การหาโปรตีนบนผิวเซลล์ทั้งหมดของเชื้อเลปโตสไปรา
สายพันธุ์ก่อโรค *Leptospira interrogans* serovar Pomona

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Surface proteomics of *Leptospira interrogans* serovar Pomona

Miss Praparat Thaibankluay

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Medical Science
Faculty of Medicine
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ประภารัตน์ ได้บ้านกวย : การหาโปรตีนบนผิวเซลล์ทั้งหมดของเชื้อเลปโตสไปราสายพันธุ์ก่อโรค *Leptospira interrogans* serovar Pomona (Surface proteomics of *Leptospira interrogans* serovar Pomona) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. พญ. กนิษฐา ภัทรกุล, หน้า.

โรคเลปโตสไปโรซิสเป็นโรคที่มีการแพร่กระจายในหลายพื้นที่รวมทั้งประเทศไทย มีสาเหตุมาจากเชื้อเลปโตสไปราสายพันธุ์ก่อโรค ซึ่งพยาธิกำเนิดของโรคยังไม่ทราบชัดเจน โปรตีนบนผิวเซลล์เป็นส่วนแรกที่เชื้อสัมผัสกับเซลล์ของโฮสต์ สามารถกระตุ้นระบบภูมิคุ้มกัน และเป็นเป้าหมายในการพัฒนาเป็นวัคซีนแอนติเจน งานวิจัยในปัจจุบันศึกษาโปรตีนบนผิวเซลล์เพียงบางตัว แต่การหาโปรตีนบนผิวเซลล์ทั้งหมดของเชื้อเลปโตรสไปรายังมีน้อย ซึ่งการโปรตีนบนผิวเซลล์ทั้งหมดจะเป็นข้อมูลสำคัญสำหรับการศึกษาพยาธิกำเนิด และการค้นหาวัคซีนตัวใหม่ งานวิจัยนี้มีจุดมุ่งหมายเพื่อจำแนกโปรตีนบนผิวเซลล์ทั้งหมดของเชื้อเลปโตสไปราสายพันธุ์ก่อโรค *Leptospira interrogans* serovar Pomona ด้วยวิธีการติดฉลากโปรตีนด้วยไบโอติน (Sulfo-NHS-SS-Biotin) และวิธีการย่อยโปรตีนบนผิวเซลล์ด้วยเอนไซม์โปรติเอส ร่วมกับแมสสเปกโตรเมทรี (LC-MS/MS) การจำแนกหาจึงมีความสำคัญ ในการศึกษานี้ใช้ 2 วิธี คัดเลือกโปรตีนบนผิวเซลล์ออกจากโปรตีนอื่น เพิ่มความเข้มข้นของโปรตีน ทำบริสุทธิ์โปรตีนด้วยเจลอิเล็คโตรโฟริซิส SDS-PAGE และจำแนก (ระบุ) ชนิดโปรตีนด้วยแมสสเปกโทรเมทรี ตามด้วยการทำนายตำแหน่งของโปรตีนด้วยเครื่องมือทางชีวสารสนเทศ (bioinformatics) เทียบกับฐานข้อมูลโปรตีน (protein database). เชื้อเลปโตสไปราที่มีสภาพสมบูรณ์ถูกติดฉลากด้วยไบโอติน ตัวอย่างโปรตีนถูกทำบริสุทธิ์โดยผ่านคอลัมน์ที่มีอะวิดิน (avidin) จากนั้นชนิดโปรตีนจะถูกวิเคราะห์ด้วยเครื่องแมสสเปกโทรมิเตอร์ สำหรับวิธีการย่อยโปรตีนบนผิวเซลล์ด้วยเอนไซม์ เชื้อเลปโตสไปราที่มีสภาพสมบูรณ์ (intact cells) ถูกย่อยด้วยเอนไซม์โปรติเนสเค (proteinase K) ที่ความเข้มข้นที่เหมาะสม หลังจากโปรตีนบนผิวเซลล์ถูกย่อยไปอยู่ในส่วนสารละลาย (supernatant) โปรตีนในส่วนนี้ถูกนำไปวิเคราะห์ชนิดโปรตีนด้วยเครื่องแมสสเปกโทรมิเตอร์ ความสมบูรณ์ของผนังเซลล์ถูกตรวจสอบโดยวิธีย้อมฟลูออเรสเซนต์ (Fluorescent staining) และ เวสเทิร์น บล็อททิง (Western blotting) ตรวจสอบโปรตีน FlaA1 ซึ่งอยู่ระหว่างผนังเซลล์ชั้นนอกกับชั้นในของเชื้อและโปรตีน OmpL1 หรือ OmpL47 ซึ่งเป็นโปรตีนที่อยู่ผนังเซลล์ชั้นนอกและมีส่วนที่ยื่นออกมานอกเซลล์ จากโปรตีนที่ผ่านการทำบริสุทธิ์ด้วยวิธีติดฉลากด้วยไบโอติน และโปรตีนส่วนที่ไม่ได้ถูกย่อยด้วยเอนไซม์ (Pellet cells). หลังจากโปรตีนถูกจำแนกชนิดโปรตีนด้วย LC-MS/MS โปรตีนถูกทำนายตำแหน่งในที่อยู่เชื้อโดยเครื่องมือทางชีวสารสนเทศ 3 เครื่องมือ ได้แก่ PSORTb CELLO และ SOSUI-GramN ทำให้ได้โปรตีนที่มีตำแหน่งอยู่ที่ผนังเซลล์ชั้นนอก 510 โปรตีน ได้แก่ 214 โปรตีนได้จากทั้ง 2 วิธี 222 โปรตีนได้จากวิธีติดฉลากด้วยไบโอติน และ 74 โปรตีนจากวิธีย่อยโปรตีนด้วยเอนไซม์ นอกจากนี้ยังพบว่ามี 8 โปรตีนที่พบเจอในทุกครั้งของทั้ง 2 วิธี ได้แก่ โปรตีน LipL71 โปรตีน DUF3383 domain containing/ Phage-related protein โปรตีน LipL45 โปรตีน LolA โปรตีน LipL41 โปรตีน cheA1 โปรตีน FlaA1 และโปรตีน conserved hypothetical protein (LIC10175) ในงานวิจัยนี้ยังพบโปรตีนที่มีรายงานว่าเป็นโปรตีนบนผิวเซลล์ เช่น โปรตีน OmpL1 โปรตีน OmpL47 โปรตีน OmpL37 โปรตีน OmpL41 โปรตีน LipL71 และโปรตีน LigA เป็นต้น ดังนั้นงานวิจัยนี้ทำให้ได้ข้อมูลโปรตีนบนผิวเซลล์ของเชื้อเลปโตสไปราสายพันธุ์ก่อโรค *Leptospira interrogans* serovar Pomona รวมทั้งยังมีอีกหลายโปรตีนที่ยังไม่ถูกตรวจสอบซึ่งอาจเป็นโปรตีนบนผิวเซลล์ จึงควรตรวจสอบต่อไปในอนาคต

สาขาวิชา วิทยาศาสตร์การแพทย์	ลายมือชื่อนิสิต ....
ปีการศึกษา 2560	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....

5774048430 : MAJOR MEDICAL SCIENCE
KEYWORDS: LEPTOSPIRA / LEPTOSPIROSIS / SURFACEOME / PROTEOMIC

PRAPARAT THAIBANKLUAY: Surface proteomics of *Leptospira interrogans* serovar Pomona. ADVISOR: ASSOC. PROF. KANITHA PATARAKUL, M.D., Ph.D., pp.

Leptospirosis is a re-emerging zoonosis of global distribution including Thailand. It caused by pathogenic *Leptospira*. The pathogenesis is not clear. Surface-exposed proteins (PSEs) are first part for interactions with host cells, immune system and should be potential targets for vaccine development. To date, many researches study only individual PSEs but the studies of surface proteomic is less. The study of surface proteomic will be important information for pathogenesis studies and search for vaccine candidate. This study aimed to identify surface proteomics of pathogenic *Leptospira* by surface biotinylation and surface shaving using proteinase K that has been used to identify surface proteins in several bacteria combined with Liquid chromatography tandem-mass spectrometry (LC-MS/MS). Intact leptospiral proteins were labeled with biotin (Sulfo-NHS-SS-Biotin). Biotinylated proteins were purified though avidin column before further identification by LC-MS/MS. For surface shaving, Intact leptospiral proteins were treated with proteinase K (ProK) at optimal concentration. The supernatant (shaved protein) were identified by LC-MS/MS. The membrane integrity of leptospires were investigated during experiment in both methods by fluorescence and western blot of periplasmic protein FlaA1 and outer membrane protein OmpL1 or surface-exposed protein OmpL47 in eluted protein sample from biotinylation and in pellet cells of shaving. Proteins were predicted localization by subcellular localization tools including PSORTb, CELLO and SOSUI-GramN. These tools predicted as total 510 outer membrane proteins (OMPs) including 214 OMPs from both of surface biotinylation and shaving method, 66 of which were identified as hypothetical proteins, 222 OMPs from biotinylation only and 74 OMPs from surface shaving method. 8 OMPs. All replicate experiments of both methods identified 8 proteins in common including LipL71 (LIC11003), DUF3383 domain-containing protein/Phage-related protein (LIC12615), LipL45 (LIC11643), LolA outer membrane lipoprotein carrier protein (LIC12545), LipL41 (LIC12966), cheA1 chemotaxis protein histidine kinase-like kinase (LIC13522), FlaA-1 flagellar filament sheath protein (LIC10788), and conserved hypothetical protein (LIC10175). In addition, known PSEs including OmpL1, OmpL47, OmpL37, OmpL41, LipL71 and LigA were found in this study. Therefore, OMPs were found by surface biotinylation and surface shaving. Therefore, this study obtained surface-OMPs information of *Leptospira interrogans* serovar Pomona. There were at least 66 hypothetical proteins identified as putative surface-exposed OMPs. These proteins are interesting targets to be confirmed as PSEs and further study on their roles in pathogenic leptospires.

Field of Study: Medical Science Student's Signature ....

Academic Year: 2017 Advisor's Signature ....

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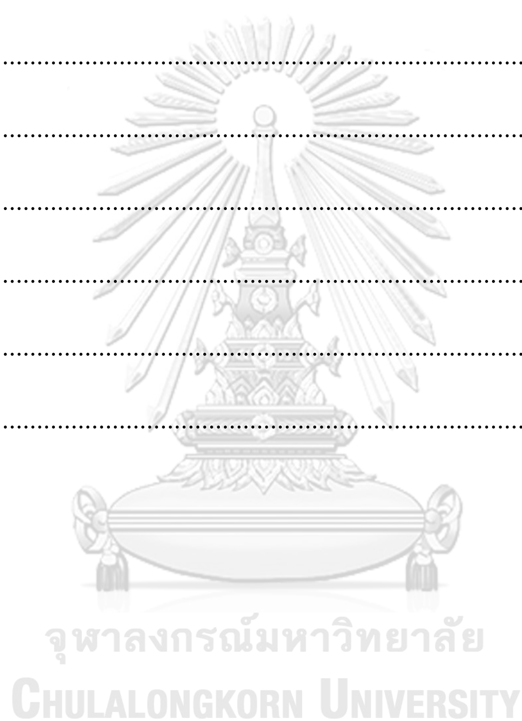

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LIST OF ABBREVIATIONS

OMPs	Outer Membrane Proteins
PSEs	Surface-Exposed Proteins
OM	Outer Membrane
OM3	OMPs were predicted as OMP by at 3 tools.
OM2	OMPs were predicted as OMP by at 2 tools
OM1	OMPs were predicted as OMP by at least 1 tools
OM (in final prediction)	OMPs were predicted as OMP at a low score by CELLO
IM	Inner Membrane
EC	Extracellular
PER	Periplasm
CYT	Cytoplasm
UNK	Unknown
IEM	Immunoelectron Microscopy
T-X-114	Triton X-114 fractionation
SPA	Surface Proteolysis Assay
SIFA	Surface Immunofluorescence Assay
SB	Surface Biotinylation
MAA	Membrane Affinity Analysis
SIP	Surface Immunoprecipitation
B	Sample from cell surface biotinylation

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S	Sample from cell surface shaving
NB	Non-biotinylated intact
InB	Biotinylated intact leptospires
F	Flow through
W	Wash
E	Elute
MS	Mass spectrometry
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis

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CHAPTER I

INTRODUCTION

Leptospirosis is caused by pathogenic *Leptospira* spp. It is the most prevalent zoonosis in the world particularly in tropical and subtropical regions including Southeast Asia, Oceania, the Indian subcontinent, Caribbean, and Latin America (1). There are annually 1.03 million cases and 58,900 deaths due to leptospirosis worldwide (January 1970 to October 2008) with The large majority occurred in tropical regions and the world's poorest countries (2). Currently, more than 300 serovars and over 20 species of pathogenic leptospires distribute worldwide (3). Most wild and domestic mammals can be reservoir hosts harboring *Leptospira* in their kidneys. Humans are considered as accidental hosts. Symptoms of leptospirosis range from asymptomatic infection to severe manifestations causing multiorgan dysfunction, such as renal and hepatic failure, pulmonary hemorrhage, and death. Pathogenesis of leptospirosis has not been well understood. After host entry, pathogenic leptospires are able to adhere to host cells and extracellular matrix proteins of target organs during the initial steps of infection. Leptospiral surface-exposed outer membrane proteins (PSEs) required for the interactions with host components are potential virulence factors.

Identification of surface-exposed OMPs that mediate the interactions with the hosts including target cells and the immune system is essential for understanding the

pathogenesis of leptospirosis and the development of diagnostic tests and vaccines for leptospirosis (4). The whole genome sequencing with gene annotations of many pathogenic microorganisms make the prediction of OMPs possible through bioinformatics analysis. However, *in silico* prediction cannot definitely inform whether the proteins are surface-exposed. Therefore, experimental strategies are required to identify proteins expressed on the cell surface and accessible to host interactions (5).

Several experimental procedures have been described for identification of bacterial surface-associated proteins (6), such as subcellular fractionation, cell surface shaving, and cell surface labelling followed by separation of the surface proteins by one or two-dimensional gel electrophoresis and identification of the proteins by mass spectrometry analysis. Subcellular fractionation has been used to isolate membrane proteins but has low reproducibility, low yields, and high contamination of cytoplasmic proteins. Cell surface biotinylation using Sulfo-NHS-SS-Biotin with hydrophilic membrane impermeable properties is commonly used to label proteins expressed at the cell surface of various organisms by reacting with primary amines (-NH2), such as lysine side-chains or the amino-terminus of polypeptides on intact whole cells (7). Biotinylated proteins are purified by highly specific biotin-avidin interactions through avidin-coated columns followed by protein identification by mass spectrometry. Cell surface shaving is a method for identifying bacterial surface-exposed proteins (8) by shaving the bacterial surface proteins (5, 6, 9) with proteolytic enzymes under conditions that preserve the integrity and viability of bacterial cells. After digestion, the

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released peptides are separated from the shaved bacteria and are subsequently identified by mass spectrometry. Proteinase K has been successfully applied for surface protein identification of Gram-positive bacteria such as group A (8) and group B *Streptococcus* (10) and Gram-negative bacteria, such as *Escherichia coli* (11, 12), *Borrelia burgdorferi, Borrelia garinii,* and *Borrelia afzelii* (13). In addition, proteinase K treatment was also used for confirmation of individual surface proteins in *Leptospira* (14, 15). Proteinase K was selected for this study because it has very broad cleavage sites (16) resulting in obtaining most coverage of surface proteins.

However, these methods have limitations. One method may not yield proteins to cover total surface-exposed proteins and both may yield contaminations of cytoplasmic proteins. Cell lysis could occur during surface biotinylation procedure and the hydrophilic biotinylation reagents can passively cross the OM through porins (17-19). Cell surface shaving may cause cell lysis during protease treatment, especially in gram-negative bacteria because theirs cells walls are relatively fragile (5, 17). To study surface proteomics of pathogenic *Leptospira,* the present study selected two different approaches, cell surface biotinylation and cell surface shaving with proteinase K, to obtain the most coverage of putative surface-exposed OMPs.

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CHAPTER II

OBJECTIVE

To identify surface proteomic of *Leptospira interrogans* serovar Pomona by cell surface biotinylation and cell surface shaving methods.

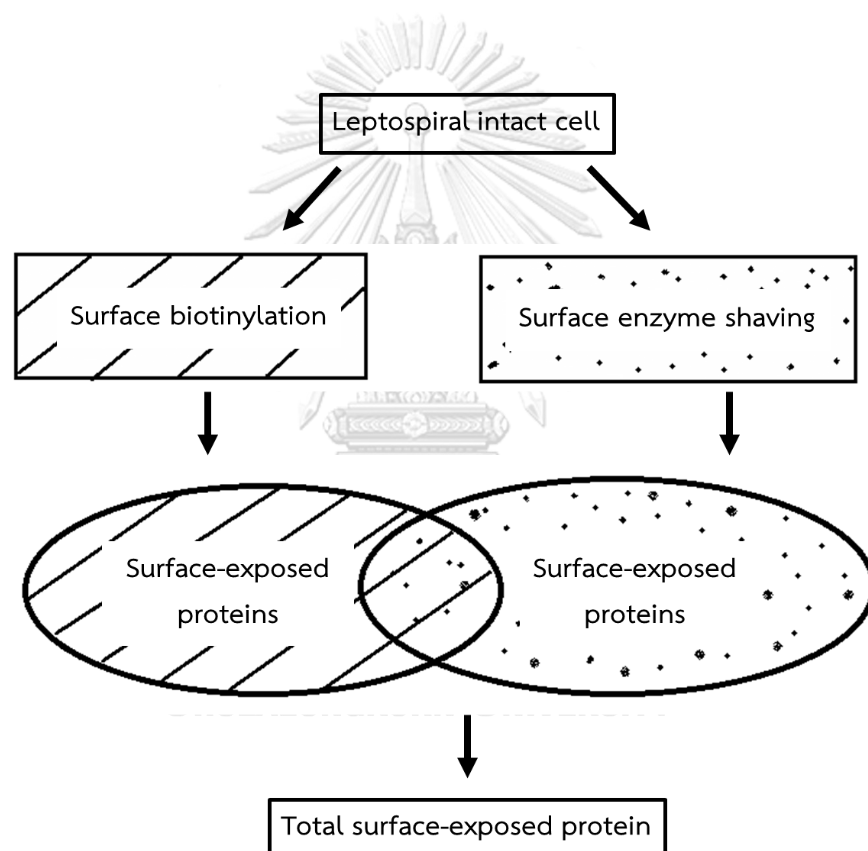

Figure 1. Conceptual Framework

CHAPTER III

REVIEW OF RELATED LITERATURES

Leptospira

Pathogenic *Leptospira* spp. is the causative agent of leptospirosis. This genus was formerly divided into *L. biflexa*, which include non-pathogenic (saprophytic) strains and *L. interrogans* that comprise pathogenic *Leptospira* spp. (20). *Leptospira* strains are classified into serogroups that contain antigenically related serovars. There are currently over 60 saprophytic and more than 250 pathogenic serovars. Serovars classified based on variations in the lipopolysaccharide (LPS) component of the leptospiral outer membrane are important for epidemiological studies. Serovars are related to common animal hosts, e.g., serovar Icterohaemorrhagiae, serovar Pomona, and serovar Canicola are associated with rats, pigs, and dogs, respectively. However, the genospecies classified based on DNA sequence similarity do not correspond to the serology-based system. *Leptospira* are Gram-negative bacteria, about 0.1 μm in diameter and 6–12 μm in length. This spirochete has double membrane comprising an outer membrane (OM) and inner (or cytoplasmic) membrane (IM), and a peptidoglycan that is associated with the IM. The principal antigenic component of the OM is lipopolysaccharide (LPS). Although the LPS is similar to that of other Gram-negative bacteria, it is not as endotoxic. Leptospires are highly motile using endoflagella

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containing two hooks located at each polar end within the periplasmic space. Leptospires can be observed under dark-field or immunofluorescence microscopy (20, 21). *Leptospira spp.* are aerobic bacteria with optimal growth at 28–30°C in enriched media containing long-chain fatty acids as their main energy source. The pathogenic serovars can survive for extended periods in the environment outside the host.

Whole genome sequences are available in several *Leptospira* species, such as *L. biflexa*, *L. interrogans* (serovars Lai and Copenhageni) and *L. borgpetersenii* (two strains of serovar Hardjo) (22-25). The Leptospiral genomes are composed of one large and one small chromosome, approximately 2,000 genes are conserved among different species, while there are over 1,000 pathogen-specific genes.

