
78	Dermonecrotic toxin	BP3439	161 kDa		CM	-	Bacterial toxins	19	20	-0,1468
79	D-fructose-6-phosphate amidotransferase	BP0666	67 kDa		C	-	Carbohydrate metabolism	3	7	0,7431
80	Dihydrodipicolinate reductase	BP2509	28 kDa		C	-	Amino acid metabolism	4	2	-0,9084
81	Dihydrodipicolinate synthase	BP1570	31 kDa		C	-	Amino acid metabolism	8	8	-0,2160
82	Dihydrolipoamide dehydrogenase	BP0995	62 kDa		C	-	Carbohydrate metabolism	12	19	0,3988
83	DNA polymerase III subunit beta	BP0490	41 kDa		C	-	Nucleotide metabolism	7	12	0,4699
84	DNA topoisomerase IV subunit A	BP1266	84 kDa		C	-	DNA replication	2	6	0,9438
85	DNA-directed RNA polymerase subunit alpha	BP3642	36 kDa		C	-	Nucleotide metabolism, transcription	7	7	-0,2159
86	DNA-directed RNA polymerase subunit beta	BP0015	153 kDa		C	-	Nucleotide metabolism;	33	29	-0,3987
87	Electron transfer flavoprotein alpha-subunit	BP0962	31 kDa		C	-	Unknown	6	6	-0,2158
88	Electron transfer flavoprotein beta-subunit	BP0961	27 kDa		C	-	Unknown	12	10	-0,4532
89	Elongation factor G	BP3610	77 kDa		C	-	Translation	28	33	0,0125
90	Elongation factor Ts	BP1420	31 kDa		C	-	Translation	9	9	-0,2160
91	Elongation factor Tu	BP3611	43 kDa	2 (M)	C	-	Translation	15	19	0,1028
92	Enolase	BP2386	46 kDa		C	-	Carbohydrate metabolism, energy metabolism, xenobiotics biodegradation and metabolism, folding, sorting and degradation	11	11	-0,2162
93	Enoly-coa hydratase	BP1446	42 kDa		C	-	Carbohydrate metabolism,	2	4	0,4772
94	Enoyl-coa hydratase	BP3277	30 kDa		C	-	Amino acid metabolism, Carbohydrate metabolism, lipid metabolism, metabolism of terpenoids and polyketides	6	5	-0,4304
95	Enoyl-coa hydratase/isomerase	BP0627	28 kDa		C	-	Unknown	3	5	0,3417
96	Extracellular solute-binding protein	BP0121	47 kDa		P	+	Membrane transport	13	15	-0,0259
97	F0F1 ATP synthase subunit alpha	BP3286	55 kDa	2 (C-M)	C	-	Energy metabolism	23	19	-0,4789
98	F0F1 ATP synthase subunit beta	BP3288	51 kDa		C	-	Energy metabolism	23	23	-0,2170
99	Filamentous hemagglutinin/adhesin	BP1879	368 kDa	2 (M)	OM	+	Unknown	94	86	-0,3525
100	Fimbrial adhesin	BP1883	39 kDa		P	+	Infection related, secretion system, cell motility	4	4	-0,2157

Table 3. 4. Continued

101	**Fim X**	**BP2674**	**21 kDa**	**P**	**+**	**Unknown**	**0**	**5**	**2,1088**
102	Fold bifunctional protein [includes: methylenetetrahydrofolate dehydrogenase and methylenetetrahydrofolate cyclohydrolase]	BP0990	29 kDa	C	-	Metabolism of cofactors and vitamins	3	2	-0,6031
103	Fructose-1,6-bisphosphate aldolase	BP1519	38 kDa	C	-	Carbohydrate metabolism	6	9	0,2851
104	Fumarate hydratase	BP0248	50 kDa	C	-	Carbohydrate metabolism	2	4	0,4772
105	Glutamate dehydrogenase	BP1857	46 kDa	C	-	Energy metabolism	5	6	-0,0012
106	**Glutamate synthase subunit beta**	**BP3754**	**54 kDa**	**C**	**-**	**Energy metabolism**	**12**	**2**	**-2,2482**
107	Glutamine ABC transporter ATP-binding protein	BP1575	27 kDa	C	-	Membrane transport	2	2	-0,2155
108	Glutamine binding periplasmic protein precursor	BP1573	27 kDa	P	+	Membrane transport	9	11	0,0420
109	Glutamine synthetase	BP1596	52 kDa	C	-	Energy metabolism, amino acid metabolism, signal transduction	7	5	-0,6173
110	Glutamyl-trna synthetase	BP2978	51 kDa	C	-	Metabolism of cofactors and vitamins, translation	11	9	-0,4742
111	Glutathione reductase	BP2120	49 kDa	C	-	Amino acid metabolism	5	10	0,6345
112	Glyceraldehyde-3-phosphate dehydrogenase	BP1000	36 kDa	C	-	Carbohydrate metabolism	6	6	-0,2158
113	Glycerol-3-phosphate-binding periplasmic protein precursor	BP1281	48 kDa	P	+	Membrane transport, secretion system	23	26	-0,0474
114	Glycine dehydrogenase	BP0197	102 kDa	C	+	Amino acid metabolism	7	5	-0,6173
115	Hemolysin activator-like protein	BP1884	64 kDa	OM	-	Infection related	6	6	-0,2158
116	Homoserine dehydrogenase	BP2784	46 kDa	C	-	Amino acid metabolism	7	13	0,5753
117	Hypothetical protein BP0101B	BP0101B	23 kDa	C	-	Unknown	3	2	-0,6031
118	Hypothetical protein BP0116	BP0116	14 kDa	C	-	Unknown	3	3	-0,2156
119	Hypothetical protein BP0279	BP0279	28 kDa	C	-	Unknown	4	5	0,0363
120	Hypothetical protein BP0334	BP0334	34 kDa	C, CM, P	+	Unknown	6	6	-0,2158
121	Hypothetical protein BP0682	BP0682	35 kDa	P	+	Unknown	2	3	0,1719
122	Hypothetical protein BP1087	BP1087	17 kDa	C	-	Membrane transport, signal transduction	2	2	-0,2155
123	Hypothetical protein BP1204	BP1204	29 kDa	C	-	Unknown	3	5	0,3417
124	Hypothetical protein BP1435	BP1435	31 kDa	C	-	Unknown	5	7	0,1857
125	Hypothetical protein BP1440	BP1440	33 kDa	CM	-	Unknown	12	13	-0,1108
126	Hypothetical protein BP1535	BP1535	55 kDa	C	-	Carbohydrate metabolism, amino acid metabolism, metabolism of cofactors and	3	19	2,0439