Leptospirosis

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Leptospirosis, caused by pathogenic members of the genus *Leptospira,* is one of the most widely spread zoonotic diseases in the world (26). It is estimated to cause 1.03 million cases and 58,900 deaths each year (2). The disease is more prevalent in tropical and subtropical regions of both developed and developing countries, mainly during the rainy season and flooding (27). The disease is a hazard for some occupations and activities, such as farmers, campers, some sports, and animal production (cropping, dairy farming, pig production, abattoirs). Severe urban leptospirosis outbreaks tend to be associated with serovars carried by rats and other rodents while rural outbreaks can

involve different serovars because of the greater diversity of the reservoir hosts (27-29) especially prevalent in tropical countries, where many serovars may be present in a locality (26). Many mammals can be reservoirs of *Leptospira,* such as rodents, dogs, cattle, and swine, and at least 150 mammalian species have been reported to be principal reservoirs for this spirochete (30). Because of the diversity of *Leptospira*, many animals can be reservoir of *Leptospira* resulting in difficulty to control leptospirosis (31). Moreover, the pathogenic mechanisms of leptospirosis are still not clearly revealed (32). In humans, leptospirosis is commonly transmitted by exposure to water or soil contaminated with the urine of infected animals or by direct contact with urine, blood, or tissue from infected animals (33). The bacteria can enter through broken skin or through the mucosa of the mouth, nose, or eyes and penetrate into blood stream. The incubation period is usually 10 days but can range from two to 21 days. Leptospirosis presents two phases: the acute phase when the leptospires replicate and

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rapidly disseminate throughout the organs, followed by the immune phase when antibodies are produced and clear the leptospires from the bloodstream (20). The patients with severe leptospirosis can suffer acute renal and hepatic failure, pulmonary hemorrhage that is associated with lesions in the vascular endothelium leading to death. In human, leptospirosis typically presents as a nonspecific, acute febrile illness with wide range of symptoms including high fever, headache, chills, muscle aches, conjunctivitis, diarrhea, vomiting, kidney insufficiency, liver dysfunction causing jaundice, anemia, and rashes. These symptoms are similar to other acute febrile illness,

such as dengue, influenza, and rickettsial diseases (28) resulting in misdiagnosis of these diseases. Some infected persons may have no symptoms at all. If patients are not diagnosed and treated early, leptospirosis can progress to more severity characterized by hepatic, renal or pulmonary dysfunction, hemorrhagic manifestations, meningitis, and even death (34). Severity of disease depends on the *Leptospira* strain or serovar involved, inoculum size for at least some strains, as well as the age, health and immune status of the infected individual (35). Leptospires may be isolated from the blood (days 0–7) and cerebrospinal fluid (days 4–10) during the acute illness, and from the urine after day 10. Leptospirosis is diagnosed using culture isolation, the standard method microscopic agglutination test (MAT) (36), PCR amplification of bacterial DNA from the blood during the first week after the onset of symptoms, and by detection of antibodies during the second week of the disease (37). Severe leptospirosis has also been associated with high numbers of leptospires ($\geq 10^5$ leptospires/gram) in patient tissue, including lung, liver, kidney and muscle (38). Mortality of patients with severe disease form, Weil's disease (jaundice, renal failure, and haemorrhage) and severe pulmonary haemorrhage syndrome, is high (>10% and >50%, respectively) even when optimal treatment is provided (39). Treatment of the disease varies according to its severity. Mild forms may resolve spontaneously, while severe leptospirosis requires antibiotics, such as traditionally penicillin or doxycycline and more recently ceftriaxone (40), and appropriate supportive care.

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Current vaccines for leptospirosis

Presently, the vaccines against leptospirosis have limitations. The heat-killed whole-cell vaccine preparations (bacterins) have many drawbacks including severe side effects (pain, nausea, fever), short-term immunity, and serovar-restricted protection (20). This type of vaccine has been used for agricultural or companion animals and high-risk human populations in certain countries such as Japan (41, 42), China (43), and Cuba (44), other countries in Asia (45) but there is a lack of universal vaccines due to serovar-specific protection. In addition, short-term immunity remains a major problem requiring regular yearly booster immunizations for maintenance of protective antibody levels (20).

Significance of outer membrane protein (OMPs)/ surface-exposed proteins (PSEs) of leptospires

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1. Pathogenesis

There are several PSEs that have been investigated as virulent factors such as LipL32, LipL41, LipL21, OmpL1 and LipL45. These proteins increase their expression during infection or only expressed in pathogenic serovar, and induce host immunity. Common OMPs present in pathogenic *Leptospira* include LipL32 (46), Loa22 (47), Lsa21 (48), LenA , LigA and LigB (49, 50). These proteins can function as adhesins by binding with host extracellular matrix proteins (ECM) such as fibronectin, fibrinogen, collagen

and laminin. For example, Endostatin-like protein A (LenA) can bind to human plasminogen leading to plasminogen conversion into plasmin and fibrin degradation, and entry of leptospires into host tissue (51). LigA and LigB of pathogenic leptospires are factor H binding proteins used to evade the host complement system (52, 53). Surface-exposed proteins in several pathogenic bacteria (54) and *Leptospira* (55) are surface adhesins to initiate interactions with host cells. Therefore, surface-exposed proteins have an important role in several steps of interactions between pathogenic *Leptospira* and host including adhesion, colonization, and invasion to host tissue (55). Therefore, PSEs are crucial for leptospiral pathogenesis (16).

2. Vaccine development

Currently available vaccines for leptospirosis are kill whole cell vaccines or bacterins. The immunodominant component is lipopolysaccharides (LPS), which produce antibodies specific against serovar, induce shot-term immunity, and cause several side effects. Many researchers have focused on PSEs as vaccine candidates because proteins are T-cell dependent antigens that can induce memory cells resulting in long-term immunity. OMPs that are conserved in different pathogenic serovars may confer cross protection among heterologous serovars. PSEs are accessible or easily binding to antibodies that can rapidly eliminate leptospires. Several PSEs have been investigated for their vaccine potential in animal models, such as LigA (56-59), LipL32

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(59-64), and LipL41 (65, 66). No OMP vaccine antigens have been reported to induce complete protection because target organs of challenged animals had bacterial invasion and pathology. LipL32, the most abundant OMP, was shown to be subsurface protein (15) and showed no or partial protection. Since PSEs are important for host-microbe interactions, they may be potential vaccine candidates.

3. Identification of surface-exposed proteins

Several methods have been used to identify PSEs including membrane extraction by Triton X-114 fractionation (15), cell surface biotinylation (15, 67), cell surface shaving (15), sucrose gradient centrifugation, proteomic analysis of outer membrane vesicle (OMV), surface immunofluorescence (15), surface immunoelectron microscopy (67), whole cell enzyme-linked immunosorbent assay (whole cell-ELISA), and flow cytometry. These methods were used in several bacteria and *Leptospira* (14, 15, 68-70).

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Subcellular fractionation methods, including Triton X-114 detergent extraction-phase partitioning and the isolation of OM vesicles (71-74) work well for the differentiation of OM from inner membrane lipoproteins (66, 71, 75). However, these methods are not applicable for assessment of surface-exposed proteins. A comprehensive surface-localization strategy involving several complementary methods have been used to identify and characterize proteins located on the

leptospiral surface. Cell surface biotinylation (15, 67) and cell surface shaving (15) have been widely used to identify the PSEs.

Cell surface biotinylation is the process of covalently attaching biotin to proteins or other molecules (Figure 1). Biotinylation is highly rapid, specific, and is unlikely to disturb the natural function of the molecules due to its small size (MW = 244.31 g/mol). Biotin binds to avidin with an extremely high affinity, fast on-rate, and high specificity. The interactions are exploited to isolate biotinylated molecules of interest including purification of biotinylated proteins. Surface biotinylation method was used to identify the PSEs in several organisms and *Leptospira.* The biotinylation reagents developed for surface labeling of PSEs such as Sulfo-NHS-SS-Biotin, which is a hydrophilic reagent containing a sulfonate group on the N-hydroxysuccinimide ring that reacts with primary amines (-NH2), such as lysine side-chains or the amino-termini of polypeptides on the surface proteins. Cullen et al. (2005) studied surfaceome of pathogenic leptospires by surface biotinylation followed by two-dimensitional gel electrophoresis (2-DE), and proteins were identified by matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF MS). This approach identified some previously reported leptospiral surface-exposed proteins such as LipL21 (70) and LipL41 (76), and some cytoplasmic proteins, such as GroEL, and FlaB1. However, proteins such as O8F8Q0 (32), OmpL1 (76), and other transmembrane OMPs were not detected by mass spectrometry because 2-DE has a limitation to detect proteins with lower intensity and hydrophobic proteins (66, 67)

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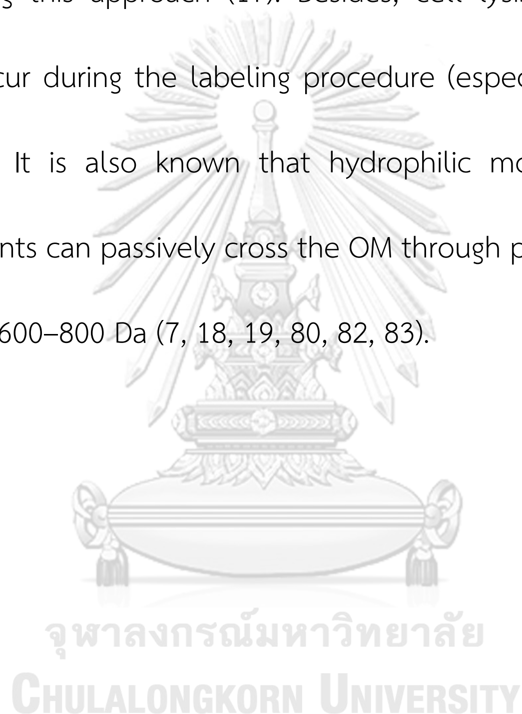
Sulfo-NHS-SS-Biotin is the most commonly used reagent to investigate the proteins expressed at the cell surface of various organisms, such as *Ehrlichia chaffeensis* (77), uropathogenic *E. coli* (12), *Neorickettsia sennetsu* (78), *Rickettsia* spp. (79, 80), and *Bacteroides fragilis* (81). However, some periplasmic proteins, inner membrane lipoproteins, integral membrane proteins, and cytoplasmic proteins are identified following this approach (17). Besides, cell lysis that causes non-surface labeling could occur during the labeling procedure (especially in centrifugation and incubation steps). It is also known that hydrophilic molecules including surface biotinylation reagents can passively cross the OM through porins, with a size exclusion limit estimated at 600–800 Da (7, 18, 19, 80, 82, 83).



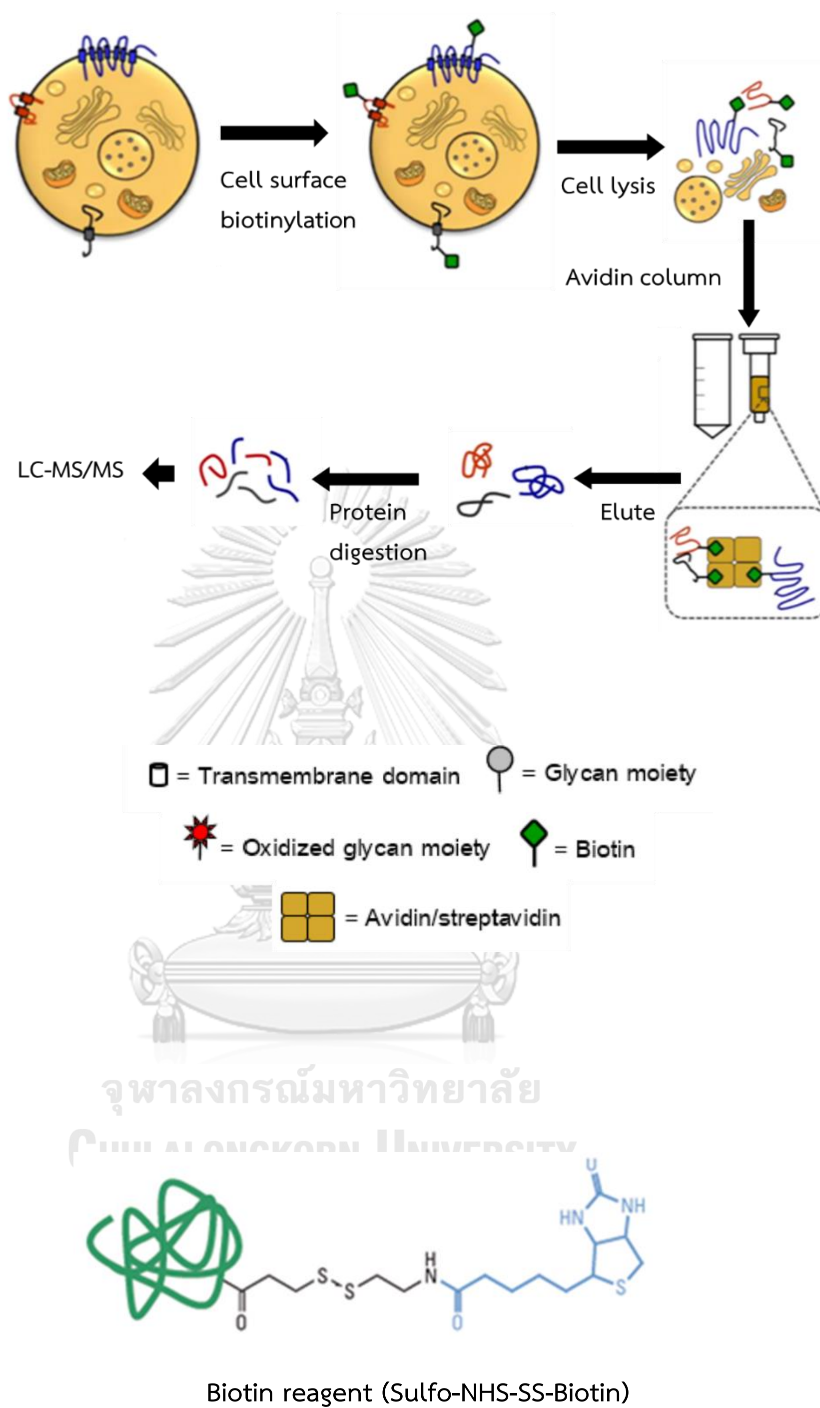


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Figure 2. Cell surface biotinylation (84)

For surface enzyme shaving, proteolytic enzymes are used to cleave or digest surface proteins into the supernatant that is subsequently used for protein identification (Figure 2). Trypsin and proteinase K are widely used enzymes in several bacteria, such as *Staphylococcus aureus* (85, 86), *Streptococcus pyogenes* (87), *Streptococcus suis* (88), *Streptococcus pneumoniae* (89).

Trypsin is a serine protease that specifically cleave at C-terminal side of positively charged lysine or arginine residues. Peptide fragments of 7-20 amino acids are generated (90). This enzyme is well active in both in-gel and in-solution, so it is suitable for peptide preparation in mass-spectrometry analysis. Moreover, C-terminal side of peptides always has an extra positively charged group from lysine and arginine that is suitable for mass-spectrometry (16). However, trypsin has limitations. For example, proteins which are tightly folded are difficult to be digested by trypsin. Moreover, membrane proteins are usually resistant to trypsin because cleavage sites are less. The post-translational modifications (PTMs) such as acetylation or demethylation and trimethylation of lysine and arginine residues can reduce trypsin digestion (91).

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Proteinase K is a non-specific protease with broad cleavage sites. The predominant site of cleavage is the peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids. Peptide fragments of 7-20 amino acids are generated. Proteinase K is also stable over a wide pH range (4–12), with a pH optimum at 8.0 (92) and wide temperature range from 37 °C to 50–60 °C. Some proteins that do

not expose cleavage sites for trypsin can be digested by proteinase K. For example, 11 new proteins of *S. pyogenes* were identified by surface shaving with proteinase K since their transmembrane domains are not enough exposed for digestion by trypsin (10). PSEs of *S. aureus* were digested by trypsin or proteinase K and then identified by MS showing 10 proteins found by both enzymes, 16 proteins found by proteinase K only, and 13 proteins found by trypsin only (85). Due to non-specific property of enzyme, digestion with proteinase K may get variable results so the experiments should be performed in replicate.

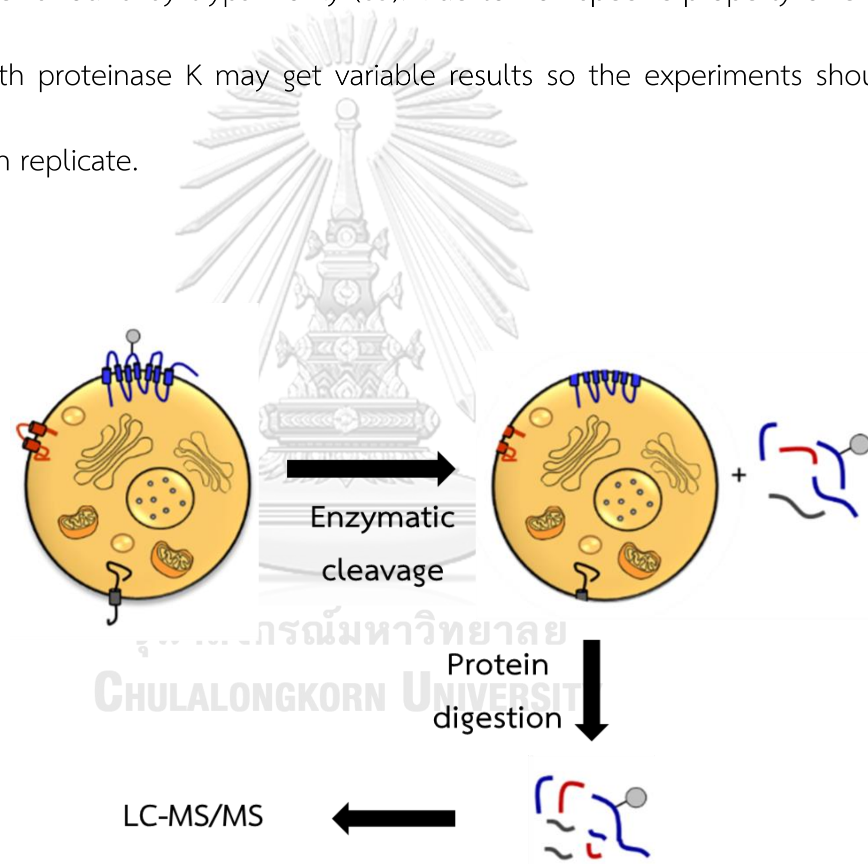

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Figure 3. Cell surface shaving (84)

Proteomic analysis

Gel-based methods are proteomic analyses of complex protein samples involving the resolution of proteins using two-dimensional gel electrophoresis followed by the identification of resolved proteins by mass spectrometry. This approach has limitations for membrane proteins because many hydrophobic membrane proteins are not solubilized in the non-detergent isoelectric focusing sample buffer and solubilized proteins are prone to precipitate at their isoelectric point. Limited dynamic range of detection is also an issue because membrane proteins are typically lower abundant than soluble proteins.

Mass spectrometry (MS) is a technique that widely used for proteomics study. This technique can rapidly identify proteins. The bottom-up proteomics LC-MS approach generally involves protease digestion and denaturation using trypsin as a protease, urea to denature the tertiary structure, and iodoacetamide to modify the cysteine residues. After digestion, LC-MS/MS (tandem MS) is used to derive sequence of individual peptides. (93) LC-MS/MS is most commonly used for proteomic analysis. Proteins were digested by trypsin into peptides at C-terminus of lysine and arginine. Peptides are separated by high performance liquid chromatography (HPLC) connected with ionization chamber followed by m/z analysis of peptides by mass spectrometry 1 (MS1) and ions by (MS2). Data are shown in a form of mass spectra and are analyzed by bioinformatics tools and available protein databases to identify amino acid

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components of peptides leading to identification of proteins that are matched to the database.

OMPs is a challenge for analysis by mass spectrometry because it has hydrophobic property resulting in low accessibility by enzyme digestion and difficulty for ionization. Moreover, OMPs express at low abundance. Analysis by MS, proteins which are highly abundant and ionized will provide good signals. Therefore, low abundant proteins may not be detected.

Study of surface-exposed proteins in *Leptospira*

Pinne and Haake, et al (2009) predicted 4 OMPs (OmpL36, OmpL37, OmpL47 and OmpL54) of *L. interrogans* serovar Fiocruz from database to be integral membrane proteins and then investigated their location by several techniques including cell surface biotinylation, cell surface proteinase K shaving, surface immunofluorescence, Triton X-114 and membrane affinity. The results showed that these 4 OMPs were surface labeled with biotin, but OmpL36 was not digested with proteinase K and was not identified by surface immunofluorescence. (14).

Cell surface biotinylation and cell surface proteinase K shaving methods are suitable for high-throughput screening and identification of PSEs. Other methods are limited because they cannot obtain all PSEs but often use to confirm the location of PSEs and require antibodies specific against interested proteins. Pinne (2013) (15) used

surface proteolysis method, immunofluorescence assay, and membrane affinity analysis to study the location of LipL32 of *L. interrogans* serovar Copenhageni strain Fiocruz L1130. Surface proteolysis by proteinase K revealed that LipL32 of intact cells was not digested while LipL32 of permeabilized cells was digested indicating that LipL32 have position inside of outer membrane. This result was consistent with that by immunofluorescence method showing antibody binding to LipL32 mostly in permeabilized cells but less immunofluorescent staining signals in intact cells. Membrane affinity analysis found LipL32 in the membrane fraction. These results indicated that LipL32 is a subsurface protein, which is different from previous study (67). Surface biotinylation and surface shaving combined with LC-MS/MS for high-throughput identification of PSEs in *Leptospira interrogans* serovar Pomona have never been reported.