vitamins

Table 3. 4. Continued

127	Hypothetical protein BP1601	BP1601	33 kDa	C, P	-	Unknown	4	3	-0,5210
128	Hypothetical protein BP1722	BP1722	29 kDa	C	-	Unknown	3	4	0,0897
129	Hypothetical protein BP1843	BP1843	16 kDa	OM	-	Unknown	4	3	-0,5210
130	Hypothetical protein BP1849	BP1849	29 kDa	C	-	Unknown	2	2	-0,2155
131	Hypothetical protein BP2068	BP2068	34 kDa	P	+	Membrane transport	18	18	-0,2167
132	Hypothetical protein BP2233	BP2233	25 kDa	C	-	Unknown	4	4	-0,2157
133	Hypothetical protein BP2308	BP2308	26 kDa	C	-	Unknown	5	6	-0,0012
134	Hypothetical protein BP2402	BP2402	17 kDa	C	-	Enzyme families	3	5	0,3417
135	Hypothetical protein BP2452	BP2452	29 kDa	P	-	Unknown	2	3	0,1719
136	Hypothetical protein BP2535	BP2535	41 kDa	OM	+	Metabolism of cofactors and vitamins	2	4	0,4772
137	Hypothetical protein BP2755	BP2755	189 kDa	P, OM	-	Unknown	3	4	0,0897
138	Hypothetical protein BP2788	BP2788	62 kDa	P	-	Unknown	4	5	0,0363
139	Hypothetical protein BP2815	BP2815	81 kDa	C	-	Unknown	12	9	-0,5879
140	Hypothetical protein BP2909	BP2909	51 kDa	C	-	Enzyme families	11	14	0,1012
141	Hypothetical protein BP2936	BP2936	37 kDa	C	-	Unknown	6	7	-0,0290
142	Hypothetical protein BP2937	BP2937	31 kDa	C	-	Unknown	5	6	-0,0012
143	Hypothetical protein BP2953	BP2953	24 kDa	P, E	-	Unknown	4	5	0,0363
144	Hypothetical protein BP2964	BP2964	49 kDa	OM	+	Enzyme families	3	7	0,7431
145	Hypothetical protein BP3013	BP3013	26 kDa	C	-	Unknown	6	8	0,1365
146	Hypothetical protein BP3084	BP3084	42 kDa	C	-	Unknown	6	3	-0,9877
147	Hypothetical protein BP3440	BP3440	35 kDa	P	+	Unknown	11	13	0,0029
148	Hypothetical protein BP3481	BP3481	37 kDa	P	+	Membrane transport	4	7	0,4377
149	Hypothetical protein BP3819	BP3819	26 kDa	P	+	Unknown	3	4	0,0897
150	Hypothetical protein BP3827	BP3827	35 kDa	P	+	Unknown	8	9	-0,0674
151	Hypothetical protein BP3867	BP3867	36 kDa	P	+	Signal transduction	9	15	0,4515
152	Inorganic polyphosphate/ATP-NAD kinase	BP2505	32 kDa	C,CM	-	Metabolism of cofactors and vitamins	5	3	-0,7730
153	Inosine-5'-monophosphate dehydrogenase	BP2625	52 kDa	C	-	Nucleotide metabolism	18	16	-0,3758
154	Isocitrate dehydrogenase	BP2488	46 kDa	C	-	Carbohydrate metabolism	9	16	0,5381

Table 3. 4. Continued

155	Isoleucyl-trna synthetase	BP1753	106 kDa	C	-	Translation	14	13	-0,3147
156	Ketol-acid reductoisomerase	BP0791	36 kDa	C	-	Amino acid metabolism	8	10	0,0673
157	Leu/Ile/val-binding protein precursor	BP1277	39 kDa	P	+	Membrane transport, secretion system	13	15	-0,0259
158	Leu/Ile/val-binding protein precursor	BP1285	40 kDa 4 (C)	P	+	Membrane transport, secretion system	15	13	-0,4068
159	Leucine-responsive regulatory protein	BP0554	19 kDa	C	+	Unknown	2	3	0,1719
160	Leucyl-trna synthetase	BP2044	99 kDa	P	-	Translation	14	19	0,1950
161	Lipoprotein	BP2750	23 kDa	P	+	Unknown	0	4	1,8568
162	Lysr family transcriptional regulator	BP1613	35 kDa	C	-	Transcription	2	3	0,1719
163	Lysyl-trna synthetase	BP1102	57 kDa	C	-	Translation	13	13	-0,2163
164	Malate synthase G	BP3680	79 kDa	C	-	Carbohydrate metabolism	17	24	0,2549
165	Malic enzyme	BP1120	82 kDa	C	-	Carbohydrate metabolism	10	19	0,6359
166	Methionyl-trna synthetase	BP0180	77 kDa	C	+	Amino acid metabolism	7	16	0,8522
167	Molecular chaperone dnak	BP2499	70 kDa	C	-	Folding, sorting and degradation	26	42	0,4563
168	Molybdate-binding periplasmic protein precursor	BP3095	26 kDa	P	+	Membrane transport	4	4	-0,2157
169	N-acetyl-gamma-glutamyl-phosphate reductase	BP2960	38 kDa	C	-	Amino acid metabolism	11	9	-0,4742
170	NADH dehydrogenase subunit G	BP0847	81 kDa	C	-	Energy metabolism	3	7	0,7431
171	Nitrogen regulatory protein	BP2699	12 kDa	C	-	Signal transduction	4	5	0,0363
172	Oligopeptidase A	BP0989	76 kDa	C	-	Enzyme families	8	15	0,6001
173	Osmolarity response regulator	BP3222	28 kDa	C	-	Unknown	8	9	-0,0674
174	Outer membrane lipoprotein	BP2508	20 kDa	P	+	Unknown	0	4	1,8568
175	Outer membrane lipoprotein lolb	BP3127	21 kDa	P	-	Unknown	2	3	0,1719
176	Outer membrane porin protein precursor	BP0840	41 kDa 3 (C-M)	OM	+	Unknown	17	20	0,0043
177	Outer membrane protein A precursor	BP0943	21 kDa	P	+	Unknown	5	3	-0,7730
178	Outer membrane usher protein precursor	BP1882	93 kDa	OM	-	Infection related, secretion system, cell motility	2	6	0,9438
179	Outer-membrane lipoprotein carrier protein	BP2472	22 kDa	P	+	Unknown	3	4	0,0897
180	Oxidoreductase	BP0900	30 kDa	C	-	Unknown	3	3	-0,2156
181	Peptidyl-prolyl cis-trans isomerase B	BP1906	19 kDa 3 (C-M)	C, P	-	Folding, sorting and degradation	6	6	-0,2158
182	Peptidyl-prolyl cis-trans isomerase D	BP1732	70 kDa	OM	+	Folding, sorting and degradation, Enzyme families	2	10	1,5794