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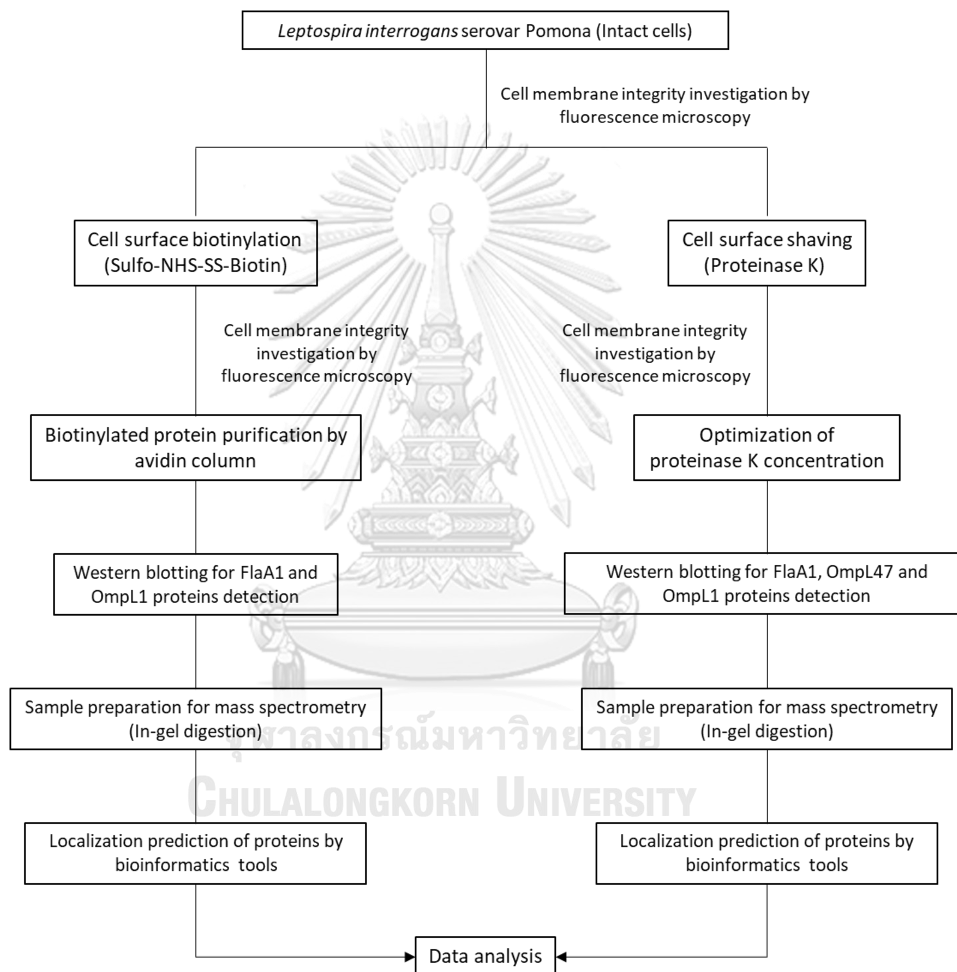
CHAPTER IV

MATERIALS AND METHODS

Research design



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Growth of *Leptospira*

Low-passage *Leptospira interrogans* serovar Pomona (originally obtained from Professor Ben Adler, Monash University, Australia and maintained at the Faculty of Medicine, Khon Kaen University) were cultivated at 30°C in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco™ *Leptospira* Medium Base EMJH) contained 10% albumin fatty acid (AFA) until reaching a log phase at a density of $5x10^8$ cells/ml and harvested by centrifugation before use. The cell density of leptospires was determined by counting cells using Petroff-Hausser counting chamber under dark-field microscope.

Dark-field microscopy and leptospiral cell count

Leptospires were diluted 1:20 with phosphate buffer saline (PBS) and 10 µl was dropped onto a Petroff-Hausser counting chamber (Hausser Scientific). Cells were counted under a dark-field microscope and used for calculation of cell density.

To determine viability of leptospires before and after both biotin labeling and proteinase K treatment. Each sample was diluted 1:20 with PBS pH 7.2-7.4. Sample of 5 µl was dropped on to a slide with a cover slip and then observed under a dark-field microscope.

Live/Dead fluorescence viability staining

SYTO9 and propidium iodide (PI) in the Live/Dead BacLight Bacterial Viability kit (Invitrogen, Thermo Fisher Scientific, Product No. l7007) were mixed at equal volumes.

The mixed dyes were diluted 1: 3,000 in phosphate buffer saline (PBS) pH 7.2-7.4 and stored on ice in the dark before use. Each leptospiral sample of 10 µl was mixed with 90 µl mixing dyes in the dark for 15 min. For compromised membrane control, leptospires were treated with 10 µl of 99.8% cold methanol followed by mixing and standing on ice 5 min before adding 90 µl of the mixed dyes. Each sample (5 µl) was put on to a slide one by one at a time. Sample slides were observed under a fluorescence microscope at excitation/emission wavelength about 480/500 nm for SYTO9 stain and 490/635 nm for propidium iodide at 400X magnification.

Cell surface biotinylation

To optimized concentration of biotin, leptospires were harvested by low-speed centrifugation at 2,000 xg for 7 min at room temperature. Cells were gently washed twice with EMJH base and used at a density of 1×10^{10} cells/ml to resuspend in phosphate buffer saline (PBS) pH 7.2 (cell surface protein isolation kit; Pierce) containing Sulfo-NHS-SS-Biotin at a final concentration of 0 (PBS alone) were used as a negative control, 0.2, 0.4, 0.6, 0.8 and 1 mg/ml. After incubation for 30 min at room temperature, the reaction was stopped by a quenching buffer (cell surface protein isolation kit; Pierce). Inactivated Sulfo-NHS-SS-Biotin was removed by two washes with Tris-buffered saline (TBS) buffer (cell surface protein isolation kit; Pierce). SDS sample buffer was added to each sample followed by heating at 100°C for 15 min, centrifugation 5,000 xg for 1 min. The supernatant of each sample was subjected to

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SDS-PAGE and western blot detected biotinylated proteins by streptavidin staining compare to cells resuspended in PBS alone (negative control). The concentration at 0.4 mg/ml was selected for this experiment. To determine cell integrity, the biotinylated cells at 0.4 mg/ml were investigated the membrane integrity under fluorescence microscopy and streptavidin stain western blot. Lysed cells resuspended in the same concentration of biotin were used as a positive control and cell resuspended in PBS alone was negative control. Biotin-labeled proteins were purified by Neutral Avidin Agarose column as the manufacturer's instruction. The biotinylated leptospiral cells was lysed in 500 µl of lysis buffer (cell surface protein isolation kit; Pierce) containing 1X protease inhibitors cocktail (Complete™ ULTRA Tablets, Mini, Easypack Protease Inhibitor, Roche) and then disrupted by sonication on ice. Cell lysates were centrifuged at 10,000xg for 2 min at 4°C and the supernatant was transferred to the column, incubated for 1h at room temperature, centrifuged at 1,000xg for 1 min, and the flow-through was collected. The column was washed with 500 µl of washing buffer (cell surface protein isolation kit; Pierce) containing 1x protease inhibitors cocktail for three time, centrifuged at 1,000xg for 1 min. Biotinylated proteins were eluted by adding 1X SDS-PAGE sample buffer containing 50 mM dithiothreitol (DTT), incubated for 60 min at room temperature and centrifuged at 1,000xg for 2 min. Each sample was resuspended with 1X sample buffer containing Coomassie brilliant blue R dye, for eluted sample was only added dye. Each sample was subjected to SDS-PAGE and western blot detected FlaA1 and OmpL1 protein.

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Cell surface shaving with proteinase K

Leptospires were harvested by low-speed centrifugation at 2,000 xg for 7 min at room temperature. Cells were gently washed twice with EMJH base and resuspended in phosphate buffer saline (PBS) pH 7.4 to a final concentration of $1x10^{10}$ cells/ml. Proteinase K in proteolysis buffer (10 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$) was added to final concentration ranges of 0-100 µg/ml. For a negative control, proteolysis buffer alone without proteinase K was added to the cell suspension. After incubation for 30 min at 37°C, the reactions were stopped by 100 µl 1x protease inhibitors cocktail (Complete™ ULTRA Tablets, Mini, Easypack Protease Inhibitor, Roche). The supernatant was collected and subjected to SDS-PAGE for further mass spectrometry analysis. The cell pellets were centrifuged at 9,000 xg for 5 min and washed twice with PBS. Then, proteins were subjected to SDS-PAGE and western blot detect OmpL1 or OmpL47 and FlaA1. In addition, pellet cells were investigated membrane integrity under fluorescence microscopy.

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Measurement of protein concentration

Protein standard and Micro BCA working reagent (WR) was prepared as the manufacturer’s instruction (Thermo Scientific™ BCA Protein Assay Kit Product No. 23235). Bovine serum albumin (BSA) Standard 2mg/ml was diluted with phosphate buffer saline (PBS) pH 7.2-7.4 to final BSA concentration at 0-200 µg/ml. Micro BCA working reagent was prepared with mixing 25 parts of Micro BCA reagent A (MA) and 24

parts Micro BCA reagent B (MB) with 1 part of Micro BCA reagent C (MC) (25: 24: 1, reagent MA: MB: MC) sufficient for number of samples and standards on two replicates. Each standard or unknown samples were replicate pipetted for 150 µL into a microplate well (Pierce™ 96-Well Plates Product No. 15041. Working reagent was added for 150 µL to each well and mix plate thoroughly on a plate shaker for 30 seconds. Plate was covered using Sealing Tape for 96-Well Plates (Thermo Scientific™ Sealing Tape for 96-Well Plates Product No. 15041) and incubated at 37°C for 2 h. After incubation, plate was cool to room temperature. Protein concentrations were measure the absorbance at 562 nm on a plate reader (Thermo Scientific™ Varioskan™ Flash Multimode Reader Product Code: MIB#5250030). Standard curve was plotted from average blank-corrected 562 nm reading for each BSA standard compared with its concentration in µg/ml. The standard curve was used to determine the protein concentration of each unknown sample.

SDS-PAGE

Each protein sample was prepared at the same concentration. The sample was resuspended in 1X SDS sample buffer and heated at 100°C for 15 min follow by centrifugation 5,000 xg for 1 min. Each sample was loaded up to 40 µl in the well of 15% polyacrylamide gel assembled in electrophoresis running systems (Mini-PROTEAN Tetra Cell, Bio-Rad) under 1x running buffer. Protein samples were separated at 120

volts for 90 min. The gel was stained with Coomassie Brilliant Blue for 30 min and destained with destaining buffer until the background was clear.

Western blot

Protein samples in the SDS-PAGE gel were electrophoretically transferred to nitrocellulose membranes with semi-dry transfer cell (SemiDry Transblot, Bio-Rad) at 15 volts for 30 min using blotting buffer. The membranes were blocked with 1% bovine serum albumin (BSA) for 1 h and washed three times 5 min with phosphate buffer saline containing 0.05%Tween 20 (PBST). After blocking, The membrane were incubation with streptavidin-horseradish peroxidase (HRP) conjugate or primary antibodies (gifts from David Haake, UCLA) specific against known outer membrane proteins OmpL1 (1: 2,000) or surface protein OmpL47 (1: 500) and a known periplasmic protein using FlaA1 (1: 2,000) in 1% BSA for 1 h at room temperature and washed with PBST three times for 5 min. Membranes were incubated with a secondary antibody: HRP-conjugated streptavidin or HRP-conjugated goat anti-rabbit IgG (1: 5,000) in 1%BSA for 1 h at room temperature and washed with PBST three times 5 min. Then the blots were stained with ECL chemiluminescent HRP-conjugate substrate (Western blotting detection reagent, Amersham™ ECL™ Prime, RPN22323, GE Healthcare) and detected by chemiluminescence detection system (ChemDoc, BioRad).

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In-gel digestion procedure for mass spectrometry

Each gel slice was diced into small pieces (approximately 0.5-1 mm^3) and placed into 1.5 ml eppendorf tubes. Two hundred microliters of 25 mM NH_4HCO_3 (Ambic) containing 50% acetonitrile (ACN) (BAKER ANALYZED HPLC ULTRA Gradient Solvent, J.T.Baker™) were added into the tubes followed by vortexing for 10 min, and the supernatant was discarded. This step was repeated once. Next, one mililiter of 25 mM Ambic containing 50% ACN was added and incubated overnight until the gel pieces were colorless. 100% ACN was added to cover the gel pieces and incubated for a few minutes until the gel pieces shrinked and turned white, and ACN was removed. The gel pieces were subjected to speed vacuum to complete dryness (about 10 min). Then, the gel pieces were rehydrated in 10 mM DTT at 56°C for 45 min, and the supernatant was removed. The gel pieces were incubated with 55 mM Iodacetamide (GE Healthcare, RPN6302) at room temperature for 30 min in the dark and supernatant was removed. 100% ACN were added followed by vortexing and incubation until the gel pieces shrink and turned white. ACN was removed and the gel pieces were subjected to speed vacuum to complete dryness. Gel pieces were completely covered by an excess of trypsin solution for digestion for 60 min on ice. After 60 minutes, any trypsin solution which was not absorbed into the gel was discarded. The gel pieces were incubated in 25 mM NH_4HCO_3 at 37°C overnight (12-16 h). The peptides were extracted by spinning down and the supernatant (water extract) was aspirated into eppendorf tubes. 50% ACN/ 0.1% formic acid (FA) in water was added to the gel pieces, followed

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by vortexing 15 min and centrifugation. The supernatant (organic extract) was combined with the water extract taken from the previous step. Extraction step was repeat, 50% ACN/ 0.1% formic acid (FA) in water was added to the gel pieces, followed by vortexing 15 min and centrifugation. The supernatant was combined with the water extract in the previous step. Peptide extracts were reduced to a final volume to approximately 10 µl by speed vacuum before applying to mass spectrometric analysis. Peptides were desalted with C-18 column. Column was calibrated with 70 µl of 100% ACN following by centrifugation 1500 rpm for 5 min at room temperature. Column was equilibrated with 100 µl of 0.1% FA following by centrifugation 1500 rpm for 5 min at room temperature. Sample was loaded into column following by centrifugation 1500 rpm. Column was wash three times with 70 µl of 0.1% FA following by centrifuged and discarded flow though. Sample was eluted with 70 ul of 0.1% FA containing 50% ACN by centrifugation 1500 rpm into low bind eppendorf tube and repeat elution step three times. Peptides were concentrated and dried by speed vacuum. Peptides were resuspended with 0.1% FA before load to mass spectrometer.

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Measurement of peptide concentration

The kit components stan at room temperature before opening and loading. Peptide Digest Assay Standard (1 mg/mL) were diluted to 0-1000 µg/ml as the manufacturer's instruction (The Thermo Scientific™ Pierce™ Quantitative Fluorometric Peptide Assay Product No. 3290). Each standards and samples 10 µl were pipetted

into well of the fluorescence-compatible microplate (Thermo Scientific™ 96-Well Black Plates Product No. 88378). Fluorometric peptide assay buffer 70 µl were added to each well. Fluorometric peptide assay reagent 20 µl were added to each well and covered by sealing tape (Thermo Scientific™ Sealing Tape for 96-Well Plates Product No. 15036) to cover plate for incubation following by incubation at room temperature for 5 min. Sealing tape was remove before making fluorescence measurements. Sample were measured the fluorescence using excitation/emission wavelength at 390 nm/ 475 nm. The standard curve was plotted and used to determine the peptide concentration of each unknown samples.

LC/MS-MS and Bioinformatics analysis

Peptide mixtures were analyzed by LC/ MS/MS using an EASY-nLC1000 system coupled to a Q-Exactive Orbitrap Plus mass spectrometer equipped with a nano-electrospray ion source (Thermo Scientific, San Jose, CA). The 5 µl of the peptide mixture was injected onto EASY-Spray PepMap RSLC C18 Column (Thermo Fisher Scientific, 2um,100 Å, 50 um x 25 cm) C18 nanoAcquity UPLC trap column (Waters, 0.18 × 20 mm, 5 µm, 100 Å) with a flow rate of 300 nl/min for 2 min and separated on a BEH300C18 nanoAcquity UPLC column (Waters, 0.075 × 250 mm, 1.7 µm, 300 Å) using a linear gradient of 5–40% of solvent B in 60 min. The peptides were eluted with 5-20% acetonitrile containing 0.1%FA for 43 min followed by 20-40% acetonitrile containing 0.1% FA for 10 min at a flow rate of 300 nl/min. The full scan measured in

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the Orbitrap mass analyzer at a mass resolution of 70,000 were followed by 10 data-dependent MS2 scans at a resolution of 17500. The normalized collision energy of higher-energy collision dissociation (HCD) fragmentation was set at 27. An MS scan range of 400-1600 m/z were selected and monoisotopic precursor ion with unassigned charge states, a charge state of +1 or a charge state of greater than +8 were excluded. Dynamic exclusion was set for 30s. Peak list generating software used was Thermo Xcalibur 3.0.63.3 (August 27, 2013). Mass spectra data from LC-MS/MS were matched with peptide sequences by X! tandem software. The MS raw data (MGF file) were advance searched by The Global Proteome Machine (GPM) database of prokaryotes bacteria *Leptospira interrogans* serovar Copenhageni Fiocruz L1130 and *Leptospira interrogans* serovar Lai 56601 containing the forward and reversed peptide sequences. The parameter was set as follow: For measurement errors, fragment mass error was 10 ppm, parent mass error was ±10 ppm, fragment type was monoisotopic. The search parameter included trypsin as the proteolytic enzyme with 1 missed cleavage. Cleavage C-terminal change +17.002735 Da and Cleavage N-terminal change +1.007825 Da. The identifier number (GI number) were uploaded for retrieve the FASTA file in UniprotKB (http://www.uniprot.org/uploadlists/). FASTA files were exported to bioinformatic localization tools including PSORTb v3.0.2 (http://www.psort.org/psortb/), CELLO v.2.5 (http://cello.life.nctu.edu.tw/), and SOSUI-GramN (http://harrier.nagahama-i-bio.ac.jp/sosui/sosuigramn/sosuigramn_submit.html). To prediction of subcellular localization base on gram-negative bacteria.

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Data analysis

Proteins were identified by liquid chromatography–tandem mass spectrometry (LC-MS/MS) and three bioinformatics tools including PSORTb v.3.0.2, CELLO v.2.5, and SOSUI-GramN were used to predict their localization. Predicted subcellular localization of identified proteins were categorized as follows:

- Outer membrane (OM) if predicted by at least 1 of 3 tools: by all 3 tools (OM3), by 2 tools (OM2), by 1 tool (OM1), or have multiple location sites with a low score at the outer membrane (OM)
- Localization sites other than OM if predicted by at least 2 of 3 tools: extracellular (EC), periplasm (PER), inner membrane (IM), and cytoplasm (CYT)
- Unknown localization (UNK) if predicted to be at different localization sites by all 3 tools or predicted as unknown localization.

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CHAPTER V

RESULTS

Cell surface biotinylation of *Leptospira interrogans* serovar Pomona

Optimization of Sulfo-NHS-SS-Biotin concentration for cell surface biotinylation

This experiment used a cell-impermeable amine-reactive biotin, Sulfo-NHS-SS-Biotin, to label surface-exposed proteins on intact bacterial cells. First, the concentration of biotin was optimized for surface labeling. Intact leptospires were incubated with the biotin at various concentrations of 0, 0.2, 0.4, 0.6, 0.8 and 1 mg/ml for 30 minutes. Unlabeled intact cells were used as a negative control. SDS-PAGE showed that approximately equal amount of total proteins from 10^8 cells/lane was used for each concentration of biotin (Fig. 4A). The biotin-labeled proteins were detected with streptavidin-horseradish peroxidase (HRP) (Fig. 4B). Only weakly streptavidin-binding proteins were seen in the unlabeled control (lane 0 mg/ml). The intensity of biotinylated proteins reached the highest signal at 0.4 mg/ml biotin. Therefore, we further used the biotin at a concentration of 0.4 mg/ml for surface labeling of leptospires in the next step.

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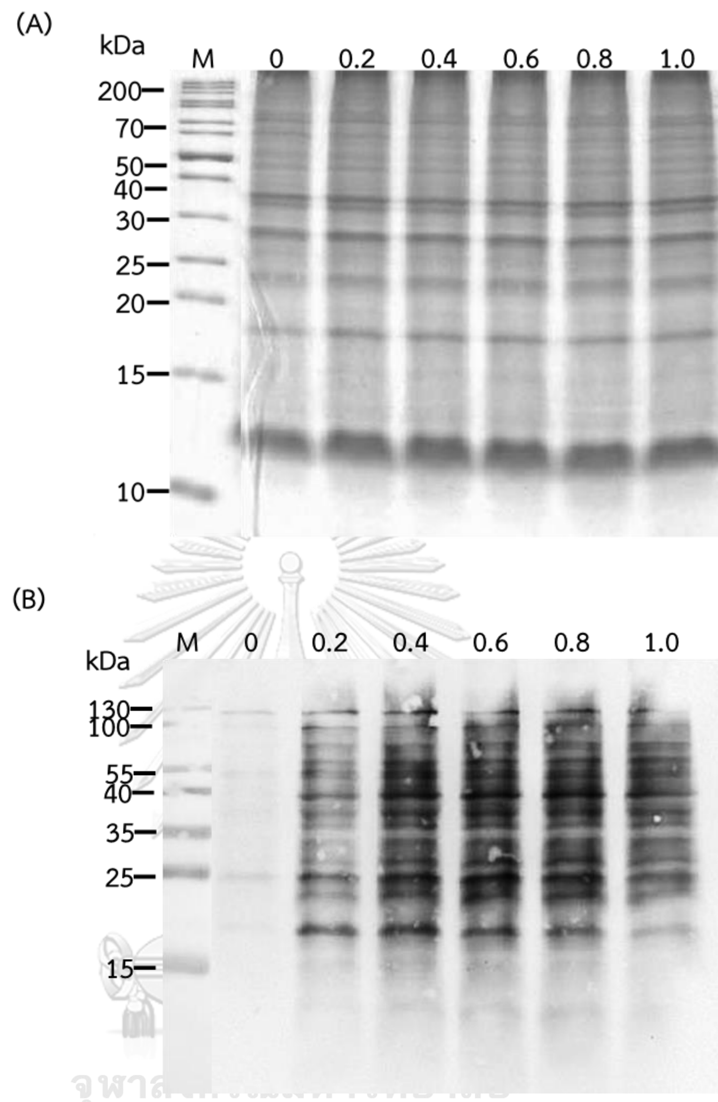

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Figure 4. Optimization of surface biotinylation. Intact 1×10^{10} leptospiral cells were incubated with different concentrations of Sulfo-NHS-SS-Biotin at a final concentration of 0.2, 0.4, 0.6, 0.8 and 1 mg/ml. Proteins from approximately 10^8 leptospires were loaded per lane and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue (A), transferred to a nitrocellulose membrane, and stained with 1:5000 HRP-conjugated streptavidin, and detected with ECL chemiluminescence detection system (B). Intact leptospires in

PBS (0 mg/ml biotin) were used as a negative control. The molecular mass standards (in kilodalton, kDa) are indicated on the left (lane M).