Table 3. 4. Continued

183	Periplasmic solute-binding protein	BP0128	36 kDa		P	+	Membrane transport	4	3	-0,5210
184	Periplasmic solute-binding protein	BP3674	34 kDa		P	+	Membrane transport	6	11	0,5431
185	Pertactin precursor	BP1054	93 kDa	4 (C)	OM, E	+	Membrane transport, secretion system	28	30	-0,1211
186	Pertussis toxin subunit 1	BP3783	30 kDa		OM, E	+	Bacterial toxins	12	11	-0,3299
187	**Pertussis toxin subunit 2**	**BP3784**	**25 kDa**		**OM, E**	**+**	**Bacterial toxins**	**3**	**19**	**2,0439**
188	Pertussis toxin subunit 3	BP3787	25 kDa		CM, P, E	+	Bacterial toxins	4	9	0,7518
189	Pertussis toxin subunit 4	BP3785	17 kDa		P	+	Bacterial toxins	5	3	-0,7730
190	Phosphate-binding periplasmic protein precursor	BP1071	37 kDa		P	+	Membrane transport	13	18	0,2198
191	Phospho-2-dehydro-3-deoxyheptonate aldolase	BP2908	39 kDa		C	-	Amino acid metabolism	5	7	0,1857
192	Phosphoenolpyruvate synthase	BP1436	86 kDa		C	-	Carbohydrate metabolism, energy metabolism	11	16	0,2799
193	Phosphoglyceromutase	BP0607	28 kDa		C	-	Carbohydrate metabolism	5	6	-0,0012
194	Phosphoribosylformylglycinamidine synthase	BP2668	145 kDa		C	-	Nucleotide metabolism	9	6	-0,7169
195	Phosphoribosylglycinamide formyltransferase 2	BP0702	43 kDa		C	-	Nucleotide metabolism	4	5	0,0363
196	P-protein [includes: chorismate mutase and prephenate dehydratase]	BP0946	39 kDa		C	-	Amino acid metabolism	2	6	0,9438
197	Preprotein translocase subunit seca	BP3014	103 kDa		C	-	Folding, sorting and degradation, membrane transport	18	16	-0,3758
198	Preprotein translocase subunit secd	BP1046	68 kDa		CM	-	Folding, sorting and degradation; membrane transport; bacterial secretion sys	3	6	0,5563
199	Prolyl-trna synthetase	BP3348	64 kDa		C	-	Translation	12	16	0,1661
200	Putative ABC transport protein, substrate-binding component	BP2418	43 kDa		P	+	Membrane transport	4	4	-0,2157
201	Putative ABC transport solute-binding protein	BP2747	41 kDa		P	+	Membrane transport	15	18	0,0293
202	Putative ABC transporter ATP-binding subunit	BP2397	69 kDa		C	-	Membrane transport	3	7	0,7431
203	Putative ABC transporter periplasmic amino acid-binding protein	BP3831	37 kDa	4 (C)	P, OM	+	Membrane transport	15	16	-0,1299
204	Putative ABC transporter substrate binding protein	BP0301	42 kDa		P	+	Membrane transport, secretion system	9	9	-0,2160
205	Putative ABC transporter, ATP-binding protein	BP3757	30 kDa		C	-	Membrane transport	3	3	-0,2156
206	Putative aconitate hydratase	BP2014	98 kDa		C, P	-	Carbohydrate metabolism	19	22	-0,0161
207	Putative aldehyde dehydrogenase	BP3624	52 kDa		C	-	Carbohydrate metabolism, amino acid metabolism	13	8	-0,8420
208	Putative amino acid ABC transporter ATP-binding protein	BP3828	28 kDa		C	-	Membrane transport	5	4	-0,4677
209	Putative amino acid-binding periplasmic protein	BP1532	28 kDa		P	+	Membrane transport	8	13	0,4097

Table 3. 4. Continued

210	Putative amino-acid ABC transporter, periplasmic amino acid-binding protein	BP1364	28 kDa	P	+	Membrane transport, secretion system	14	13	-0,3147
211	Putative aminopeptidase	BP2391	64 kDa	C	-	Enzyme families	2	4	0,4772
212	Putative aminotransferase	BP3870	43 kDa	C	-	Enzyme families	2	5	0,7292
213	Putative autotransporter	BP2224	91 kDa	OM, E	+	Unknown	0	4	1,8568
214	Putative bacterioferritin comigratory protein	BP1307	19 kDa	P	+	Unknown	4	6	0,2509
215	Putative bifunctional protein	BP3744	43 kDa	CM, P	+	Energy metabolism	4	3	-0,5210
216	Putative binding-protein-dependent transport periplasmic protein	BP2396	58 kDa	P	+	Membrane transport	20	24	0,0338
217	Putative binding-protein-dependent transport protein (periplasmic)	BP3237	59 kDa	P	+	Membrane transport	5	5	-0,2157
218	Putative carbohydrate kinase	BP2993	33 kDa	C	-	Nucleotide metabolism	3	6	0,5563
219	Putative carboxy-terminal processing protease precursor	BP0609	51 kDa	P	-	Enzyme families	15	14	-0,3085
220	Putative cell surface protein	BP2219	35 kDa	P	+	Unknown	5	6	-0,0012
221	Putative chromosome partition protein	BP3558	131 kDa	C, OM	-	Unknown	12	13	-0,1108
222	Putative cyclase	BP3130	36 kDa	C	-	Unknown	9	12	0,1557
223	Putative D-isomer specific 2-hydroxyacid dehydrogenase	BP3543	34 kDa	C	-	Unknown	4	7	0,4377
224	Putative electron transfer flavoprotein-ubiquinone oxidoreductase	BP2403	59 kDa	P	-	Enzyme families	4	3	-0,5210
225	Putative exported protein	BP0205	19 kDa	P	+	Unknown	8	8	-0,2160
226	Putative exported protein	BP0250	35 kDa	P	+	Unknown	15	8	-1,0325
227	Putative exported protein	BP0387	25 kDa	P	+	Unknown	5	9	0,4997
228	Putative exported protein	BP0452	33 kDa	P	+	Unknown	4	3	-0,5210
229	Putative exported protein	BP0461	38 kDa	P	-	Membrane transport, secretion system	4	7	0,4377
230	Putative exported protein	BP0561	34 kDa	P	+	Unknown	12	12	-0,2162
231	Putative exported protein	BP0562	17 kDa	P	+	Unknown	3	5	0,3417
232	Putative exported protein	BP0612	34 kDa	P	+	Unknown	2	4	0,4772
233	Putative exported protein	BP0664	36 kDa	CM	+	Unknown	10	10	-0,2161
234	Putative exported protein	BP0735	21 kDa	P, C	+	Unknown	4	5	0,0363
235	Putative exported protein	BP0782	40 kDa	P	+	Membrane transport	6	7	-0,0290
236	Putative exported protein	BP0807	34 kDa	CM	+	Unknown	5	6	-0,0012
237	Putative exported protein	BP0819	35 kDa	P	+	Unknown	2	3	0,1719
238	Putative exported protein	BP0913	35 kDa	C, P	+	Membrane transport	7	6	-0,4027