Determination of membrane Integrity after cell surface biotinylation

To test if leptospiral cells remained intact after biotin labeling, the membrane integrity of the bacteria was determined by fluorescent dyes, SYTO9 and propidium iodide (PI), used for viability staining. Leptospires with intact membranes were stained with SYTO9 (green) and the ones with compromised membranes were stained with PI (red). The results indicated that before labeling leptospires were mostly intact (99%) (Fig. 5A). After the process of surface labelling with 0.4 mg/ml biotin, most leptospires (approximately 79%) remained intact cells (Fig. 5B). All leptospires treated with methanol used as control cells with damaged membranes were stained red as expected (Fig. 5C). Furthermore, biotinylated proteins of intact cells detected by streptavidin-HRP were different from and had lower signals than those of the lysate cell control (Fig. 6B). No bands were detected in the unlabeled cell control. The results indicated that most cells of leptospires remained intact after surface labeling with biotin. The surface biotinylated leptospires were subsequently used for the next purification step.

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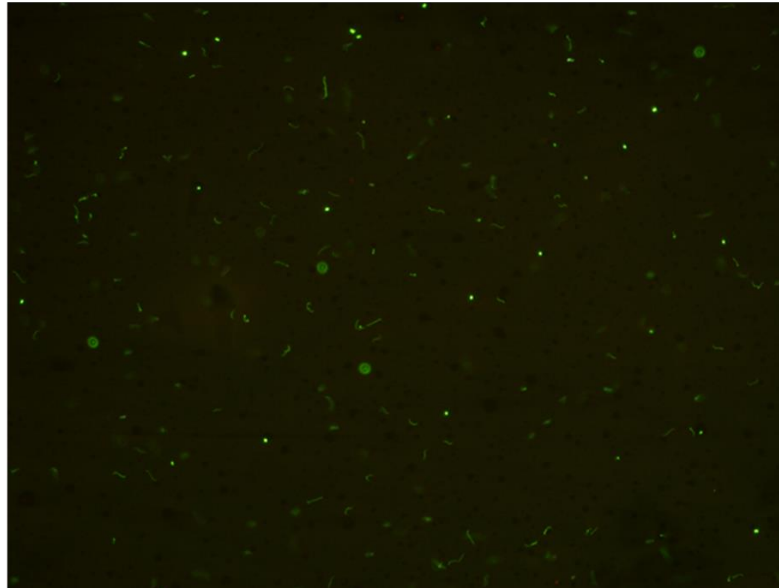
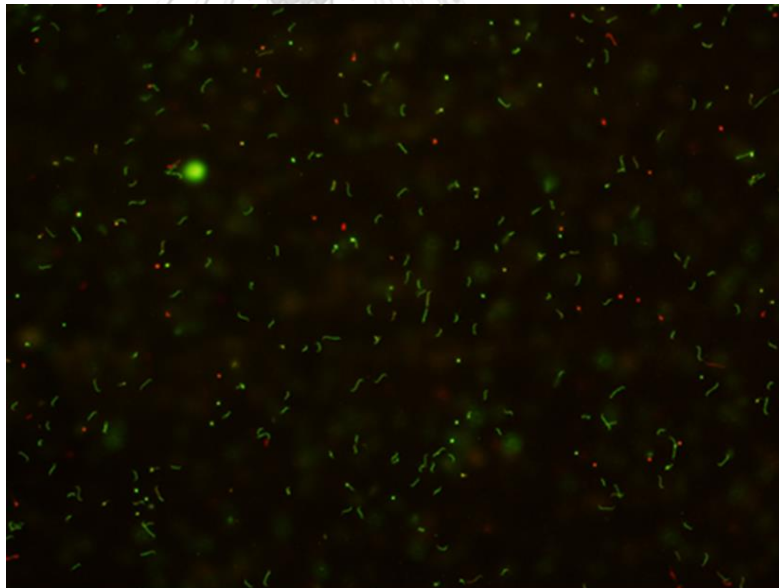
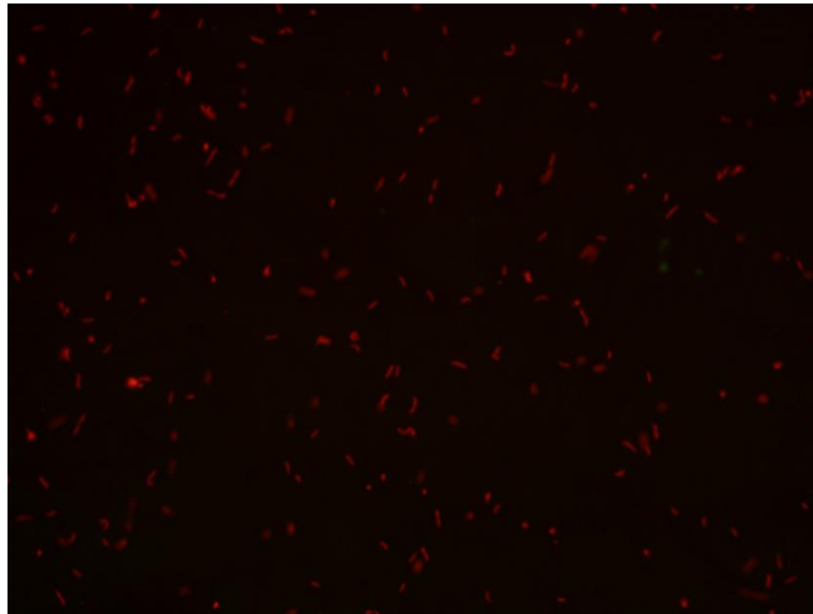


(A) Before biotin labeling (99%)



(B) After biotin labeling (79%)



(C) Non-intact cell control (methanol treatment)

Figure 5. Determination of membrane integrity after cell surface biotinylation. Leptospiral membrane integrity was determined by SYTO9/PI fluorescence staining before surface labeling 99%, (93±37 intact cells/field) (A), after labeling with 0.4 mg/ml Sulfo-NHS-SS-Biotin 79% (154±29 intact cells/field) (B). Leptospires treated with methanol were used as a non-intact cell control (100% cell lysis) (C). The green (SYTO9) and red (PI) colors indicated intact and lytic cells, respectively. (three counts of 3 fields per sample)

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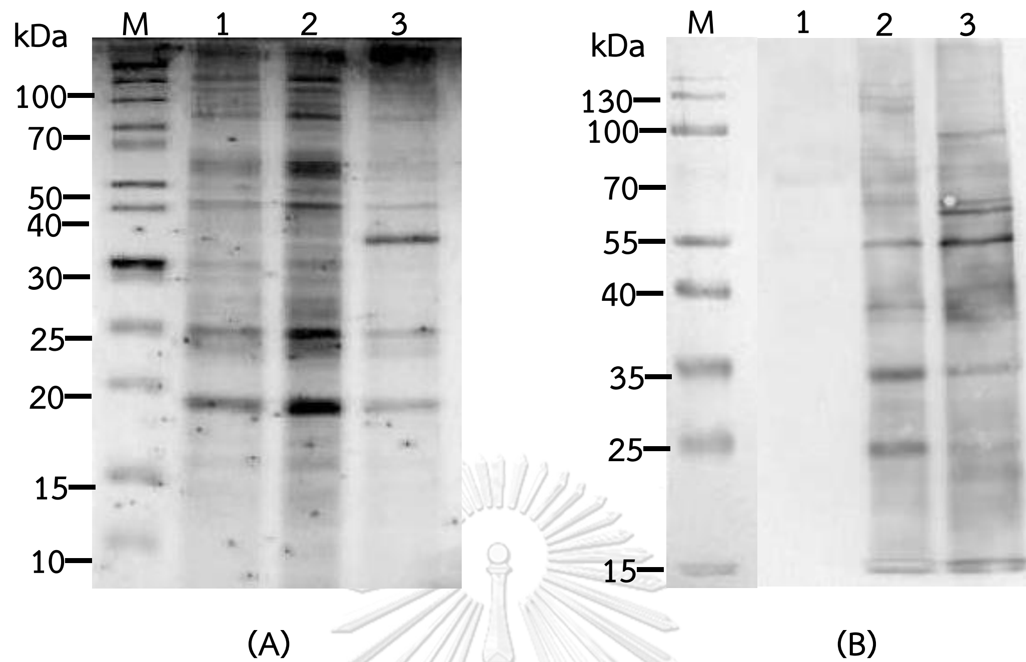

Figure 6. Cell surface biotinylation of leptospires. Leptospiral proteins of unlabeled intact cells (lane 1), labeled intact cell (lane 2), and labeled lysate cells (lane 3) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie brilliant blue staining (A) and transferred to a nitrocellulose membrane, and then were detected with streptavidin-HRP and ECL chemiluminescence system (B).

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Purification of surface proteins after cell surface biotinylation

The biotinylated proteins were purified by avidin agarose columns and eluted by a reducing agent, Dithiothreitol (DTT) (Fig. 7A, 7C). Before purification biotinylated proteins of intact leptospires were observed on the blot. The purified surface proteins in the eluted fraction were hardly detected on the membrane because the biotin had been cleaved previously by DTT (Fig. 7B) and low amount of the eluted proteins were obtained as shown on the corresponding SDS-PAGE gel (Fig. 7C).

To demonstrate the presence of OMPs without periplasmic/cytoplasmic contamination in the eluted fraction, immunoblotting using antisera against OmpL1 (known OMP) and FlaA1 (known periplasmic protein) was performed. OmpL1 was detected whereas FlaA1 was not found in the eluted fraction (Fig. 7D).

Surface biotinylation of intact leptospires was performed in triplicate to obtain 3 samples in the eluted fractions as detected by SDS-PAGE and Coomassie blue staining (Fig. 7E). After in-gel trypsin digestion, the resulting peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify leptospiral proteins in the purified fractions.

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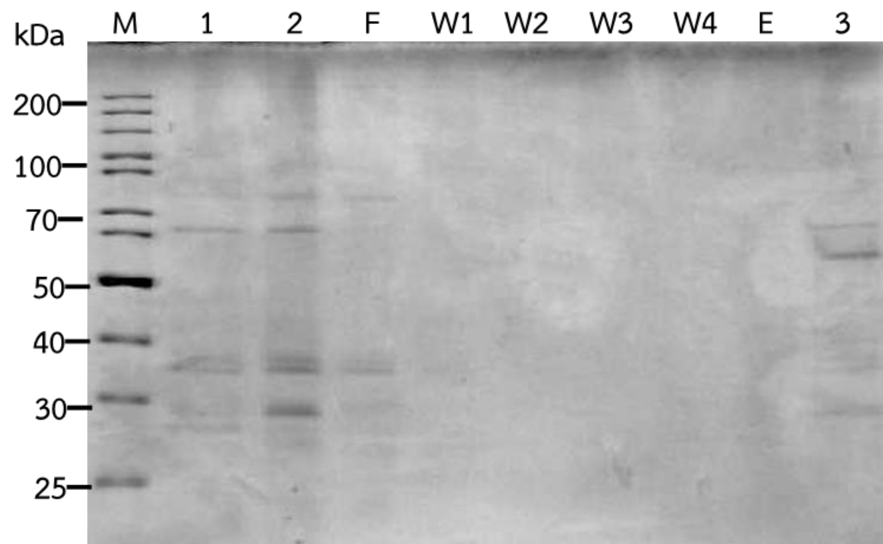
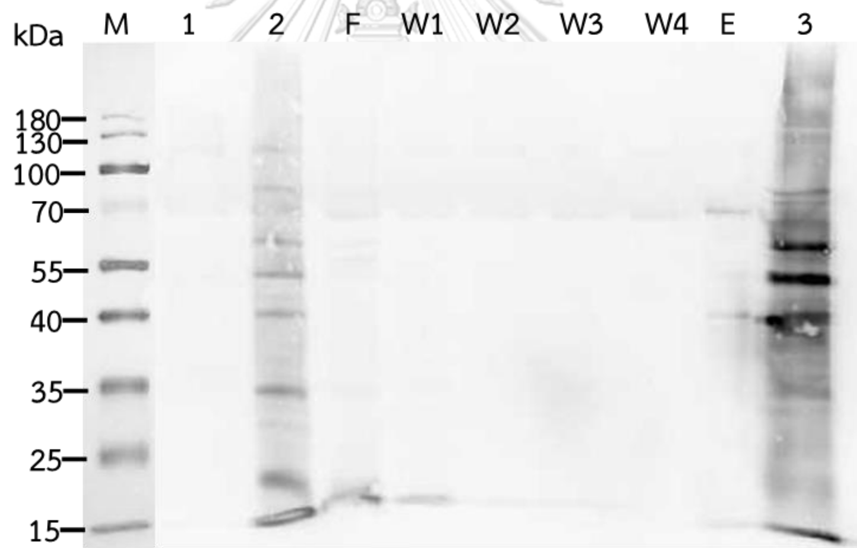


(A)



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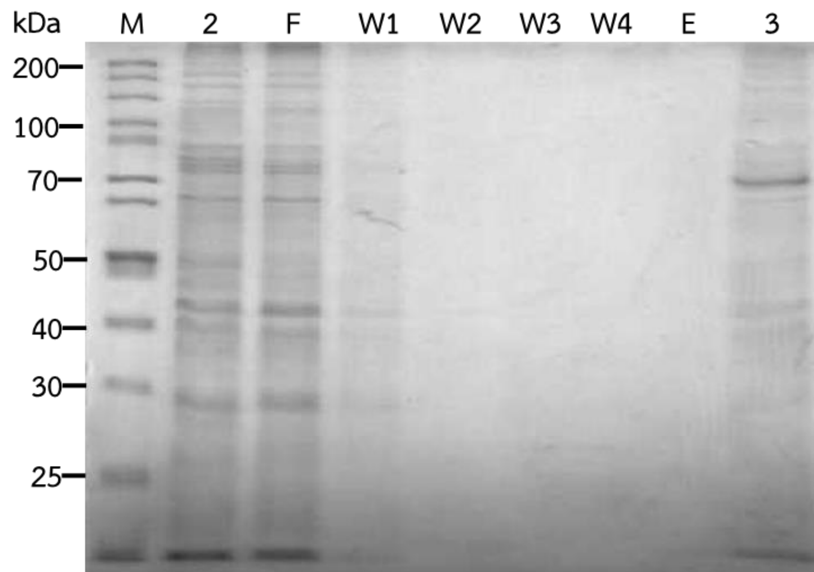
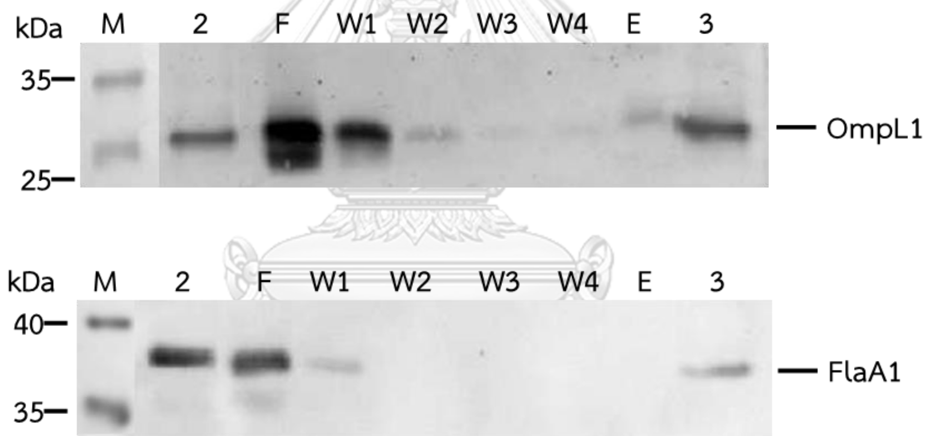


(C)



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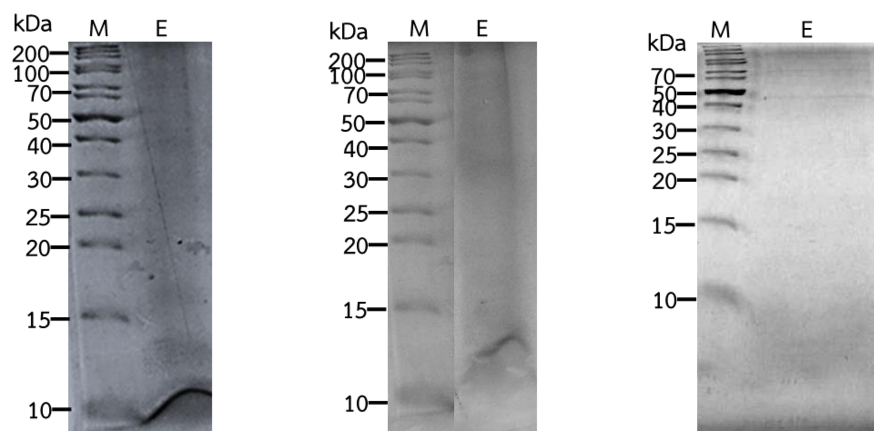
(D)



(E)

Figure 7. Purification of surface proteins after cell surface biotinylation. Biotinylated intact leptospires (lane 2) were added to an avidin column for purification. Non-biotinylated proteins were discarded from the column (flow through, F). The column was washed 4 times (W1-W4) and the purified proteins were eluted (E). Equal volumes of each fraction were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (A and C), transferred to a nitrocellulose membrane, and then detected with streptavidin-HRP and ECL chemiluminescence system (B) or probed with polyclonal rabbit antisera against OmpL1 (known outer membrane protein) or FlaA1 (known periplasmic protein) (D). Non-biotinylated intact (lane 1) was used as a negative control and Biotinylated lysate cells were used as positive control (lane 3). Purified proteins from three independent experiments were concentrated and separated by SDS-PAGE and Coomassie Brilliant Blue staining (E) before in-gel trypsin digestion for mass spectrometry analysis. The molecular mass standards (in kilodalton, kDa) are indicated on the left (lane M).

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Prediction of subcellular localization of leptospiral proteins from cell surface biotinylation approach.

Leptospiral proteins obtained from surface biotinylation and identified by Liquid chromatography tandem-mass spectrometry (LC-MS/MS) were analyzed to predict their subcellular localization by three bioinformatics tools including PSORTb v.3.0.2, CELLO v.2.5, and SOSUI-GramN. Examples of the purified proteins and their predicted localization are shown in Table 6. (shown in the Appendix section)

Total number of putative outer membrane proteins (combination of OM3, OM2, OM1 and OM) from three independent experiments were 463 proteins (Fig 8). These proteins were proteins of interest in this study due to their higher possibility to be surface-exposed proteins than those predicted to be at other localization sites. Proteins obtained from three independent experiments were categorized into various predicted localizations (Table 1), some of which were distinct or overlapped as shown in Fig 8.

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Table 1. Total number of leptospiral proteins with predicted localization from three independent experiments of cell surface biotinylation method.

Predict localizations	Experiment 1	Experiment 2	Experiment 3
Extracellular (EC)	16 (1%)	17 (1%)	0 (0%)
Outer membrane 3 tools (OM3)	7 (0%)	10 (1%)	2 (1%)
Outer membrane 2 tools (OM2)	38 (3%)	36 (2%)	4 (2%)
Outer membrane 1 tools (OM1)	245 (16%)	250 (17%)	24 (12%)
Outer membrane low score (OM)	91 (6%)	99 (7%)	8 (4%)
OM3+OM2+OM1+OM	**381 (25%)**	**395 (27%)**	**38 (19%)**
Periplasm (PER)	20 (1%)	17 (1%)	7 (4%)
Inner membrane (IM)	146 (10%)	154 (10%)	15 (8%)
Cytoplasm (CYT)	860 (57%)	824 (56%)	128 (65%)
Unknown (UNK)	86 (6%)	73 (5%)	8 (4%)
Total	**1509 (100%)**	**1480 (100%)**	**196 (100%)**

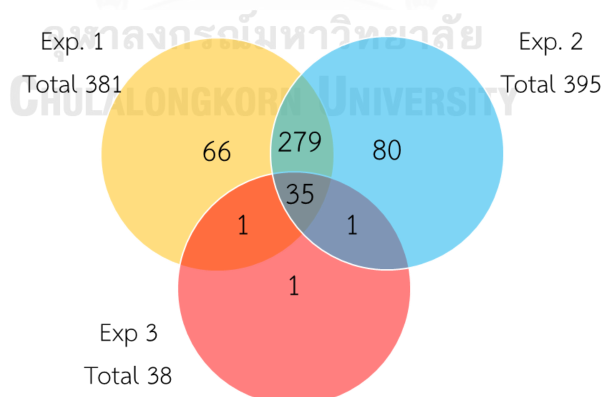

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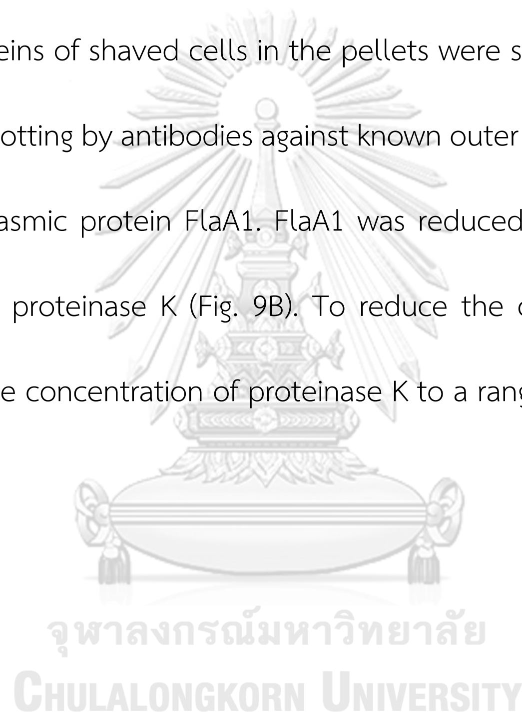
Figure 8. The diagram showing the number of leptospiral proteins predicted as putative OMPs (OM3+OM2+OM1+OM) obtained from three independent experiments of the surface biotinylation method.

Cell surface shaving with proteinase K of *Leptospira interrogans* serovar Pomona

Optimization of proteinase K concentration for cell surface shaving

To determine the optimal proteinase K concentration for surface protein shaving, viable intact leptospires (10^{10} cells) were first treated with proteinase K (ProK) at a final concentration of 0, 12.5, 25, 50, and 100 µg/ml (Fig. 9). After proteolytic treatment, digested surface proteins were separated by centrifugation into the supernatant. Proteins of shaved cells in the pellets were separated by SDS-PAGE (Fig. 9A) and immunoblotting by antibodies against known outer membrane protein OmpL1 and known periplasmic protein FlaA1. FlaA1 was reduced after treatment with 12.5 µg/ml or more of proteinase K (Fig. 9B). To reduce the cell damage, we therefore further reduced the concentration of proteinase K to a range of 0-25 µg/ml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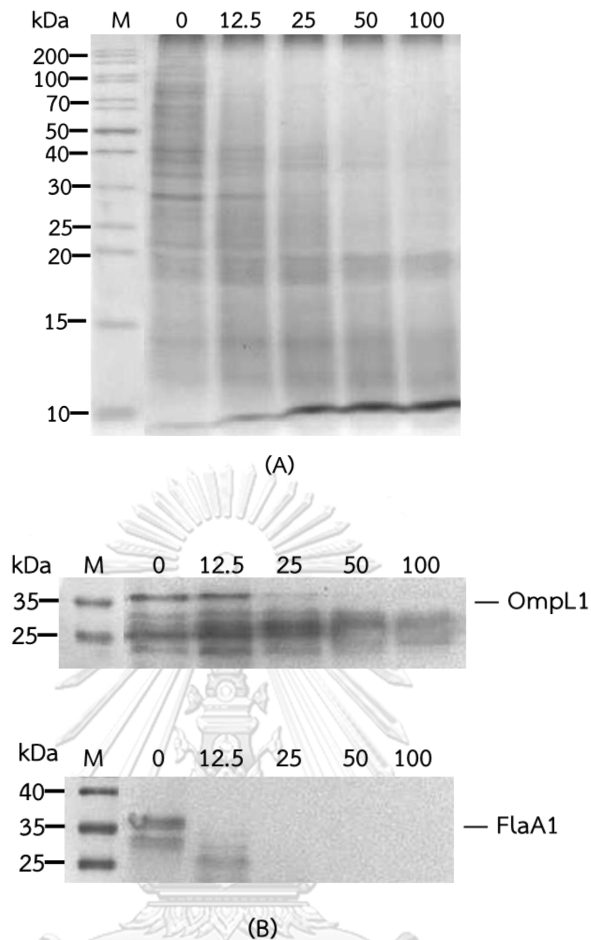

Figure 9. Optimization of proteinase K concentrations at 0-100 μg/ml for cell surface shaving. Intact leptospires were incubated with various concentrations of proteinase K at 0, 12.5, 25, 50 and 100 μg/ml. Equivalents of 10^8 leptospires per lane were separated by sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) (A), transferred to a nitrocellulose membrane, and probed with polyclonal rabbit antisera against OmpL1 used as a known outer membrane protein control, and FlaA1 used as a known periplasmic protein (B). The expected position of proteins are indicated on the right, and the molecular mass standards (in kilodaltons, kDa) are indicated on the left.

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Next, 10^{10} leptospires were treated at 0, 5, 10, 15, 20, 25 µg/ml (Fig. 9). The immunoblotting revealed the OmpL47 (known surface-exposed proteins), and FlaA1 were reduced at 5 µg/ml or more of proteinase K (Fig 10B). The results indicated that proteinase K at a concentration of 5 µg/ml or more caused cell lysis. Therefore, the concentration of proteinase K were reduced to 0-5 µg/ml for the next optimization.

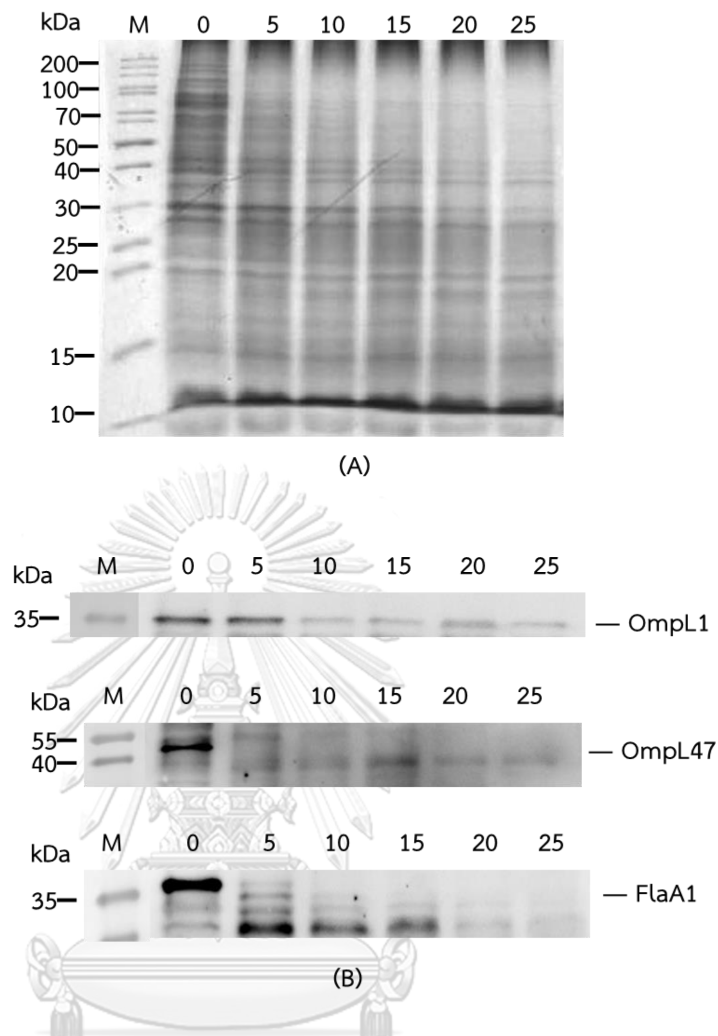

Figure 10. Optimization of proteinase K concentrations at 0-25 μg/ml for cell surface shaving. Intact leptospires were incubated with various concentrations of proteinase K at 0, 5, 10, 15, 20, 25 μg/ml. Equivalents of 10^8 leptospires per lane were separated by sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) (A), transferred to a nitrocellulose membrane, and probed with polyclonal rabbit antisera against OmpL1, OmpL47 (known surface-exposed protein) and FlaA1 (known periplasmic protein) (B). The expected position of proteins are indicated on the right, and the molecular mass standards (in kilodaltons, kDa) are indicated on the left.

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Finally, 10^{10} leptospires were treated with proteinase K at 0, 0.0625, 0.125, 0.25, 0.5, 1, 2, 5 μg/ml (Fig 11). The immunoblotting revealed that OmpL47 started to reduce while FlaA1 was stable at 1 μg/ml proteinase K. This indicated that proteinase K at 1 μg/ml was optimal to digest only surface-exposed protein with no effect on the periplasmic protein. Therefore, the supernatant of the sample treated with 1 μg/ml proteinase K was subjected to identify surface exposed proteins by LC-MS/MS.

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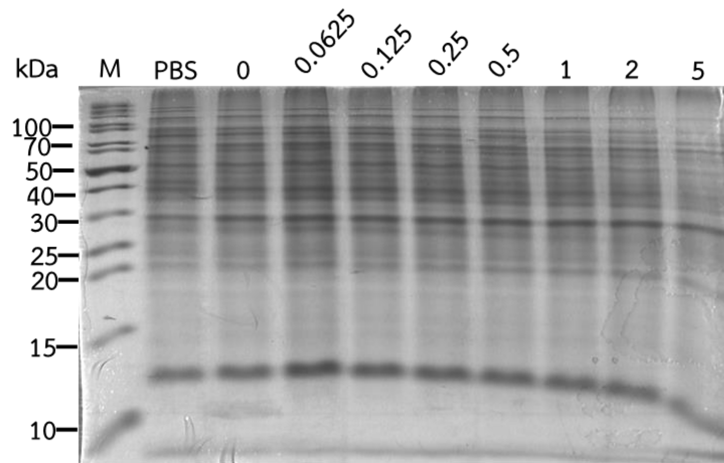
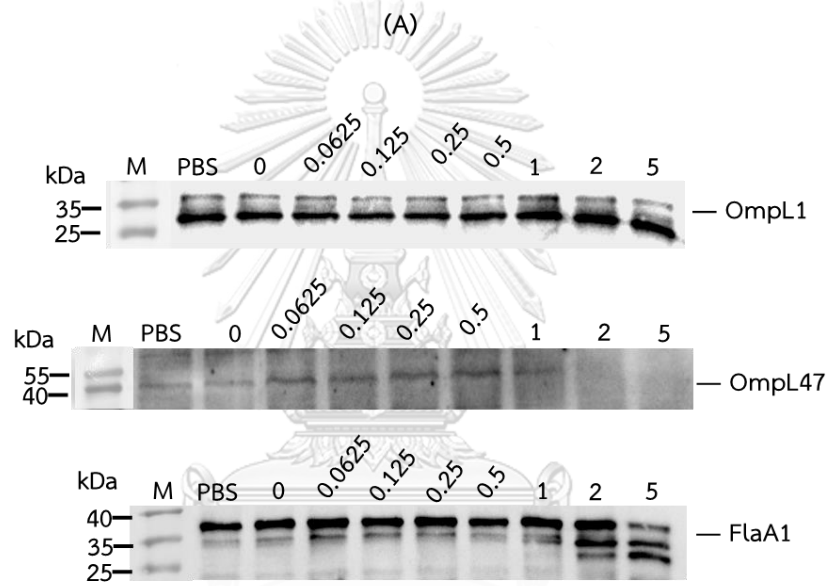


(A)



(B)

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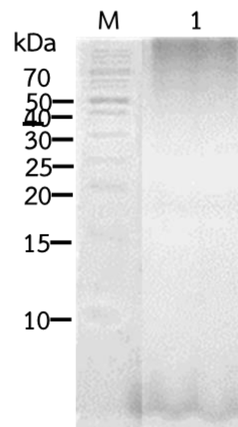

(C)

Figure 11. Optimization of proteinase K concentrations at 0-5 μg/ml for cell surface shaving. Intact leptospires were incubated with various concentrations of proteinase K at 0, 0.0625, 0.125, 0.25, 0.5, 1, 2 and 5 μg/ml. Equivalents of 10^8 leptospires per lane were separated by sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) (A), transferred to a nitrocellulose membrane, and probed with polyclonal rabbit antisera against OmpL1, OmpL47 (known surface-exposed protein) and FlaA1 (known periplasmic protein) (B). The supernatant containing cleaved proteins after cell surface shaving at 1 μg/ml proteinase K proteins. The proteins were concentrated and separated by SDS-PAGE and Coomassie Brilliant Blue staining (C) before in-gel trypsin digestion for mass spectrometry analysis. The expected position of proteins is indicated on the right, and the molecular mass standards (in kilodaltons, kDa) are indicated on the left.

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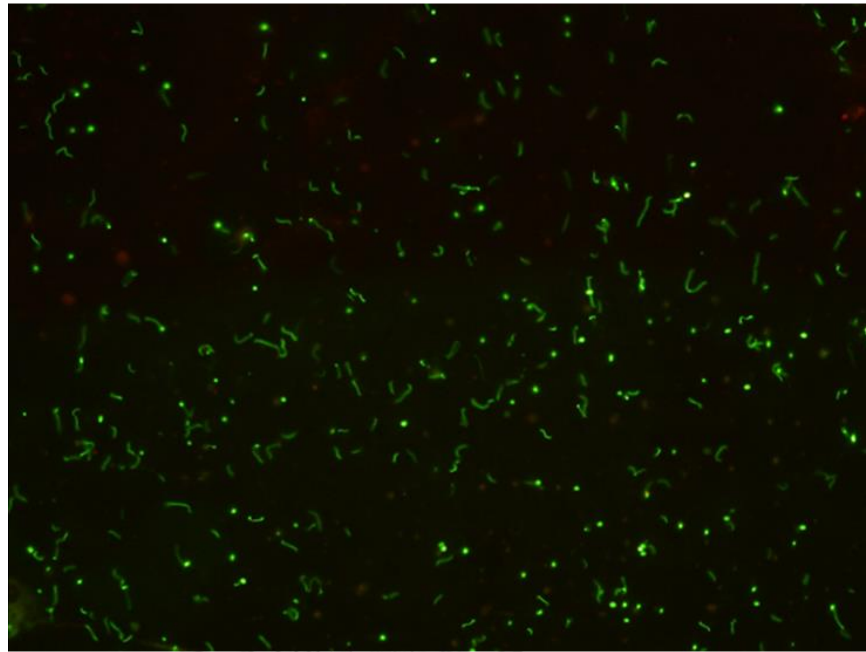
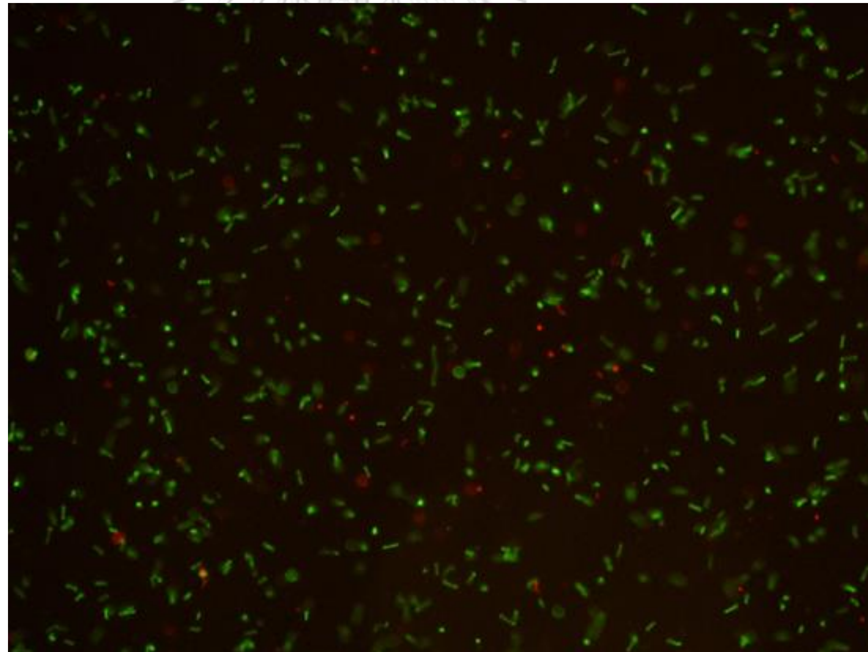
Determination of membrane Integrity after cell surface shaving

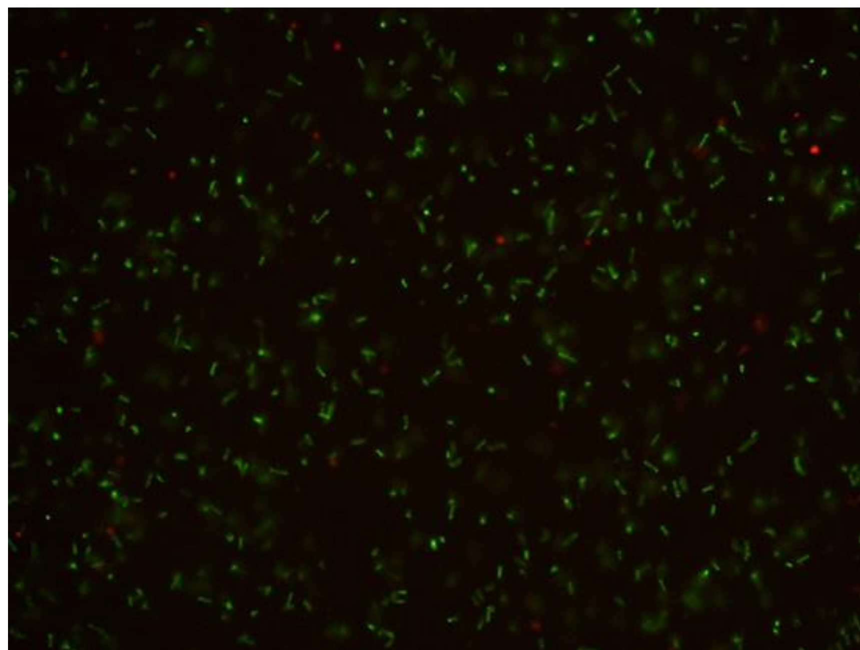
SYTO9/PI fluorescence viability staining showed that lytic cells were increased as a dose dependent manner. Most leptospiral cells (greater than 90% intact cells) remained intact (green) after treatment with proteinase K at a concentration lower than 5 μg/ml including 1 μg/ml proteinase K treated sample used for LC-MS/MS. The fluorescence staining was similar to intact cells before treatment (green) but different from methanol-treated lytic cell control (red).