Table 3. 4. Continued

239	Putative exported protein	BP1056	26 kDa		P	+	Unknown	4	11	1,0098
240	Putative exported protein	BP1211	29 kDa		P	+	Unknown	2	3	0,1719
241	Putative exported protein	BP1838	20 kDa		P	+	Unknown	6	8	0,1365
242	Putative exported protein	BP3467	88 kDa		P, OM	+	Unknown	3	5	0,3417
243	Putative exported protein	BP3501	33 kDa		P	+	Unknown	4	2	-0,9084
244	Putative exported protein	BP3568	40 kDa		P	+	Membrane transport	10	16	0,4032
245	Putative exported protein	BP3575	43 kDa	5 (C)	P	+	Membrane transport	13	17	0,1424
246	Putative exported protein	BP0385	29 kDa		P	+	Membrane transport, secretion system	10	9	-0,3508
247	Putative exported protein	BP0454	33 kDa		P	+	Unknown	11	14	0,1012
248	Putative exported protein	BP1292	30 kDa		C, CM, P	+	Unknown	4	2	-0,9084
249	Putative exported protein	BP1358	34 kDa		CM, P	+	Unknown	5	10	0,6345
250	Putative exported protein	BP1480	29 kDa		P	+	Membrane transport, secretion system	15	11	-0,6259
251	Putative exported protein	BP1506	41 kDa		C, CM, P	+	Membrane transport	9	10	-0,0813
252	Putative exported protein	BP1887	36 kDa		P	+	Unknown	11	10	-0,3395
253	Putative exported protein	BP1900	35 kDa		P	+	Unknown	6	7	-0,0290
254	Putative exported protein	BP1920	35 kDa		P	+	Unknown	2	4	0,4772
255	Putative exported protein	BP2231	43 kDa		C, CM	+	Unknown	4	10	0,8865
256	Putative exported protein	BP2652	17 kDa		C, P	-	Unknown	5	5	-0,2157
257	Putative exported protein	BP2653	33 kDa		P	+	Unknown	3	6	0,5563
258	Putative exported protein	BP2661	39 kDa		P	+	Unknown	6	7	-0,0290
259	Putative exported protein	BP2802	34 kDa		P	+	Unknown	5	8	0,3512
260	Putative exported protein	BP2818	29 kDa		P	+	Membrane transport	6	8	0,1365
261	Putative exported protein	BP3761	23 kDa		P	+	Unknown	4	6	0,2509
262	Putative exported solute binding protein	BP2963	40 kDa		P	+	Membrane transport	17	18	-0,1392
263	Putative extracellular solute-binding protein	BP1529	37 kDa		C, P	+	Membrane transport	9	11	0,0420
264	Putative extracellular solute-binding protein	BP3159	57 kDa		P	+	Membrane transport	7	8	-0,0504
265	Putative extracellular solute-binding protein	BP3802	59 kDa		P	+	Membrane transport	11	4	-1,4417
266	Putative extracellular solute-binding protein	BP3862	57 kDa		P	+	Membrane transport	18	18	-0,2167
267	Putative gamma-glutamyltranspeptidase	BP2693	59 kDa		P	-	Amino acid metabolism, lipid metabolism	6	9	0,2851

Table 3. 4. Continued

268	Putative glutamine-binding periplasmic protein precursor	BP1852	27 kDa	P	+	Membrane transport	4	7	0,4377
269	Putative glutathione transferase	BP1300	26 kDa	C	-	Amino acid metabolism	2	3	0,1719
270	Putative grpe chaperone	BP2501	20 kDa	C, P	-	Folding, sorting and degradation	2	3	0,1719
271	Putative hemolysin	BP3040	42 kDa	C	-	Amino acid metabolism, metabolism of cofactors and vitamins	3	9	1,0572
272	Putative hydrolase	BP2810	43 kDa	C	-	Amino acid metabolism	4	5	0,0363
273	Putative hydrolase	BP3857	43 kDa	C	-	Unknown	2	3	0,1719
274	Putative inner membrane protein	BP3326	26 kDa	CM, P	+	Unknown	4	2	-0,9084
275	Putative inner membrane-anchored protein	BP2190	33 kDa	C, CM, P	-	Enzyme families	7	7	-0,2159
276	Putative iron binding protein	BP1605	37 kDa	P	+	Membrane transport	10	16	0,4032
277	Putative L-asparaginase	BP2398	34 kDa	C	-	Enzyme families	2	6	0,9438
278	Putative lipoprotein	BP0178	28 kDa	P, OM	+	Unknown	4	4	-0,2157
279	Putative lipoprotein	BP1296	30 kDa	P	+	Unknown	3	3	-0,2156
280	Putative lipoprotein	BP1569	41 kDa	OM	+	Unknown	7	8	-0,0504
281	Putative lipoprotein	BP2072	22 kDa	P, OM	+	Unknown	6	6	-0,2158
282	Putative lipoprotein	BP2564	15 kDa	C, P	+	Unknown	3	3	-0,2156
283	Putative lipoprotein	BP2992	17 kDa	E	+	Unknown	5	4	-0,4677
284	Putative lipoprotein	BP3454	34 kDa	P	+	Unknown	0	3	1,5515
285	Putative lipoprotein	BP3760	28 kDa	P, E	+	Unknown	3	3	-0,2156
286	Putative membrane protein	BP1485)	52 kDa	OM	-	Unknown	8	5	-0,7829
287	Putative membrane protein	BP2191	47 kDa	P	-	Enzyme families	5	10	0,6345
288	Putative membrane-bound transglycolase	BP3268	47 kDa	P	+	Enzyme families	4	3	-0,5210
289	**Putative outer membrane (permeability) protein**	**BP3329**	**89 kDa**	**OM**	**+**	**Unknown**	**0**	**5**	**2,1088**
290	Putative outer membrane ligand binding protein (BipA)	BP1112	137 kDa 2 (C)	OM	-	Unknown	47	53	-0,0470
291	Putative outer membrane protein	BP1428	21 kDa	P, OM	-	Unknown	2	2	-0,2155
292	Putative outer membrane protein	BP3755	23 kDa	OM	+	Unknown	0	2	1,1640
293	**Putative outer protein D (BopD)**	**BP2253**	**32 kDa**	**OM**	**+**	**Unknown**	**1**	**11**	**2,2338**
294	Putative oxidoreductase	BP2454	27 kDa	C	-	Carbohydrate metabolism	5	5	-0,2157
295	Putative oxidoreductase	BP2906	51 kDa	C	-	Carbohydrate metabolism	2	7	1,1306