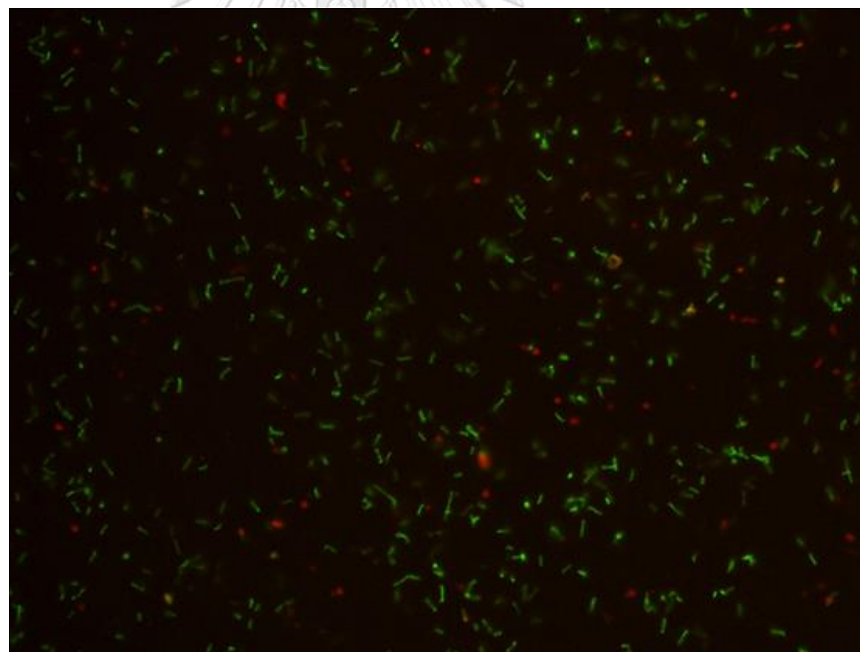
Before proteinase K treatment (96.90%)



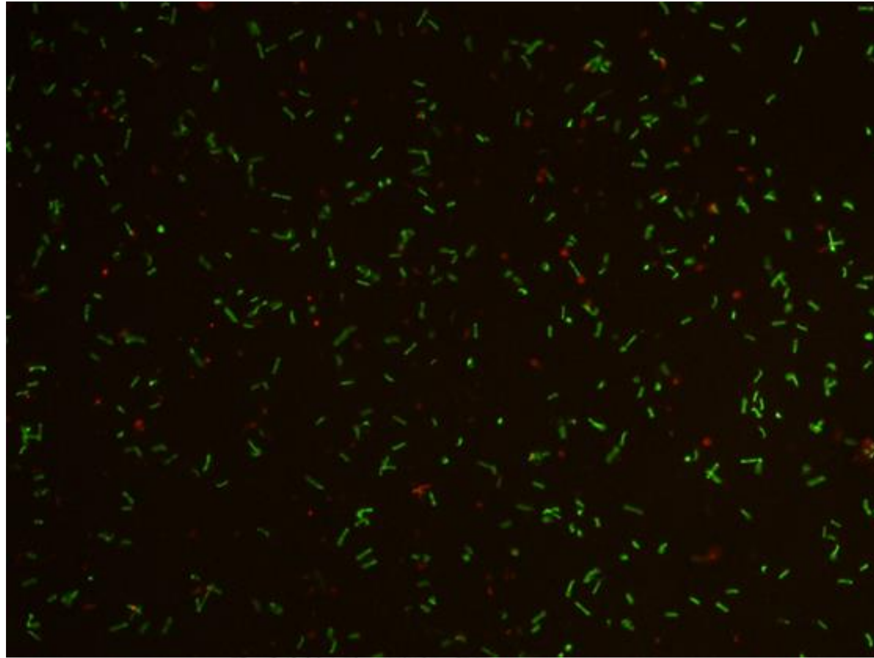
Proteinase K 0 μg/ml (97%)



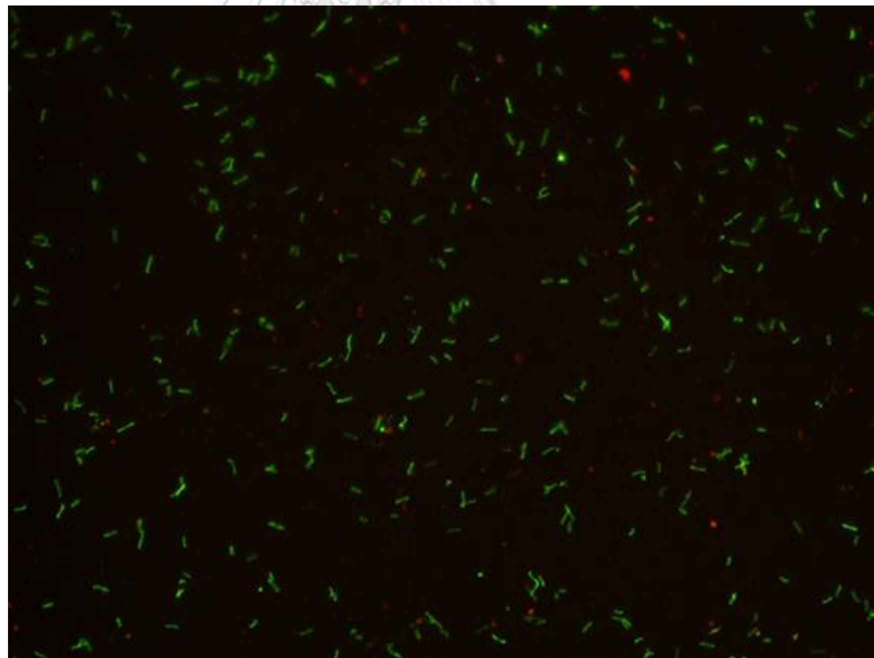
Proteinase K 0.0625 μg/ml (96%)



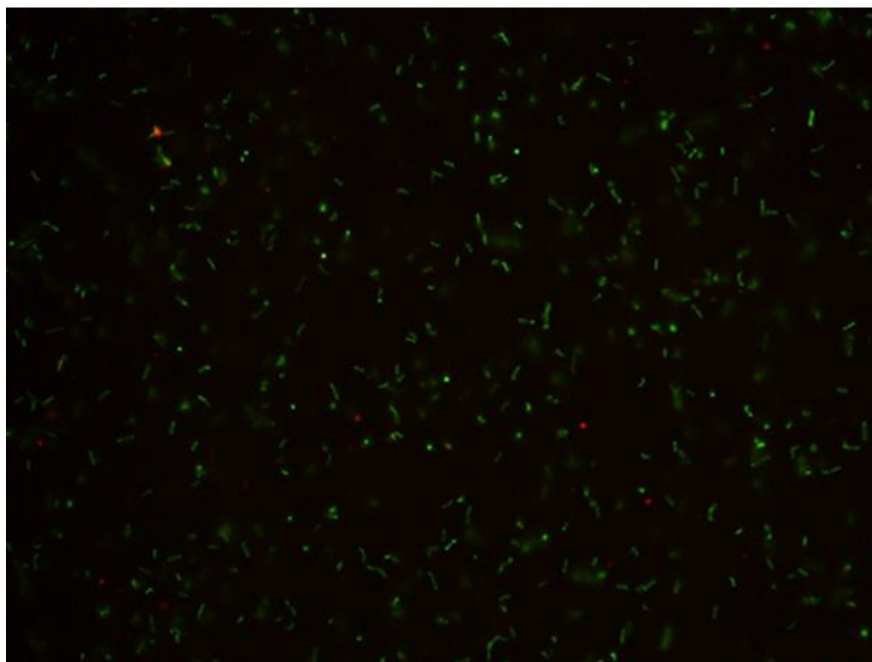
Proteinase K 0.125 μg/ml (96%)



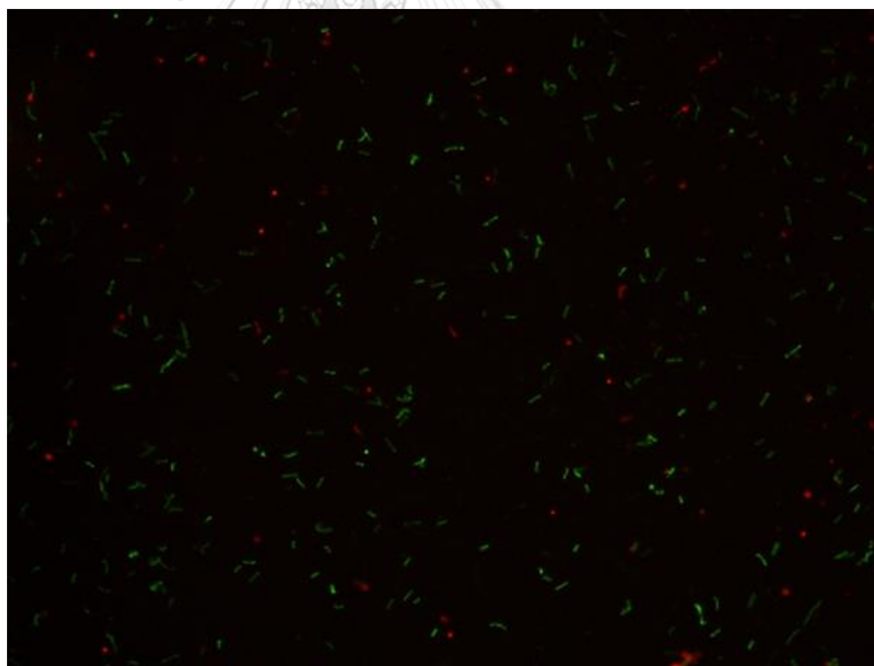
Proteinase K 0.25 μg/ml (95%)



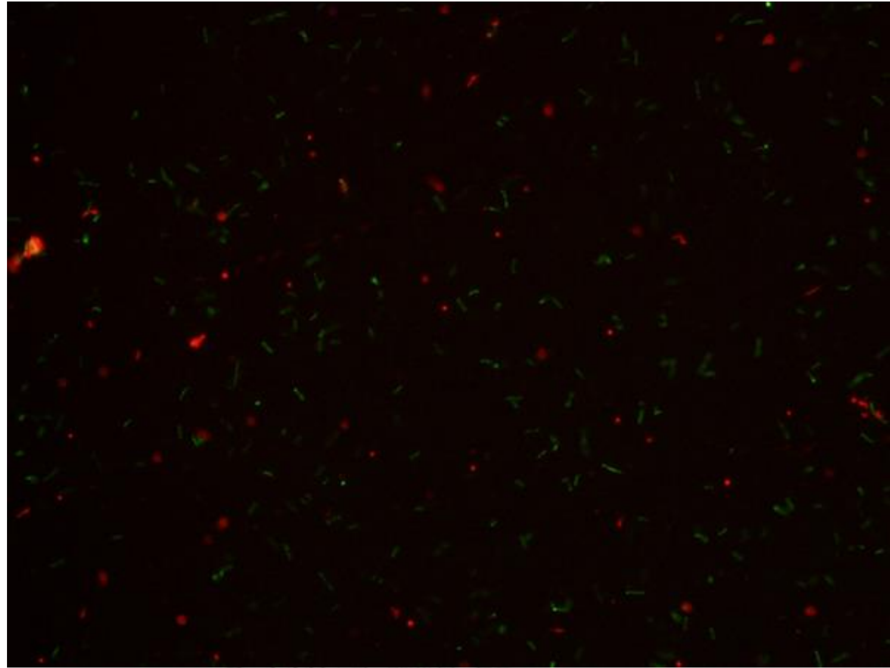
Proteinase K 0.5 μg/ml (95%)



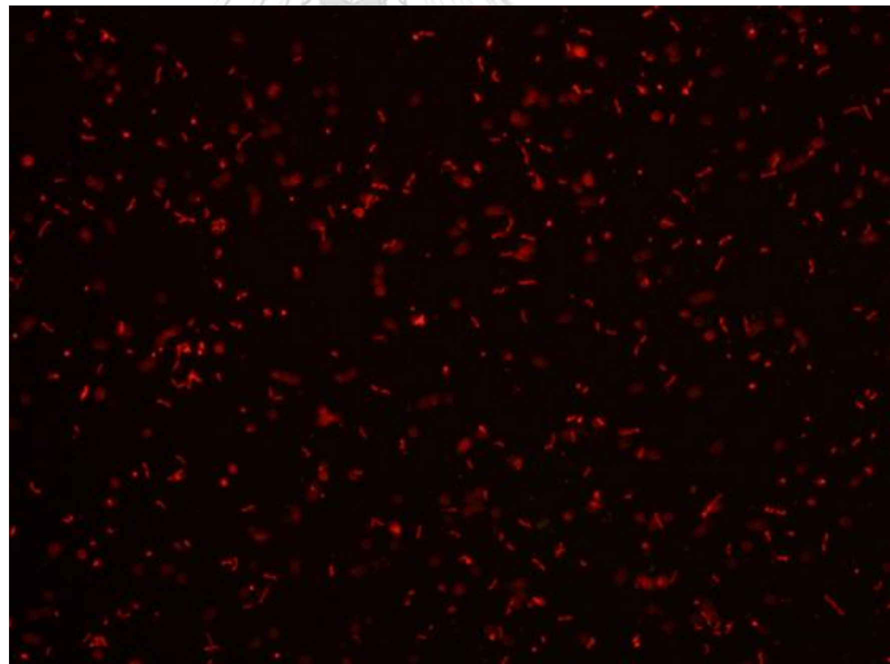
Proteinase K 1 μg/ml (93%)



Proteinase K 2 μg/ml (92%)



Proteinase K 5 μg/ml (91%)



Non-intact cell control (methnol treatment)

Figure 12. Determination of membrane Integrity after cell surface shaving. Leptospires were stained with SYTO9/propodium iodide (PI) fluorescence dyes before and after treatment by Proteinase K at various concentrations including 0, 0.0625, 0.125, 0.25, 0.5, 1, 2 and 5 µg/ml. Leptospires were treated by methanol as lytic cell control. Intact cells were stained by SYTO9 (green) and lytic cells were stained by PI (RED). A number of intact cells at 0, 0.0625, 0.125, 0.25, 0.5, 1, 2 and 5 µg/ml. are 97% (210±52), 96% (189±38.43), 96% (283±21), 95% (229±77), 95% (191±50), 93% (165±49), 92% (192±29), 91% (147±35) respectively. (three counts of 3 fields per sample)

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Prediction of subcellular localization of leptospiral proteins from cell surface shaving approach

All protein data obtained from LC-MS/MS were analyzed for their predicted localization using 3 bioinformatics tools, PSORTb, CELLO, and SOSUI-GramN. Total number of leptospiral proteins shaved into the supernatant after proteolytic treatment was 1,176 proteins from two idenpendent experiments (Table 2). The number of putative outer membrane proteins (OM3, OM2, OM1 and OM) obtained from two independent experiments was 356 proteins (Fig. 13).

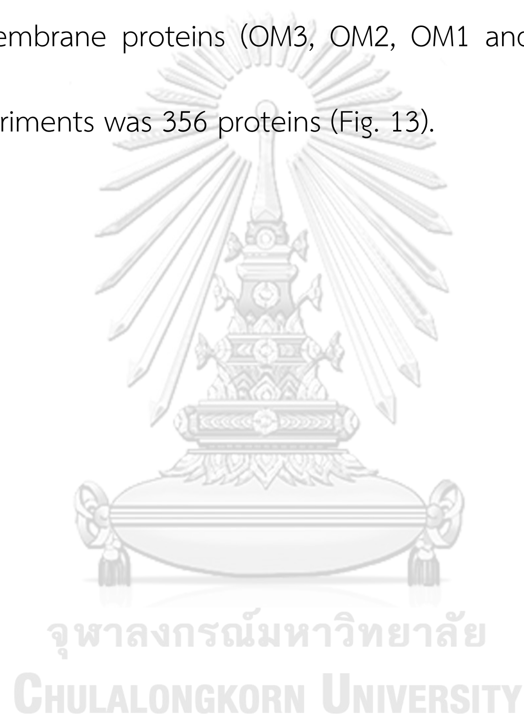
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Table 2. Total number of leptospiral proteins with predicted localization from two independent experiments of cell surface shaving method.

Predicted localizations	Experiment 1	Experiment 2
Extracellular (EC)	22 (2%)	9 (5%)
Outer membrane 3 tools (OM3)	10 (1%)	4 (2%)
Outer membrane 2 tools (OM2)	32 (3%)	7 (4%)
Outer membrane 1 tool (OM1)	177 (18%)	41 (22%)
Outer membrane low score (OM)	70 (7%)	15 (8%)
OM3+OM2+OM1+OM	**289 (29%)**	**67 (36%)**
Periplasm (PER)	18 (2%)	12 (6%)
Inner membrane (IM)	28 (3%)	3 (1%)
Cytoplasm (CYT)	579 (59%)	87 (46%)
Unknown (UNK)	51 (5%)	11 (6%)
Total	987 (100%)	189 (100%)

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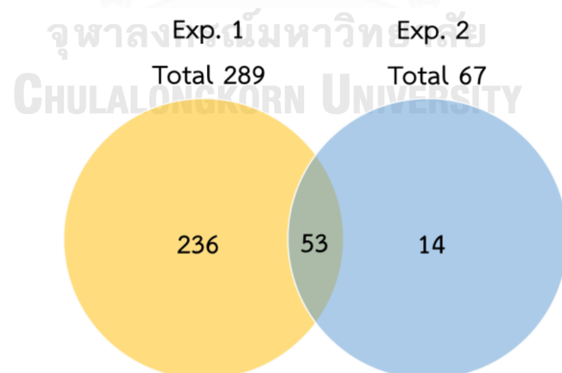

Figure 13. The diagram showing the number of leptospiral proteins predicted as putative OMPs (OM3+OM2+OM1+OM) obtained from two independent experiments of cell surface shaving method.

In this study, putative surface-exposed OMPs were further analyzed by combination of data from two different methods. The total number of putative OMPs was 514 proteins: 223 proteins from cell surface biotinylation only, 77 proteins from cell surface shaving only, and 214 proteins found in both methods (Fig. 14). All proteins and putative OMPs obtained from each approach were shown in the Appendix section.

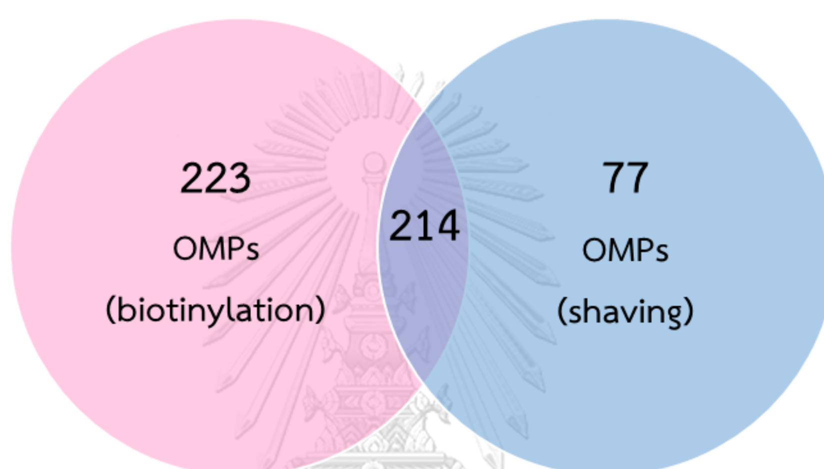

Figure 14. Diagram showing the number of putative OMPs (combination of OM3, OM2, OM1, and OM) obtained from cell surface biotinylation and cell surface shaving methods.

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Of 214 proteins found in both surface biotinylation and protease shaving methods, 8 proteins were predicted to be OMPs by all three bioinformatics tools (PSORTb, CELLO, and SOSUI-GramN) (Table 3). LipL71 (LIC11003), Cysteine protease (LIC20197), TonB-dependent receptor (LIC10714), ImpL63 (LIC11815), and OmpA-family protein (LIC10050) are known OMPs. Three proteins; LIC12048, LIC10439, and LIC10711; are predicted as hypothetical proteins.

Table 3. Eight proteins obtained from both cell surface biotinylation and cell surface shaving methods are predicted as putative OMPs by all three bioinformatics tools, PSORTb, CELLO, and SOSUI-GramN

LIC number	Description	PSORTb	CELLO	SOSUI GramN	Final Prediction
LIC11003	LipL71 lipoprotein	OM	OM	OM	OM3
LIC20197	Cysteine protease	OM	OM>PER>EC	OM	OM3
LIC10714	Outer membrane receptor for Fe3+-dicitrate/TonB-dependent receptor	OM	OM	OM	OM3
LIC11851	ImpL63	OM	OM	OM	OM3
LIC10439	Conserved hypothetical protein	OM	OM	OM	OM3
LIC10050	OmpA-family protein	OM	OM	OM	OM3
LIC10711	Conserved hypothetical protein	OM	OM	OM	OM3
LIC12048	Hypothetical lipoprotein	OM	OM	OM	OM3

outer membrane was predicted by all 3 tools (OM3), outer membrane (OM), periplasm (PER), extracellular (EC)

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Moreover, 27 putative OMPs were found in all three independent experiments of cell surface biotinylation method only, and 44 putative OMPs were found in two independent experiments of cell surface shaving method only. Eight OMPs were in all replicate experiments of both methods: LipL71 (LIC11003), Phage-related protein (LIC12615), Lipoprotein LipL45 (LIC11643), outer membrane lipoprotein carrier protein

LolA (LIC12545), LipL41 (LIC12966), chemotaxis protein histidine kinase-like kinase cheA1 (LIC13522), and flagellar filament sheath protein FlaA-1 (LIC10788) (Table 4.)

Table 4. Eight OMPs were in all replicate experiments of both methods.

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B&S	LIC11003	LipL71	OM	OM	OM	OM3
B&S	LIC12615	Phage-related protein	UNK	OM	EC	OM1
B&S	LIC11643	LipL45	CYT	PER>CYT	OM	OM1
B&S	LIC12545	LolA: outer membrane lipoprotein carrier protein	UNK	OM>PER	IM	OM1
B&S	LIC12966	LipL41	UNK	CYT	OM	OM1
B&S	LIC13522	CheA1: chemotaxis protein histidine kinase-like kinase	CYT	CYT>OM	UNK	OM
B&S	LIC10175	Conserved hypothetical protein	CYT	CYT>OM	EC	OM
B&S	LIC10788	FlaA-1, flagellar filament sheath protein	CYT	PER>OM>EC	CYT	OM

outer membrane was predicted by all 3 tools (OM3), outer membrane 1 (OM1), outer membrane (OM), periplasm (PER), extracellular (EC), unknown (UNK), cytoplasm (CYT), cell surface biotinylation method (B), cell surface shaving (S)

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CHAPTER VI

DISCUSSION

Bacterial outer membrane proteins (OMPs) are of great interest because of their location on the cell surface where pathogens interact with the host. OMPs often play key roles in pathogenesis such as adhesins, porins, targets for antibodies, and receptors for various host molecules. OMPs are likely to be crucial for adaptation to host tissues and their response to changes in environmental conditions during infection. Surface-exposed proteins of pathogenic leptospires are essential for recognizing host molecules, counteracting host defense mechanisms, and promoting invasion and colonization of various target tissues (94). Identification of their surface OMPs as potential virulence factors is useful for understanding pathogenic mechanisms and development of diagnostic tests and vaccines.

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Several methods have been used to identify OMPs, such as in silico analysis, cell surface biotinylation (67), cell fractionation (14), sucrose density gradient membrane fractionation (95), sucrose gradient centrifugation of outer membrane vesicles (OMVs) (71). Additional techniques are required to determine whether OMPs are surface-exposed protein (PSEs), such as cell surface proteolysis, immunofluorescence, and immunoelectron microscopy using antibodies against proteins of interest.