Table 3. 4. Continued

296	Putative oxidoreductase	BP3139	50 kDa	C	-	Unknown	2	3	0,1719
297	Putative peptidase	BP0906	71 kDa	P	+	Unknown	15	26	0,5343
298	Putative peptidase	BP1721	30 kDa	P	+	Unknown	8	10	0,0673
299	Putative peptidoglycan-associated lipoprotein	BP3342	18 kDa	OM	+	Unknown	13	9	-0,6934
300	Putative peptidoglycan-binding protein	BP1746	44 kDa	P	+	Unknown	3	4	0,0897
301	Putative peptidyl-prolyl cis-trans isomerase	BP3330	57 kDa	P	+	Folding, sorting and degradation, enzyme families	18	18	-0,2167
302	Putative peptidyl-prolyl cis-trans isomerase	BP3561	29 kDa	P	+	Folding, sorting and degradation	9	9	-0,2160
303	Putative periplasmic solute-binding protein	BP1487	40 kDa	P	+	Unknown	15	17	-0,0481
304	Putative periplasmic substrate-binding protein	BP2055	37 kDa	P	+	Membrane transport	2	2	-0,2155
305	Putative periplasmic substrate-binding transport protein	BP2352	35 kDa	P	+	Membrane transport	4	7	0,4377
306	Putative polyamine transport protein	BP2348	41 kDa	P	+	Membrane transport	3	7	0,7431
307	Putative quinoprotein	BP2196	40 kDa	P, OM	+	Unknown	5	6	-0,0012
308	Putative regulatory lipoprotein	BP2271	47 kDa	P	+	Membrane transport	7	8	-0,0504
309	Putative secreted protein	BP1047	12 kDa	5 (C)	CM	-	Folding, sorting and degradation; membrane transport; bacterial secretion system	2	4	0,4772
310	Putative secretion system protein	BP3794	29 kDa	P	+	Secretion system	2	2	-0,2155
311	Putative septum site-determining protein (MinD)	BP3228	30 kDa	C	-	Unknown	6	5	-0,4304
312	Putative sigma factor regulatory protein	BP2435	39 kDa	C, CM, P	+	Unknown	3	9	1,0572
313	Putative solute-binding periplasmic protein	BP3572	36 kDa	P	+	Membrane transport	7	6	-0,4027
314	Putative solute-binding transport protein (periplasmic)	BP2293	59 kDa	P	+	Membrane transport	3	2	-0,6031
315	Putative sugar transport protein	BP2549	60 kDa	CM	-	Unknown	2	2	-0,2155
316	Putative sulfite oxidase subunit yedy	BP0479	36 kDa	P	-	Unknown	5	9	0,4997
317	Putative tolq-like translocation protein	BP3346	25 kDa	CM	-	Unknown	3	3	-0,2156
318	Putative type III secretion protein	BP2235	63 kDa	OM	+	Membrane transport; secretion system	6	3	-0,9877
319	Putative zinc protease	BP2497	101 kDa	P, OM	+	Enzyme families	31	38	0,0689
320	Putatvie membrane protein	BP3012	37 kDa	P	+	Unknown	9	9	-0,2160
321	Pyruvate dehydrogenase subunit E1	BP0993	101 kDa	C	-	Carbohydrate metabolism	13	13	-0,2163
322	Pyruvate kinase	BP3333	53 kDa	C	-	Carbohydrate metabolism, nucleotide metabolism	8	7	-0,3815

Table 3. 4. Continued

323	Regulatory protein bvgr	BP1876	32 kDa	C	-	Unknown	3	3	-0,2156
324	Ribonuclease PH	BP1588	26 kDa	C	-	Enzyme families	3	4	0,0897
325	Ribonucleotide-diphosphate reductase subunit alpha	BP2983	108 kDa	C	-	Nucleotide metabolism	14	12	-0,4201
326	S-adenosyl-L-homocysteine hydrolase	BP3068	52 kDa	C	-	Amino acid metabolism	18	19	-0,1432
327	Serine hydroxymethyltransferase	BP2952	45 kDa	C	-	Amino acid metabolism, energy metabolism, metabolism of cofactors and vitamins	4	8	0,6032
328	Serine protease	BP2434	52 kDa	P	+	Unknown	18	18	-0,2167
329	Serotype 2 fimbrial subunit precursor	BP1119	22 kDa 4 (C)	E	+	Infection related, membrane transport, secretion system, cell motility	5	4	-0,4677
330	Serotype 3 fimbrial subunit	BP1568	22 kDa	E	+	Infection related, secretion system, cell motility secretion system, cell motility	4	6	0,2509
331	Serum resistance protein	BP3494	103 kDa 22 (C-M)	OM, E	+	Infection related, secretion system	34	40	0,0116
332	Succinate dehydrogenase flavoprotein subunit	BP2361	65 kDa	C	-	Carbohydrate metabolism, energy Metabolism, xenobiotics Biodegradation and Metabolism	14	8	-0,9403
333	Succinate dehydrogenase iron-sulfur subunit	BP2360	27 kDa	C	-	Carbohydrate metabolism, energy metabolism, xenobiotics biodegradation and metabolism	4	4	-0,2157
334	Succinyl-coa synthetase subunit beta	BP2540	41 kDa	C	-	Carbohydrate metabolism	7	10	0,2329
335	Superoxide dismutase	BP2761	21 kDa 2 (M)	E	-	Unknown	3	9	1,0572
336	Surface antigen	BP1427	86 kDa	OM	+	Unknown	8	13	0,4097
337	Thiol:disulfide interchange protein dsba precursor	BP0113	23 kDa	P	+	Folding, sorting and degradation	3	4	0,0897
338	Thioredoxin 1	BP1263	15 kDa	P	-	Folding, sorting and degradation	3	4	0,0897
339	Threonine dehydratase	BP0139	55 kDa	C	-	Amino acid metabolism	7	6	-0,4027
340	Threonine synthase	BP2783	51 kDa	C	-	Amino acid metabolism, metabolism of cofactors and vitamins	3	6	0,5563
341	Tonb-dependent receptor for iron transport	BP0856	82 kDa	OM	+	Unknown	17	21	0,0711
342	Tracheal colonization factor precursor	BP1201	66 kDa	P, OM, E	+	Membrane transport, secretion system	10	17	0,4850
343	Transcription elongation factor nusa	BP1246	55 kDa	C	-	Transcription	2	8	1,2961
344	Transcription termination factor Rho	BP1262	47 kDa	C	-	Folding, sorting and degradation, transcription	14	14	-0,2164
345	Translation initiation factor IF-2	BP1247	106 kDa	C	-	Translation	14	11	-0,5337

Table 3. 4. Continued

346	Translocation protein tolb	BP3343	47 kDa		OM	+	Unknown	18	17	-0,2941
347	Trifunctional transcriptional regulator/proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase	BP2749	137 kDa		C	-	Amino acid metabolism	16	11	-0,7126
348	Trigger factor	BP1774	48 kDa		C	-	Unknown	21	11	-1,0824
349	Triosephosphate isomerase	BP0801	26 kDa		C	-	Carbohydrate metabolism	4	9	0,7518
350	UDP-N-acetylglucosamine synthesis bifunctional protein	BP3730	48 kDa		C	-	Carbohydrate metabolism	17	18	-0,1392
351	Vag8	BP2315	95 kDa	2 (C)	OM, E	+	Unknown	27	33	0,0632
352	Valyl-trna synthetase	BP2203	107 kDa		C	-	Translation	2	9	1,4447
353	Virulence factors transcription regulator	BP1878	23 kDa		C	-	Signal transduction	4	13	1,2289
354	Virulence sensor protein	BP1877	135 kDa		C, CM	+	Signal transduction	8	5	-0,7829
355	Zinc-binding dehydrogenase	BP0659	34 kDa		C	-	Unknown	4	6	0,2509
356	Zinc-binding dehydrogenase	BP0777	36 kDa		C	-	Carbohydrate metabolism	3	4	0,0897
357	Zinc-binding dehydrogenase	BP0800	34 kDa		C	-	Unknown	3	2	-0,6031

[a] Protein numbers also correspond to the numbers shown on the gel (Fig. 1A).
[b] C, charge modification; M, mass modification; C–M, both charge and mass modification.
[c] Predicted location of proteins by CELLO 2.5. C: Cytoplasm, OM: Outer membrane, CM: Cell membrane, P: Periplasm and E: Extracellular.
[d] Spectral counrs of proteins of strains. Th: Tohama I and Sa : Saadet..
[e] R_{SC} values greater than 2 fold were accepted as significant which are shown in bold characters.