Previous studies in pathogenic *Leptospira* focused on OMPs that are highly abundant proteins (14, 15, 67, 70), host ligand-binding proteins (94), and highly antigenic and conserved proteins (96, 97). Reverse vaccinology has been used for high-throughput screening of leptospiral OMPs/PSEs as new vaccine candidates. For example, novel pan-genomics analysis with negative-selection strategy by removing predicted cytoplasmic proteins and inner membrane proteins was used to identify PSEs of 17 *L. interrogans* strains (98). Reverse and three-dimensional structural vaccinology predicting conserved β-barrel transmembrane proteins and outer membrane lipoproteins was employed to screen for surface-related vaccine candidates (99). However, currently available bioinformatics tools cannot accurately predict surface-exposed OMPs. After bioinformatics prediction, only a selected number of leptospiral OMPs have been further confirmed to be surface-exposed using experimental methods, such as surface immunofluorescence, surface biotinylation, and surface proteolysis (14).

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The present study used two different techniques, surface proteolytic shaving and surface biotinylation, for high-throughput isolation of leptospiral surface-exposed OMPs followed by LC-MS/MS as a highly sensitive identification of derived proteins. These techniques have been widely used to identify surface-exposed proteins of other bacteria (8, 18, 67, 70, 85, 89, 100) because they are easy and rapid for direct isolation of surface proteins. To identify global surface proteins (surfaceome) of pathogenic

Leptospira, the combination of two different techniques were employed in this study to enhance the efficiency of total protein coverage and to increase reliability of true surface OMPs. LC-MS/MS is a high-throughput and high-resolution method to identify proteins in our samples. This technique is better than the classical proteomic approach, such as Two-dimensional gel electrophoresis (2DE) and matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) commonly used in previous studies (67), which mainly identified more abundant proteins and is less efficient to identify highly hydrophobic or membrane proteins.

The main drawback of these techniques are possibility of cell lysis resulting in contamination of cytoplasmic proteins. In this study, cell integrity at each experimental step of protein preparation were determined by viability fluorescence staining using SYTO9 and propidium iodide (PI) and counting under fluorescent microscopy to ensure that leptospiral cells remained intact after protease shaving and surface biotinylation. Despite gentle manipulation, cell lysis is unavoidable. Therefore, each technique was performed in duplicate or triplicate to improve the reliability of our results.

In this study, surface proteomics of low-passage *Leptospira interrogans* serovar Pomona was performed using surface biotinylation with Sulfo-NHS-SS-Biotin and proteinase K shaving followed by LC-MS/MS. This leptospiral strain is a clinical isolate and highly virulent (LD_{50} = 10). Low-passage virulent leptospires were used in this study because after high-passage in vitro culture, pathogenic leptospires usually lose their virulence and change their protein expression, such as leptospiral bacterial-

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immunoglobulin-like outer membrane proteins LigA and LigB (101, 102). However, protein expression of in vitro grown leptospires is different from that of in vivo leptospires during infection. It is possible that some in vivo expressed OMPs were not detected in the culture condition used in this study.

Cell surface biotinylation is a method to label proteins exposed on the surface of intact cells using membrane impermeable biotinylation reagents, such as Sulfo-NHS-SS-Biotin used in this study. This biotin is a hydrophilic reagent containing sulfonate group on the N-hydroxysuccinimide ring that reacts with primary amines (-NH2), such as lysine side-chains, or the amino-termini of polypeptides on the surface proteins. The biotinylated proteins were purified by specific binding with avidin and then the biotin was cleaved from proteins at the disulfide bond by a reducing agent. Cell viability fluorescence staining showed that at least 99% of leptospires were intact before labelling. Although leptospires were handled as gently as possible, intact cells were reduced to approximately 77% after surface labelling (Fig. 5). The protein profiles of biotinylated proteins of labeled intact cells separated by SDS-PAGE and detected by streptavidin staining showed lower amount than those of lysate cell controls (Fig. 6) indicating that proteins of intact cells were partially biotinylated compared to total biotinylated proteins of cell lysate. After purifying by avidin agarose columns and eluting by a reducing agent (dithiothreitol), the purified proteins were not detected by streptavidin because biotin had been cleaved. Western blot revealed that the purified fraction contained known outer membrane protein OmpL1 but did not detect

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periplasmic protein FlaA1 (Fig. 7D) suggesting that surface proteins were obtained in the eluted samples, which were further used for protein identification by LC-MS/MS. However, FlaA1 was subsequently identified in the purified fraction possibly due to high sensitivity of LC-MS/MS to detect even trace amount of proteins.

Cell surface proteolytic shaving is a method to digest surface-exposed portion of OMPs by protease enzymes at an optimal concentration to prevent cell lysis. This study used proteinase K (ProK) because this enzyme is potent, active at wide pH range, and low peptide bond specificity adjacent to the carboxyl group of aliphatic and aromatic amino acids resulting in a broad range of surface proteins in the shaving fraction. However, the results may be variable so the experiments were performed in duplicate. The concentration of proteinase K was optimized from broad to narrow scales to refine the concentration at which cells were least disintegrated. Membrane integrity of leptospires were determined by SYTO9/PI florescence staining showed 97% of leptospires were intact before proteinase K treatment and 93% of leptospires remained intact after treating with 1 μg/ml proteinase K (Fig. 12). The immunoblotting of shaved cells in the cell pellet revealed that known surface-exposed protein OmpL47 was reduced while periplasmic protein FlaA1 and OmpL1 was stable compared to untreated cells (Fig. 11B). Therefore, surface proteins released into the supernatant at this proteinase K concentration were subjected to LC-MS/MS. The result of OmpL1 is consistent with previous study using surface immunofluorescence assay showing that major portion of OmpL1 may be integrated into the outer membrane

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(14). However, OmpL1 was subsequently identified in the shaving fraction by mass spectrometry.

Proteins obtained from surface biotinylation and proteinase K shaving were identified by LC-MS/MS using available databases of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 and *L. interrogans* serovar Lai. To determine the accuracy of our results, three bioinformatics tools including PSORTb v.3.0 (103), CELLO (104), and SOSUI-GramN (105), which have been widely used and well accepted to predict subcellular localization of Gram-negative bacteria, were used to analyze proteins obtained from both methods to determine their localization. Since these tools use different algorithms in their prediction system, the same proteins may be categorized into different locations (Table 5). For example, OmpL1 (LIC10973) and LigA (LIC10465) are predicted as proteins of unknown location by PSORTb, extracellular proteins by CELLO, and outer membrane proteins by SOSUI-GramN. LipL41 is predicted as a unknown, cytoplasmic, and outer membrane protein by PSORTb, CELLO and SOSUI-GramN, respectively. In the case of prediction discrepancy, the criterion based on at least 2 out 3 agreements was used to designate probable localization of the proteins except for OMPs. To retain OMPs as many as possible, proteins are considered as probable OMPs if they are predicted as OMP by at least 1 out 3 tools.

Based on our criteria, surface biotinylation and LC-MS/MS identified proteins whose localization are predicted to be 23.7% outer membrane (OM), 0.7% extracellular (EC), 2% periplasm (PER), 9.3% inner membrane (IM), 59.3% cytoplasmic (CYT), and 5%

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unknown (UNK). Total of 463 OMPs were obtained from three independent experiments. Proteinase K shaving and LC-MS/MS identified proteins whose localization are predicted to be 32.5% OM, 3.5% EC, 4% PER, 2% IM, 52.5% CYT, and 5.5% UNK. Total of 303 OMPs were identified from two independent experiments. Both approaches were able to identify the same 214 OMPs, 66 of which are hypothetical proteins. 222 proteins were obtained exclusively by surface biotinylation and 74 proteins were derived solely from proteinase K shaving (Table 7). Eight proteins were identified by both approaches and were predicted to be OMPs by 3 tools, including LipL71 (LIC11003), cysteine protease (LIC20197), TonB-dependent receptor (LIC10714), ImpL63 (LIC11851), OmpA-family protein (LIC10050), conserved hypothetical proteins such as LIC10439 and LIC10711 (Table 3). LipL71 conserved in pathogenic serovar (106) and strong antibody responses in patient (107). TonB-dependent receptor (LIC10714) have function as bind and transport ferric chelates called siderophores, as well as vitamin B12, nickel complexes, and carbohydrates (108). OmpA-family protein (LIC10050), the OmpA family of outer membrane proteins is related, heat-modifiable, surface-exposed, porin proteins that have high-copy number in the outer membrane of mainly Gram-negative bacteria. The important roles including bacterial adhesion, invasion, or intracellular and evasion of host defenses or stimulators of pro-inflammatory cytokine production that these pathogenic roles are most commonly associated with central nervous system, respiratory and urogenital diseases. In addition, OmpA family proteins can serve as targets of the immune system and are under

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evaluation as potential vaccine candidates (109). Both approaches used in this study also identified 66 hypothetical proteins or proteins of unknown function that have never been confirmed as PSEs (Table 8, appendix). These proteins are interesting targets to study their roles in pathogenic leptospires. However, these bioinformatics tools have their own flaws. Therefore, we cannot exclude that our proteins are not PSEs based only on these predictions. Further experimental approaches need to be performed to confirm the results.

All replicate experiments of both methods identified 8 proteins in common including LipL71 (LIC11003), Phage-related protein (LIC12615), LipL45 (LIC11643), LolA outer membrane lipoprotein carrier protein (LIC12545), LipL41 (LIC12966), CheA1 chemotaxis protein histidine kinase-like kinase (LIC13522), FlaA-1 flagellar filament sheath protein (LIC10788), and conserved hypothetical protein (LIC10175). Of these 8 proteins, LipL71, LipL45, and LipL41 are known PSEs. LipL45 expressed in many low-passage strains was not expressed in high-passage, culture-attenuated strains, suggesting that LipL45 is a virulence-associated protein (110). LolA outer membrane lipoprotein carrier protein, a protein of Lol system is responsible for the transport of lipoproteins to the outer membrane (111). LipL41 is a hemin-binding protein and a high potential for being an iron-storing protein (112). FlaA 1 involved in motility and CheA1 involved in chemotaxis (113). FlaA1 is a known periplasmic protein but is predicted to be OMP at a low score by CELLO (Table 4).

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Some surface proteins identified by our study have been previously confirmed and reported as surface-exposed protein by various methods, such as immunoelectron microscopy, Triton X-114 fractionation, surface proteolysis assay, surface immunofluorescence assay, surface biotinylation, membrane affinity analysis, and surface immunoprecipitation as shown in Table 5. Only LipL71 is predicted to be an OMP by all three tools.

In addition, 6 OMPs (from total 510 OMPs) from surface biotinylation and proteinase K shaving identified a homolog of LIC11436 (LipL45-related protein), LIC11051 (Leucine-rich repeat containing protein), LIC13066 (Conserved hypothetical protein), LIC12631 (Sphingomyelinase C precursor), LIC10714 (TonB-dependent receptor) and LIC10713 (Iron-regulated lipoprotein) of *L. interrogans* serovar Copenhageni strain Fiocruz L1–130, which was previously shown as a surface-exposed fibronectin-binding protein (94) (Table 9, appendix).

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A previous study using negative-screening strategy combined with pan-genome analysis to screen for PSEs from 17 *L. interrogans* strains showed that 121 of total 190 core (conserved in all 17 strains) PSEs were newly identified PSEs (98). Among these 121 PSEs, 49 proteins were found in this study that 37 proteins have been confirmed to be PSEs and virulent factor of pathogenic *Leptospira.* (Table 10, appendix)

Table 5. Known surface-exposed proteins found in this study. Their surface localization has been confirmed previously by different techniques. Predicted results of their subcellular localization by three bioinformatics tools are shown.

Protein	**Predicted subcellular localization**				**Previous studies**	
	PSORTb	CELLO	SOSUI-GramN	Final prediction	Surface localization method used	References
OmpL1 (B&S)	UNK	EC	OM	OM1	EM, T-X-114, SB, SIFA	(14, 114-116)
OmpL37 (B&S)	CYT	CYT>IM>OM	OM	OM1	SIFA, SB, MAA	(14) (4)
OmpL47 (B&S)	UNK	EC	OM	OM1	SPA, SIFA, SB, MAA	(4, 14)
LipL41 (B&S)	UNK	CYT	OM	OM1	SIP	(117, 118)
LigA (B&S)	UNK (EC>OM)	EC>OM	OM	OM1	IEM, SIFA	(119) (49) (120)
TonB (B&S)	OM	OM	OM	OM3	In silico	(98)
LipL71 (B&S)	OM	OM	OM	OM3	In silico	(98)
LenA (B&S)	UNK	OM>CYT>EC	IM	OM1	T-X-114	(121)

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immunoelectron microscopy (IEM), Triton X-114 fractionation (T-X-114), surface proteolysis assay (SPA), surface immunofluorescence assay (SIFA), surface biotinylation (SB), membrane affinity analysis (MAA), surface immunoprecipitation (SIP), unknown (UNK), extracellular (EC), outer membrane (OM), inner membrane (IM), cytoplasm (CYT), B (Biotin method), S (shaving method)

The present study has some limitations. This study may not detect OMPs that were not expressed or expressed at a low abundance in our in vitro culture condition as a result of their stage-specific differential expression or in vivo regulation by conditions found only in the host environment. Outer membrane of leptospires is fragile and easily disrupted so it was difficult to control cell membrane damage during experimental process resulting in cross contamination of proteins from other compartments (122). Certain degree of cell lysis was observed by fluorescence viability staining of leptospires after surface biotinylation and proteinase K shaving. Cell disintegration allowed the biotin to label non-surface proteins and caused leakage of cytoplasmic proteins into the supernatant of proteinase K shaving fraction. In addition, Sulfo-NHS-SS-Biotin used in this study can slightly pass through membrane channels to label cytoplasmic proteins (6, 17). The sample contamination may not only cause false positive findings of non-PSEs but also lead to false negative results to identify true PSEs that are expressed at very low amount or have small surface-exposed portion. In addition, some proteins may have multiple subcellular localization or moonlight between cytoplasmic and outer membrane location sites. Some proteins previously reported as cytoplasmic proteins are subsequently shown to also locate at the outer membrane, such as GroEL, DnaK, and enolase (123), which was also identified in this study. Therefore, additional methods are required to confirm the protein localization, such as immunofluorescence, confocal microscopy with specific antibodies.

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In conclusion, the present study used surface biotinylation and proteinase K shaving followed by LC-MS/MS to study surfaceome of virulent *L. interrogans* serovar Pomona. These putative PSEs should be further confirmed their localization. The knowledge will be useful to study novel virulence factors for better understanding of pathogenesis and new candidates for diagnostic tests and vaccine development of leptospirosis in the future.

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APPENDICES

APPENDIX A

Reagents for EMJH media

1. **Albumin fatty acid supplement stock solution**

Reagent	Amount	Unit
$CaCl_2 \cdot 2H_2O + MgCl_2 \cdot 6H_2O$	0.076	g
$ZnSO_4 \cdot 7H_2O$	0.04	g
$CuSO_4 \cdot H_2O$	0.03	g
Vitamin B12	0.002	g
Tween 80	1	g
Glycerol	1	g

All reagents are stored at -20°C until use. Dissolve each reagent separately in 10 ml of distilled water.

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2. **Albumin fatty acid supplement solution, ready to use (50 ml)**

Reagent	Amount	Unit
BSA	5	g
$CaCl_2 + MgCl_2 \cdot 6H_2O$	750	µl
$ZnSO_4 \cdot 7H_2O$	500	µl
$CuSO_4 \cdot 5H_2O$	50	µl
$FeSO_4$	0.025	g
Sodium pyruvate	0.02	g

Vitamin B12	500	μl
Tween 80	6.25	ml
Glycerol stock	500	μl

Dissolve in distilled water and adjust pH 7.4-7.6 with concentrated HCl. Adjust volume with distilled water to make 50 ml. Sterilize the solution by filtration 0.2 μm. Store at -20°C

3. **Basal Media (90 ml)**

Bacto *Leptospira* Media Base EMJH dehydrated	1.5	g

Dissolve in distilled water and adjust volume to 450 ml. Sterilize the solution by autoclaving at 121°C for 15 minutes.

4. **EMJH media**

Base Media	90	ml
Albumin fatty acid supplement solution	10	ml

Mix the solution and store at 4°C

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Reagents for SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. **1 M Tris-HCl pH 8.8**

The base	12.11	g

Dissolve in distilled water and adjust pH to 8.8 with concentrated HCl. Adjust volume with distilled water to make 100 ml. Sterilize the solution by autoclaving at 121°C for 15 minutes.

2. 0.5 M Tris-HCl pH 6.8

Tris base	6.055	g

Dissolve in distilled water and adjust pH to 6.8 with concentrated HCl. Adjust volume with distilled water to make 100 ml. Sterilize the solution by autoclaving at 121°C for 15 minutes.

3. 4X Tris HCl/SDS pH 8.8 (100 ml)

Tris base	18.21	g
SDS	0.4	g

Dissolve in distilled water and adjust pH to 8.8 with concentrated HCl. Store at 4°C

4. Running Buffer (1 liter)

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Tris base	15.1	g
Glycine	72	g
SDS	5.0	g

Dissolve in distilled water and adjust volume to 1,000 ml. Store at room temperature.

5. 6X sample buffer with DTT (10 ml)

4X tris HCl/SDS pH 8.8	7	ml
Glycerol	3	ml

SDS	1	g
DTT	0.93	g
Bromophenol Blue	1.2	mg

Dissolve the solution and adjust volume to 10 ml. Store at room temperature.

6. 10% Ammonium Persulfate (APS)

APS	1	g
Distilled water	10	ml

Mix the solution and store at -20°C

7. 10% Sodium lauryl sarcosine (SDS)

Sodium lauryl sarcosine	1	g
Distilled water	10	ml

Mix the solution and store at room temperature.

8. 30% Acrylamide/ 0.8% Bisacrylamide (100 ml)

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Acrylamide	30	g
Bisacrylamide	0.8	g

Dissolve the solution in distilled water and adjust volume to 100 ml. Sterilize the solution by filtration. Store in the dark at room temperature.

9. 15% SDS-PAGE

Separating gel (15 ml)

Acrylamide/ bis	6.0	ml

1 M Tris-HCl pH 8.8	3.75	ml
10% SDS	0.15	ml
10% APS	75	µl
TEMED	7.5	µl
Distilled water	2.7	ml

Stacking gel (5 ml)

Acrylamide/ Bis	0.67	ml
0.5 M Tris-HCl pH 6.8	0.5	ml
10% SDS	40	µl
10% APS	40	µl
TEMED	4.0	µl
Distilled water	2.7	ml

Reagents for Western blot

1. Blotting buffer (1 Liter)

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Tris base	2.42	g
Glycine	11.24	g
Distilled water	800	ml

Dissolve in distilled water and add 200 mL methanol. Store at room temperature.

2. 10% BSA

BSA	0.5	g

PBS + 0.05% Tween 20	50	ml

3. **10X PBS pH 7.4**

NaCl	80	g
KCl	2	g
Na_2HPO_4	14.4	g
KH_2PO_4	2.4	g
Distilled water	1	L

4. **1X PBS pH 7.4**

10X PBS pH 7.4	100	ml
Distilled water	900	ml

5. **PBS + 0.05% Tween 20**

10X PBS pH 7.4	100	ml
Distilled water	900	ml
Tween 20	500	µl

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Reagents for In-Gel Digestion

1. **1 M ammonium bicarbonate (NH_4HCO_3) stock**

NH_4HCO_3	3.95	g
Water for LC-MS	50	ml

2. **50 mM NH_4HCO_3**

1 M NH_4HCO_3	0.5	ml
Water for LC-MS	9.5	ml

3. 25 mM NH_4HCO_3

50 mM NH_4HCO_3	5	ml
Water for LC-MS	5	ml

4. 25 mM NH_4HCO_3 in 50% ACN

50 mM NH_4HCO_3	5	ml
Acetonitrile (ACN)	5	ml

5. 10 mM DTT

DTT	0.0015	g
25 mM NH_4HCO_3	1	ml

6. 55 mM iodoacetmide

Iodoacetamide	0.01	g
25 mM NH_4HCO_3	1	ml

7. Trypsin

Trypsin	20	µg
NH_4HCO_3	1	ml

8. 0.1% Formic acid

Formic acid	5	ml

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100% ACN	2.5	ml
Water for LC-MS	2.5	ml

APPENDIX B

Table 6. The demonstrate of localization prediction.

Sample	LIC Number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B3	LIC11003	LipL71	OM	OM	OM	OM3
B3	LIC11718	SppA: endopeptidase IV, Signal peptide peptidase (protease IV)	CYTM	OM	OM	OM2
B1	LIC11456	LipL31	UNK	CYT	OM	OM1
B1	LIC11643	LipL45	CYT	PER>CYT	OM	OM1
B3	LIC12966	LipL41	UNK	CYT	OM	OM1
B1	LIC12071	Hypothetical protein	UNK	IM>OM	IM	OM
B1	LIC10845	Conserved hypothetical protein	UNK	PER>OM	IM	OM
B1	LIC20192	Alpha/beta hydrolase	EC	CYT>OM	UNK	OM
B1	LIC12988	Lipase	EC	EC	EC	EC
B1	LIC13060	LipL36	UNK	EC	EC	EC
B3	LIC10011	LipL21	PER	PER	PER	PER
B3	LIC11352	LipL32	UNK	PER	PER	PER
B1	LIC13074	Efflux pump, AcrB family	CYTM	IM	IM	IM
B1	LIC20118	Hypothetical protein	CYTM	CYT	IM	IM
B3	LIC11335	Chaperonin GroEL	CYT	CYT	CYT	CYT
B3	LIC10787	FlaA-2	CYT	CYT	PER	CYT
B1	LIC10877	Conserved hypothetical protein	UNK	PER	UNK	UNK
B1	LIC11424	FKBP-type peptidyl-prolyl cis-trans isomerase	PER	CYT	IM	UNK

extracellular (EC), periplasm (PER), inner membrane (IM), cytoplasm (CYT), unknown (UNK), OM by all 3 tools (OM3), by 2 tools (OM2), by 1 tool (OM1), or have multiple location sites with a low score at the outer membrane (OM), LIC number = the predicted coding sequences are referred to according to their genome nomenclature, LIC (*Leptospira interrogans* serovar Copenhageni strain Fiocruz L1–130).