Table 3. 5. Immunogenic proteins detected in secretome of *B. pertussis* Tohama I and Saadet strains.

Protein no.	Protein name/function	Gene locus	Mass (kDa)	p*I*	Epitope prediction	Accuracy
73	Chaperonin GroES (10-kDa chaperonin)	BP3496	10, 26	5,39	AVGPGKKTEDGKILPV	0,87
91	Elongation factor Tu	BP3611	42,88	5,34	PGSINPHTDFTAEVYI	0,97
108	Glutamine binding periplasmic protein	BP1573	26,84	8,77	VKTGTATVDFMKSQVP	0,93
176	Outer membrane porin protein precursor	BP0840	41,04	5,51	YGIIDTGIGYNDVDFK	0,92
185	Pertactin	BP1054	93,81	9,23	TLTGGADAQGDIVATE	0,95
189	Pertussis toxin subunit 4	BP3785	17,97	9,24	LELIRMVECSGKQDCP	0,94
245	Putative exported protein	BP3575	43,18	5,92	INSIKAANEFGVTATM	0,95
309	Putative secreted protein	BP1047	12,18	9,05	MSAIDTAGLVLAQAAP	0,88
329	Serotype 2 fimbrial subunit	BP1119	21,93	8,39	EASAITTYVGFSVVYP	0,90
331	Serum resistance protein	BP3494	103,31	6,62	YSLAEDPKTHVWSLQR	0,95
335	Superoxide dismutase	BP2761	21,28	6,05	AYYIDYRNARPKYLEN	0,92
351	Vag8	BP2315	94,88	6,34	GISGTGTIVMNAHLPS	0,96

3.5.3. Evaluation of Secreted Proteins

The secretome includes a set of secreted proteins, which represents nearly 30% of the total proteins of an organism, and contains different classes of molecules such as antibodies, toxins, cytokines, hormones and antimicrobial peptides. Secreted proteins play an important role in biological processes like cell attachment, migration, cell-cell communication, morphogenesis, differentiation, survival and defense, virulence factors and immune responses (Bonin-Debs et al., 2004; Ranganathan and Garg, 2009). Excretory/secretory proteins (ESPs) of an organism are placed to or excreted from the cell surface, making them easy targets for drugs and/or the immune system. Because of these features of the secretome, they are attractive targets for novel vaccines and therapeutics (Serruto et al., 2004). With the advent of MS and bioinformatic analyses, proteomic approach to obtain secretome data is one of the popular methods. Main methods to obtain secretome data are gel-based and gel-free proteomics, which were also used in this study.

Of 357 proteins, PT subunit 2 and putative outer protein D (BopD) were more abundant in Saadet while one protein, glutamate synthase subunit beta expressed at a higher level in Tohama I. Moreover, 4 proteins were expressed only in Saadet (two capsular polysaccharide biosynthesis protein, protein FimX and putative outer membrane permeability protein).

3.5.3.1. Putative Outer Protein D (BopD)

During infection, many Gram-negative pathogens secrete their virulence factors via type III secretion system and *in Bordetellae* this system is active in the Bvg^{+} phase (Yuk et al., 2000). In addition to BopD, BopN, BopB, and Bsp22 are reported as type III secreted protein in *B. bronchiseptica*. Nogawa et al. (2004) reported that BopD plays a critical role in arising cytotoxity in cells infected with *B. bronchiseptica* because cytotoxicity did not occur upon infection with BopD

mutant. In their study, they also demonstrated that BopD and BopB form a complex which is required for pore formation on the plasma membrane of host cells and suggested that BopD functions as the translocator to deliver type III effectors into the host cell cytoplasm. In our study we found that putative outer protein D is expressed at higher levels in Saadet strain than in Tohama I.

3.5.3.2. Glutamate Synthase Subunit Beta (GltS subunit β)

Glutamate synthase is a complex iron-sulfur flavoprotein which contains a 162 kDa α subunit and a 52.3 kDa β subunit which form the active αβ protomer. The small β subunit of bacterial NADPH-GltS contains one FAD cofactor and a NADPH binding site. This subunit is responsible for making reducing equivalents available to α subunit for glutamate synthesis (Vanoni et al., 1996; Morandi et al., 2000). In our study we found that glutamate synthase subunit beta was expressed at a higher level in Tohama I.

3.5.3.3. Capsular Polysaccharide Biosynthesis Protein

Exopolysaccharides of pathogenic bacteria, such as capsules, lipopolysaccharides (LPS) and lipooligosaccharides are important for virulence of the microorganism. Capsule structure is a matrix which is necessary for evading host immune system, envelops the cell and protects the pathogen against environmental conditions. These capsules are composed of polysaccharide chains and their composition varies largely among bacterial species and serotypes. Polysaccharide capsules provide resistance to phagocytosis, antimicrobial peptides and complement killing (Whitfield, 2006). Earlier, *B. pertussis* was classified as a capsulated microorganism based on polysaccharide staining techniques. However, by using conventional methods like negative staining with India ink, or colony morphology on agar plates, the presence of a capsular polysachharide could not be proven in *B. pertussis* (Calder et al., 1970). It was reported much earlier that a modified Wright's staining procedure stained the capsule of this organism (Lawson, 1939).

According to another report, cacloflour also stains the the capsule from virulent phase *B. pertussis* mutants (Weiss et al., 1989; Robison and Kuhl, 1995). According to these findings, it was concluded that *B. pertussis* produces a microcapsule. Neo et al. (2010) constructed a mutant in which the whole caspule locus was deleted the polysaccharide capsule was examined in the mutant and the wild type strains in virulent and avirulent culture conditions by electron microscopy and immunostaining. They found that *B. pertussis* produced a polysaccharide capsule optimally in avirulent phase. Moreover, a capsule structure was also detected in virulent phase of the microorganism. They suggested that the presence of the capsule does not affect adherence of *B. pertussis* to mammalian cells and does not protect the bacterium from phagocytosis, complement-mediated killing or antimicrobial peptide attack (Neo et al., 2010). In our study we found two capsular polysaccharide biosynthesis proteins only in Saadet strain, but not in Tohama I.

3.5.3.4. Outer Membrane Permeability Protein

The outer membrane (OM) of Gram-negative bacteria plays important role in providing an extra layer to the organism without preventing the material exchange which is important for the microorganism. The OM acts as a selective barrier because of its hydrophobic lipid bilayer combined with pore-forming proteins. The permeability properties of this layer with its selective feature have a major impact on the susceptibility of the microorganism to antibiotics. The existence of drug-resistance because of the modifications in the OM lipid and protein composition shows the importance of OM barrier. There are different types of proteins in the OM, such as murein lipoprotein (Lpp), OmpA and general diffusion porins are found in high levels in the *E. coli* and *Salmonella* spp. (Delcour, 2009). In this study, we found a putative outer membrane permeability protein which is expressed only by Saadet strain.

3.5.3.5. Outer Membrane Porin Protein Precursor

The outer membrane is a molecular filter with a defined elimination limit for hydrophilic solutes. The majour route for this uptake is controlled by membrane located porin proteins (Nakae, 1976). Porins are beta barrel proteins which form a water-filled channel through the membrane and allow the passive diffusion of small hydrophilic molecules. The size of the diffusing molecule depends on the size of the channel (Benz et al., 1985). It was shown that during biofilm formation, the expression of outer membrane porin protein precursor BP0840 was increased in *B. pertussis* (Serra et al., 2008). Two other porin proteins, namely OmpP and OmpQ were found to be immunogenic in total membrane and extracellular protein extractions of *B. pertussis* (Zhu et al., 2010). In our study, we demonstrated the immunogenicity of outer membrane porin protein precursor BP0840 in the secretome of the both strains.

3.5.3.6. Virulence-activated Gene 8 (Vag8)

B. pertussis expresses a *bvg*-regulated 95 kDa protein, Vag8 (Autotransporter), encoded by *vag8*. Based on the conserved C termini analysis, this protein belongs to the same family with PRN, BrkA and Tcf. In common with the other members of this protein family, Vag8 contains an RGD motif (Finn and Amsbaugh, 1998). This protein had been reported as protective only if the PT level in the environment is not sufficient for protection (Monji et al., 1986; Bokhari and Blackburn, 2008). In this study, we also showed the immunogenic activity of this protein.

In spite of the widespread availability of effective vaccines and high levels of vaccination coverage, a significant resurgence in pertussis has been observed during the past 2 decades (de Carvalho and Pereira, 2006; Leung et al., 2007). Remaining non-immunized children and older individuals with waning immunity may serve as reservoirs for the infection, occasionally transmitting *B. pertussis* to

unimmunized young infants. Pertussis which has long been considered to be only a disease of childhood is now being reported in adolescents and adults. It often goes underrecognized in such individuals because their disease manifestations are frequently atypical. Contraindication to the use of whole cell pertussis vaccines in individuals over 7 years of age as well as the short-lived duration of protection afforded is well-known. The licensed adolescent and adult formulations of acellular pertussis vaccine combined with an adult formulation of diphtheria and tetanus toxoids (Tdap) are recorded to be safe, immunogenic, and well tolerated (Halperin et al., 2000; Pichichero et al., 2006). On the other hand, in view of the moderate changes that have been observed in the genomic sequences of the bacterial PRN and PT over time, there are concerns about the gradual loss of the efficacy of the current pertussis vaccines as a result of antigenic drift and continuous selection of the least vaccine-sensitive clones.

Compared with genomics and transcriptomics, proteomics has the advantage of defining (i) final active level of proteins, (ii) differentially expressed ones, not solely at gene level, and (iii) those differentially located or secreted to outside of the cell (Khan et al., 2006; Wu et al., 2008). While shotgun proteomics provides better protein coverage, the advantage of gel-based proteomics is its capacity of analyzing post-translational protein modifications that play an important role in bacterial pathogenesis.

CHAPTER 4

CONCLUSION

- Our immunoproteomics approach led to the identification of a total of 25 soluble cytoplasmic immunogenic proteins of *B. pertussis*. Besides 3 well-known antigens of *B. pertussis*; namely pertactin, serum resistance protein and Hsp60, 10 proteins that have already been shown antigenic in certain pathogenic bacteria, but not in *B. pertussis* were identified. These included ATP-dependent protease, 30S ribosomal protein S1, RNAP α-subunit, S-adenosylmethionine synthetase, EF-Tu, glutamyl t-RNA amidotransferase subunit A, ketol acid reductoisomerase, serine protease, Hsp10 and Hsp70. A more interesting group comprised 12 proteins which have not been shown to be immunogenic in any pathogen till the present study: lysyl tRNA synthetase, preprotein translocase secA subunit, aspartate-β-semialdehyde dehydrogenase, carbamoylphosphate synthetase being the most critical ones related with pathogenesis in various bacteria.

- A putative chromosome partition protein and preprotein translocase secA subunit were found only in Tohama I and carbamoyl-phosphate synthase large chain and a putative outer membrane protein were found only in Saadet. The latter protein was of particular interest with its location, very strong reactivity and specificity.

- In the surfaceome study, it was possible to identify 45 and 226 proteins, by using 2DE and geLC-MS/MS approaches, respectively. When surface proteins of the strains were separated by 2DE and analyzed by Western blotting for their reactivity, 11 different gene products were determined. Glutamine-binding

periplasmic protein, leu/ile/val-binding protein, one putative exported protein, and iron-superoxide dismutase (Fe-SOD) were found as immunogenic for the first time in *Bordetella*. Of a total of 226 proteins identified, 16 were differentially expressed in *B. pertussis* Saadet and Tohama I strains. Five proteins were expressed only in Saadet (adhesin, chaperone protein DnaJ, fimbrial protein FimX, putative secreted protein Bsp22 and putative universal stress protein), and two (ABC transporter substrate-binding protein and a putative binding protein-dependent transport periplasmic protein) only in Tohama I. As to the protein abundance, notably, PT subunits S1 and S2 were among the more abundant ones in Saadet than in Tohama I. As known Type 3 Secretion System components mediating cytotoxicity, Bsp22 and BopD were demonstrated well in the surfaceome of Saadet strain. Another protein with a higher expression level in Saadet strain was OmpQ, a porin-like protein known to play an important role in infection.

- Secretome analyses identified 40 and 357 proteins, by using 2DE and geLC-MS/MS, respectively. Western blot analysis revealed 12 immunogenic proteins. Of 357 proteins identified in secretome study, PT subunit 2 and putative outer protein D (BopD) were more abundant in Saadet while one protein, glutamate synthase subunit beta was expressed at a higher level in Tohama I. Moreover, 4 proteins were expressed only in Saadet (two capsular polysaccharide biosynthesis protein, protein FimX and putative outer membrane permeability protein). The immunogenic activity of putative secreted protein (BP1047) was detected by immunoproteomics for the first time in this pathogen.

- Besides 2D-based immuoproteome, quantitative proteomics approach employed in this study integrated data ranging from gene expression to subcellular localization. It thus permitted a detailed comparison of pathogenic components found in surfaceome and secretome of two different *B. pertussis* strains, providing important clues for evaluation of better protection afforded by the local vaccine strain Saadet than the universal vaccine strain Tahoma I.

- Cumulatively, the present study comprehensively covered almost the entire proteome of a crucial pathogen, demonstrated many novel antigens and identified hundreds of membrane-bound proteins, cell surface-associated and extracellular proteins. It can now be extended to address physiological questions in infection-relevant settings and to facilitate quantitative proteomic profiling of host-pathogen interactions, rational drug design, reverse vaccinology as well as recombinant subunit vaccine research of our group in an effort to develop much improved new generation vaccines against the pathogen.

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APPENDIX A

COLLOIDAL COOMASSIE BLUE (CCB) STAINING
AND
STOCK SOLUTIONS

1-Coomassie Brilliant Blue (CBB) Stock

Coomassie Brilliant Blue G-250	5 g
dH_2O	100 mL

2-Fixation Buffer

40 % Ethanol	125 mL
10 % Acetic Acid	25 mL
50 % dH_2O	100 mL

The gel is shaked in this solution for 1 to 2 hours

3-Washing

Gels are washed with dH_2O two times for 10 min.

4-CCB dye solution

Ammonium sulfate	100 g
85% phosphoric acid	12 mL

CBB stock solution	20 mL
Distilled water add to	1000 mL

5-CCB staining

CCB dye solution	200 mL
Methanol	50 mL

Gels are put in to 200 mL CCB dye solution. 50 mL methanol is added afterwards. Gels are kept in this solution for 24 hours.

6-Washing

The gel is washed with dH_2O until protein spots are clearly visible.

APPENDIX B

CHEMICALS AND THEIR SUPPLIERS

Chemicals	Supplier
Acetic acid	Merck
Acetone	Merck
Acrylamide	Sigma
Ammonium sulfate	Merck
Ampholines pH (3-10)	Fluka
Bis-acryamide	Sigma
Bovine Serum Albumin (BSA)	Sigma
$CaCl_2$ H_2O	Merck
CH3CN	Applichem
CHAPS	Merck
Comassie Brillant Blue G 250	Sigma
DTT	Fluka
Ethanol	Merck
Glucose	Merck
Glycerol	Merck
Glycine	Merck
H_3PO_4	Merck
HCI Merck	Merck
IPG strips	BioRad
KH_2PO_4	Merck
Methanol	Merck
$MgSO_4$ $7H_2O$	Merck

Molecular Weight Standard (14,400-116,000)	Fermentas
NaOH	Merck
NH_4Cl	Merck
NH_4HCO_3	Applichem
SDS	Sigma
TEMED	Sigma
TFA	Applichem
Thiourea	Fluka
Trichloroacetic acid (TCA)	Merck
Tris- HCI	Sigma
Urea	Fluka

APPENDIX C

CULTURE MEDIA COMPONENTS

Cohen-Wheeler Agar Medium (Sato and Arai, 1972)

Component	Concentration
Agar	30 g/L
NaCl	2.5 g/L
KH_2PO_4	0.5 g/L
$MgCl_2.6H_2O$	0.4 g/L
Soluble Starch	1.5 g/L
Yeast Extract	2 g/L
Casamino Acid	10 g/L
$CaCl_2$ (%1)	1 mL/L
$FeSO_4.7H_2O$ (%0.5)	2 mL/L
L-Cystein	2.5 mL/L
$CuSO_4.5H_2O$ (%0.5)	1 mL/L
Activated Charcoal	4 g/L

The PH was asjusted to 7.3 and the medium was sterilized at 121^0 C for 45 min (Sato et al., 1972).

Modified Morse-Bray Medium (Morse and Bray, 1969)

A solution

Component	Concentration
Casamino Acid	10 g/L
Tris	6.0 g/L
NaCl	2.5 g/L
Starch	1.5 g/L
KH_2PO_4	0.5 g/L
$MgCl_2.6H_2O$	0.4 g/L
$CaCl_2$	0.01 g/L
Glutamic Acid	0.2 g/L

Dissolved in 600 ml of dH_2O. PH was adjusted to 7.4 and volume was completed to 975 ml with dH_2O and the mixture was autoclaved.

B solution

Component	Concentration
Glutation	100 mg/mL
FeSO4.7H2O	10 mg/mL
L-Cysteine	40 mg/mL
Ascorbic Acid	20 mg/mL
Nicotonic Acid	4 mg/mL
L-Prolin	120 mg/mL

Dissolved in 10 ml of dH_2O and volume was completed to 25 ml with dH_2O. The mixture was filtered through 0.22 µm membrane and added to A.

CURRICULUM VITAE

PERSONAL INFORMATION

Name, Surname	: Burcu Emine Tefon
Nationalitiy	: Tukish
Date and Place of Birth	: 20.05.1981, Ankara
Marital Status	: Single
Phone	: +90 312 210 64 71
Fax	: +90 312 210 79 76
e-mail	: tefon@metu.edu.tr

EDUCATION

Degree	Institution	Year of Graduation
Master of Science	Ankara University, Biotechnology	2004
Master of Science	Ankara University, Educational Sicences	2004
Bachelor Science	Ankara University, Biology	2002
High School	Çankaya Anatolian High School	1998

M.Sc Thesis: Sample Preparation and Observation Techniques for Scanning Electron Microscopy

WORK EXPERIENCE

Year	Place	Enrollment
2005-	METU Department of Biology	Research Assistant

LANGUAGES

English (Advanced), German (Intermediate), Turkish (Native)

LIST OF PUBLICATIONS

Papers:

1. Tefon, B.E., Kühnel, S., Özcengiz, E., Becher, D., Hecker, M. and Özcengiz, G. (2011). A comprehensive analysis of *Bordetella pertussis* surface proteome and identification of new immunogenic proteins. *Vaccine*. 29: 3583–95.

2. Altindiş, E., Tefon, B.E., Yildirim, V., Ozcengiz, E., Becher, D., Hecker, M., Özcengiz, G. (2009). Immunoproteomic analysis of *Bordetella pertussis* and identification of new immunogenic proteins. *Vaccine*. 22: 27(4):542–8.

3. Okay, S., Tefon, B.E., Ozkan, M.and Ozcengiz, G.(2008). Expression of chitinase A (*chiA*) gene from a local isolate of *Serratia marcescens* in Coleoptera-specific *Bacillus thuringiensis*. *J. Appl. Microbiol*. 104(1):161–70.

Presentations and Meeting Abstracts:

1. Yılmaz, Ç. Okay, S., Tefon, B.E., Özcengiz, E. ve Özcengiz, G. *Bordetella pertussis* Tohama I ve Saadet Suşuna Ait PPIase, POMP ve FIMX Genlerinin Klonlanması. XVI. Ulusal Biyoteknoloji Kongresi (13-16 Aralık 2009, Antalya). Bildiri ve Poster Özetleri Kitabı sf. 71-2 (Poster presentation).
2. Tefon, B.E., Çırçır, A., Özcengiz, E. and Özcengiz, G. Cytoplasmic and outer membrane proteome of *B. parapertussis* 12822 and immunological analysis of its cytoplamic proteins, BIOTECH METU 2009, International Symposium on Biotechnology: Developments and Trends (September, 27-30, 2009 Ankara, Turkey) (Poster presentation).
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