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Table 7. The 510 OMPs, 214 proteins were found by both biotinylation and proteinase K methods, 222 OMPs from biotinylation methods only, and 74 OMPs from shaving only

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B&S 1	LIC11003	LipL71 lipoprotein	OM	OM	OM	OM3
B&S 2	LIC20197	cysteine protease	OM	OM>PER>EC	OM	OM3
B&S 3	LIC10714	Outer membrane receptor for Fe3+-dicitrate/TonB-dependent receptor	OM	OM	OM	OM3
B&S 4	LIC11851	Cytoplasmic membrane protein, ImpL63	OM	OM	OM	OM3
B&S 5	LIC10439	Conserved hypothetical protein	OM	OM	OM	OM3
B&S 6	LIC10050	OmpA-family protein	OM	OM	OM	OM3
B&S 7	LIC10711	Conserved hypothetical protein	OM	OM	OM	OM3
B&S 8	LIC12048	Hypothetical lipoprotein	OM	OM	OM	OM3
B&S 9	LIC12324	Signal transduction protein with multiple domains	CYTM	OM>CYT	OM	OM2
B&S 10	LIC12193	Conserved hypothetical protein	CYT	OM> CYT	OM	OM2
B&S 11	LIC20001	Conserved hypothetical protein	OM	OM	CYT	OM2
B&S 12	LIC12575	TolC-like protein	OM	OM	CYT	OM2
B&S 13	LIC11570	ype II secretory pathway component, protein D	OM	OM	IM	OM2
B&S 14	LIC20214	TonB dependent receptor	OM	OM	EC	OM2
B&S 15	LIC11793	sppA-2:Periplasmic serine protease (ClpP class)	CYTM	OM	OM	OM2
B&S 16	LIC13021	cAMP-dependent protein kinase	UNK	OM	OM	OM2
B&S 17	LIC20185	LipL45-related lipoprotein	UNK	OM	OM	OM2
B&S 18	LIC13194	Choline dehydrogenase	UNK	OM	OM	OM2
B&S 19	LIC11436	LipL45-related protein	OM	OM	IM	OM2
B&S 20	LIC13478	Substrate binding protein of an ABC transporter complex	PER	OM	OM	OM2
B&S 21	LIC11940	AcrA-related membrane protein	UNK	OM>CYT	OM	OM2
B&S 22	LIC10326	Hypothetical protein	UNK	OM>EC	OM	OM2
B&S 23	LIC10405	Conserved hypothetical protein containing tetratricopeptide repeat (TPR) domains	OM	OM>CYT	IM	OM2

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B&S 24	LIC10325	hlyX:p, hemolysin	UNK (CYT>EC)	OM	OM	OM2
B&S 25	LIC12669	hypothetical protein	CYTM	OM	OM	OM2
B&S 26	LIC10578	Conserved hypothetical protein	CYT	OM	OM	OM2
B&S 27	LIC11074	Conserved hypothetical protein	UNK	OM>EC	OM	OM2
B&S 28	LIC12632	Sphingomyelinase C precursor	EC	OM	OM	OM2
B&S 29	LIC13032	hypothetical protein	UNK	OM	OM	OM2
B&S 30	LIC20190	Conserved hypothetical protein	CYT	OM	OM	OM2
B&S 31	LIC10318	Conserved hypothetical protein	UNK	OM	OM	OM2
B&S 32	LIC11740	Zn-dependent protease	CYT	OM	OM	OM2
B&S 33	LIC12966	LipL41	UNK	CYT	OM	OM1
B&S 34	LIC13050	OmpL47 Conserved hypothetical protein	UNK	EC	OM	OM1
B&S 35	LIC11885	Conserved hypothetical lipoprotein	UNK	OM>CYT>PER	PER	OM1
B&S 36	LIC20172	Hypothetical lipoprotein	UNK	OM>EC	IM	OM1
B&S 37	LIC11456	lipL31:p, LipL30	UNK (CYTM>PER)	CYT	OM	OM1
B&S 38	LIC10973	ompL1:p, outer membrane protein	UNK	EC	OM	OM1
B&S 39	LIC12591	pepN:Membrane alanyl aminopeptidase	CYT	CYT>OM	CYT	OM1
B&S 40	LIC11643	LipL45	CYT	PER>CYT	OM	OM1
B&S 41	LIC12694	gltB:glutamate synthase (NADPH) alpha chain precursor	CYT	CYT	OM	OM1
B&S 42	LIC13314	Conserved hypothetical protein	CYTM	OM	IM	OM1
B&S 43	LIC11009	hypothetical protein	UNK	OM	CYT	OM1
B&S 44	LIC12841	Aspartate/tyrosine/aromatic aminotransferase	CYT	OM>CYT	CYT	OM1
B&S 45	LIC10465	LigA lipoprotein	UNK (EC>OM)	EC	OM	OM1
B&S 46	LIC11517	accA2:p, acetyl-CoA carboxylase alpha subunit	CYT	OM>CYT	CYT	OM1
B&S 47	LIC12263	hypothetical protein OmpL37	CYT	CYT>IM>OM	OM	OM1
B&S 48	LIC12359	Conserved hypothetical protein	UNK	OM	EC	OM1
B&S 49	LIC10874	Fe-S-cluster-containing hydrogenase	CYTM	OM	CYT	OM1

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B&S 50	LIC10125	TPR-repeat-containing lipoprotein	UNK	OM	CYT	OM1
B&S 51	LIC10314	ctP synthase	UNK (CYTM>PER)	OM	CYT	OM1
B&S 52	LIC12615	Phage-related protein	UNK	OM	EC	OM1
B&S 53	LIC12921	methyl-accepting chemotaxis protein	CYT	OM>IM	IM	OM1
B&S 54	LIC11489	Hypothetical protein	UNK	OM	IM	OM1
B&S 55	LIC10486	hypothetical protein	UNK	OM	IM	OM1
B&S 56	LIC10985	hypothetical lipoprotein	CYT	OM	IM	OM1
B&S 57	LIC11183	Hydrolase or acyltransferase	UNK	OM	IM	OM1
B&S 58	LIC10484	Threonine synthase	UNK	OM>CYT	CYT	OM1
B&S 59	LIC10546	lipase/ Hydrolase or acyltransferase, alpha/beta hydrolase superfamily, lipoprotein	UNK	OM>CYT	CYT	OM1
B&S 60	LIC10769	Zn-dependent peptidase	CYT	OM	IM	OM1
B&S 61	LIC13318	fabD:[Acyl-carrier protein] S-malonyltransferase	UNK	OM	CYT	OM1
B&S 62	LIC11790	Aspartate/tyrosine/aromatic aminotransferase	CYT	OM	CYT	OM1
B&S 63	LIC12303	C-terminal processing periplasmic-protease-4	CYT	OM	CYT	OM1
B&S 64	LIC13353	aofA:Amine oxidase (flavin-containing)	UNK	OM	UNK	OM1
B&S 65	LIC11935	Conserved hypothetical protein	UNK	EC	OM	OM1
B&S 66	LIC13496	ilvE:Branched-chain amino acid aminotransferase	CYT	OM>CYT	UNK	OM1
B&S 67	LIC13434	hypothetical lipoprotein	UNK	OM	PER	OM1
B&S 68	LIC12936	Hypothetical protein	CYT	OM	CYT	OM1
B&S 69	LIC11320	Hypothetical protein	UNK	OM	IM	OM1
B&S 70	LIC12446	Hypothetical protein	UNK	OM>EC>CYT	IM	OM1
B&S 71	LIC10115	Protein-disulfide isomerase	CYTM	OM	IM	OM1
B&S 72	LIC12544	Transcriptional regulator	UNK	OM	IM	OM1
B&S 73	LIC11437	adenylate/guanylate cyclase	CYTM	OM	IM	OM1
B&S 74	LIC11553	Conserved hypothetical protein	CYT	OM	IM	OM1
B&S 75	LIC10124	Hypothetical lipoprotein	UNK	OM	CYT	OM1

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B&S 76	LIC11334	Conserved hypothetical protein	UNK	OM	CYT	OM1
B&S 77	LIC10009	Hypothetical lipoprotein/ lipoprotein LenA	UNK	OM>CYT>EC	IM	OM1
B&S 78	LIC10286	Penicillin amidase or penicillin acylase	PER	OM	IM	OM1
B&S 79	LIC10763	Alanine--tRNA ligase	CYT	OM	CYT	OM1
B&S 80	LIC10226	Hypothetical protein	CYT	OM	CYT	OM1
B&S 81	LIC11813	Gamma-glutamyltransferase	PER	OM	CYT	OM1
B&S 82	LIC13176	Aryl-alcohol dehydrogenase-related oxidoreductase	CYT	OM>CYT	CYT	OM1
B&S 83	LIC12564	Histidine biosynthesis protein	CYT	OM	CYT	OM1
B&S 84	LIC13436	hypothetical protein	UNK	OM>PER>EC	PER	OM1
B&S 85	LIC11326	Endoflagellar basal body L-ring protein	UNK	OM	CYT	OM1
B&S 86	LIC10337	Conserved hypothetical protein	UNK	OM>PER	CYT	OM1
B&S 87	LIC10547	Hypothetical lipoprotein	UNK	OM	IM	OM1
B&S 88	LIC12805	hypothetical protein	UNK	OM>CYT	IM	OM1
B&S 89	LIC10123	LipL45-related lipoprotein	UNK	OM	IM	OM1
B&S 90	LIC11713	Endoflagellar motor protein	UNK (CYTM>CYT)	CYT	OM	OM1
B&S 91	LIC11966	Hypothetical lipoprotein	UNK	CYT>EC	OM	OM1
B&S 92	LIC20250	OmpA-family protein	OM	CYT>OM	IM	OM1
B&S 93	LIC11461	gatA:p, glutamyl-tRNA(Gln) amidotransferase subunit A	CTY	OM	CYT	OM1
B&S 94	LIC11224	Conserved hypothetical protein	CYTM	OM	IM	OM1
B&S 95	LIC12693	Outer membrane efflux protein related to TolC	UNK	OM	CYT	OM1
B&S 96	LIC12140	NDP-sugar dehydratase or epimerase	CYT	OM>CYT	CYT	OM1
B&S 97	LIC11602	Dehydrogenase	UNK	OM	IM	OM1
B&S 98	LIC13094	surE:Acid phosphatase	CYT	EC>OM	OM	OM1
B&S 99	LIC13089	hypothetical protein	UNK	OM	EC	OM1
B&S 100	LIC13060	LipL36, outer membrane lipoprotein	EC	EC	OM	OM1

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B&S 101	LIC11259	Cell shape-determining protein	UNK	OM	IM	OM1
B&S 102	LIC12094	tpiA:p, triosephosphate isomerase	CYTM	OM	CYT	OM1
B&S 103	LIC11370	fliN:p, flagellar motor switch protein	CYTM	OM	CYT	OM1
B&S 104	LIC12545	lolA-1: Outer membrane lipoprotein-sorting protein	UNK	OM>PER	IM	OM1
B&S 105	LIC12576	hypothetical protein	CYT	OM	IM	OM1
B&S 106	LIC20010	Delta-aminolevulinic acid dehydratase	CYT	OM	CYT	OM1
B&S 107	LIC12333	Hypothetical protein	CYTM	OM>IM	IM	OM1
B&S 108	LIC20036	hypothetical protein	UNK	OM	IM	OM1
B&S 109	LIC10382	Acyl-CoA dehydrogenase	CYT	OM>CYT	EC	OM1
B&S 110	LIC12254	OMA87 related protein	UNK	OM	EC	OM1
B&S 111	LIC12618	lysA:diaminopimelate decarboxylase	CYT	OM	CYT	OM1
B&S 112	LIC12030	Conserved hypothetical lipoprotein	UNK	OM	EC	OM1
B&S 113	LIC10231	Hypothetical protein	UNK	OM>EC>CYT	IM	OM1
B&S 114	LIC20144	Trypsin-like serine protease	CYT	OM	IM	OM1
B&S 115	LIC11982	hypothetical protein	CYT	OM	IM	OM1
B&S 116	LIC10151	Hypothetical protein	CYT	OM>CYT	IM	OM1
B&S 117	LIC11941	TolC Outer membrane protein	OM	CYT	CYT	OM1
B&S 118	LIC13464	Zn-dependent carboxypeptidase	CYT	OM	CYT	OM1
B&S 119	LIC12561	hypothetical protein	UNK	PER	OM	OM1
B&S 120	LIC12586	hypothetical protein	CYT	OM	IM	OM1
B&S 121	LIC11569	Type II secretory pathway component, protein C	UNK	OM	IM	OM1
B&S 122	LIC11616	Nucleoside-diphosphate-sugar epimerase	CYT	OM	UNK	OM1
B&S 123	LIC11612	hypothetical protein	UNK	OM	PER	OM1
B&S 124	LIC10227	Conserved hypothetical protein	CYT	OM>CYT	CYT	OM1
B&S 125	LIC13397	phoD:Phosphodiesterase I	EC	OM	CYT	OM1
B&S 126	LIC13105	pgi :Glucose-6-phosphate isomerase	CYT	OM	CYT	OM1

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B&S 127	LIC12396	Amidohydrolase	UNK	OM	CYT	OM1
B&S 128	LIC13331	Sensor histidine kinase of a two component response regulator	CYTM	OM	CYT	OM1
B&S 129	LIC11711	Hypothetical lipoprotein	CYT	OM	CYT	OM1
B&S 130	LIC10815	Conserved hypothetical protein	CYTM	OM>IM	IM	OM1
B&S 131	LIC10712	Thiol oxidoreductase	UNK	OM>EC>PER	IM	OM1
B&S 132	LIC12325	hypothetical observed protein	CYT	OM	CYT	OM1
B&S 133	LIC10955	Deoxyxylulose biosynthesis protein	CYT	OM	CYT	OM1
B&S 134	LIC20089	Serine phosphatase RsbU, regulator of sigma subunit	CYT	OM	CYT	OM1
B&S 135	LIC20274	ParA-like protein	CYTM	OM>CYT	CYT	OM1
B&S 136	LIC12275	proC:p, pyrroline-5-carboxylate reductase	CYT	OM>CYT	CYT	OM1
B&S 137	LIC11634	Short chain dehydrogenase	EC	OM>CYT	CYT	OM1
B&S 138	LIC12516	Acetyl-CoA synthetase	CYT	OM	CYT	OM1
B&S 139	LIC11823	Conserved hypothetical protein with FHA domain	UNK (CTYM>PER=EC)	OM	IM	OM1
B&S 140	LIC11540	CTP synthase	CYT	CYT	OM	OM1
B&S 141	LIC12923	Oxidoreductase	CYT	OM>CYT	CYT	OM1
B&S 142	LIC1_SPN3184	pdhC:p, dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex E2	CYT	OM	CYT	OM1
B&S 143	LIC11280	prfC:p, peptide chain release factor 3	CYT	OM>CYT	CYT	OM1
B&S 144	LIC10067	Hypothetical protein	UNK (EC>OM>PER>CYTM)	EC	OM	OM1
B&S 145	LIC12731	TPR-repeat protein	CYT	OM	IM	OM1
B&S 146	LIC11202	Sensor histidine kinase and response regulator of a two component complex	CYTM	OM	CYT	OM1

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B&S 147	LIC11468	Hypothetical protein	CYT	OM	EC	OM1
B&S 148	LIC11011	Nicotinate-nucleotide pyrophosphorylase	CYT	OM>CYT	CYT	OM1
B&S 149	LIC12101	CinA-related protein	CYT	OM	CYT	OM1
B&S 150	LIC13363	Hypothetical protein	UNK	OM	EC	OM1
B&S 151	LIC11458	Conserved hypothetical protein	OM	OM	EC	OM1
B&S 152	LIC10521	Transcriptional regulator	UNK	OM>CYT>EC	CYT	OM1
B&S 153	LIC13100	Nucleoside-diphosphate sugar epimerase	CYT	OM>CYT	CYT	OM1
B&S 154	LIC20019	Hypothetical protein	UNK	OM	CYT	OM1
B&S 155	LIC12319	Serine/threonine specific protein phosphatase	CYT	OM>CYT	CYT	OM1
B&S 156	LIC11075	NAD(+) synthase (glutamine-hydrolyzing)	CYT	OM	CYT	OM1
B&S 157	LIC20030	hypothetical protein	UNK	CYT>OM	OM	OM1
B&S 158	LIC2_SPN3827	Orotidine-5'-phosphate decarboxylase	CYT	OM	CYT	OM1
B&S 159	LIC20009	Bifunctional porphobilinogen deaminase/uroporphyrinogen synthase	CYT	OM	CYT	OM1
B&S 160	LIC13152	D-alanyl-D-alanine carboxypeptidase	CYT	OM>PER	CYT	OM1
B&S 161	LIC20109	Hypothetical protein	CYTM	CYT	OM	OM1
B&S 162	LIC10810	Hypothetical protein	UNK	OM	IM	OM1
B&S 163	LIC12635	Transcriptional regulator	CYT	OM	EC	OM1
B&S 164	LIC11027	hypothetical lipoprotein	UNK	PER>CYT	OM	OM1
B&S 165	LIC10187	Hypothetical protein	UNK	OM	EC	OM1
B&S 166	LIC10012	hypothetical protein	UNK	OM	CYT	OM1
B&S 167	LIC12631	Sphingomyelinase C precursor	EC	PER>OM	EC	OM
B&S 168	LIC13522	Chemotaxis protein histidine kinase CheA	CYT	CYT>OM	UNK	OM
B&S 169	LIC12812	Trypsin-like serine protease	PER	PER>OM	UNK	OM
B&S 170	LIC10788	flaA-1:p, flagellar filament sheath protein	CYT	PER>OM>EC	CYT	OM
B&S 171	LIC10175	Conserved hypothetical protein	CYT	CYT>OM	EC	OM
B&S 172	LIC12925	gltA-2:Citrate synthase	CYT	CYT>OM	CYT	OM

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B&S 173	LIC11630	fadD:p, long-chain-fatty-acid CoA ligase	CYT	CYT>OM	CYT	OM
B&S 174	LIC12202	Nucleoside-diphosphate-sugar epimerase	CYT	CYT> OM	UNK	OM
B&S 175	LIC11532	Endoflagellar hook-filament protein	PER	EC>OM	CYT	OM
B&S 176	LIC12075	hypothetical protein	CYT	CYT>OM	PER	OM
B&S 177	LIC10002	DNA-directed DNA polymerase, beta subunit	CYT	CYT>OM	EC	OM
B&S 178	LIC11587	nrdA:p, ribonucleotide-diphosphate reductase subunit alpha	CYT	CYT>OM	CYT	OM
B&S 179	LIC10760	Conserved hypothetical protein	UNK	EC>CYT>OM	PER	OM
B&S 180	LIC11413	Beta-glucosidase-related glycosidase	CYT	CYT>OM	IM	OM
B&S 181	LIC10842	Dihydrodipicolinate synthase/N-acetylneuraminate lyase	CYT	CYT>OM>PER	CYT	OM
B&S 182	LIC12060	Sensor histidine kinase and response regulator of a two component complex, part (N-term)	CYTM	CYT>OM	IM	OM
B&S 183	LIC10571	Homoserine dehydrogenase	CYT	CYT>OM	CYT	OM
B&S 184	LIC13345	Bifunctional glutamate--tRNA ligase/glutamine--tRNA ligase	CYT	CYT>OM	CYT	OM
B&S 185	LIC12872	rplD:50S ribosomal protein L4	CYT	PER>OM>EC	CYT	OM
B&S 186	LIC10029	Inositol monophosphatase family protein	CYT	CYT>OM	CYT	OM
B&S 187	LIC20196	Hypothetical protein	UNK	CYT>PER>OM	CYT	OM
B&S 188	LIC12563	Bifunctional glycerol-3-phosphate dehydrogenase /glycerol-3-phosphate acyltransferase	CYT	IM>CYT>OM	CYT	OM
B&S 189	LIC20208	Methylmalonyl-COA mutase	CYT	CYT>OM	CYT	OM
B&S 190	LIC11888	hypothetical protein	UNK	CYT>OM	UNK	OM
B&S 191	LIC20148	Heme oxygenase	UNK	CYT>OM	CYT	OM
B&S 192	LIC11884	Conserved hypothetical protein	PER	CYT>OM	CYT	OM
B&S 193	LIC12210	Small heat shock protein	CYT	CYT>OM	CYT	OM

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B&S 194	LIC10736	Bifunctional translation initiation inhibitor, yjgF family/endoribonuclease L-PSP	UNK	PER>CYT>OM	UNK	OM
B&S 195	LIC13433	pyrD:dihydroorotate dehydrogenase	CYTM	CYT>OM	CYT	OM
B&S 196	LIC12402	Phosphoribosylformylglycinamidine synthase	CYT	CYT>OM	CYT	OM
B&S 197	LIC10793	Surface Antigen OrfC lipoprotein	CYT	CYT>OM	CYT	OM
B&S 198	LIC12106	Membrane-associated HD superfamily hydrolase	CYTM	IM>OM	IM	OM
B&S 199	LIC10952	Phosphohydrolase	CYTM	IM>OM	IM	OM
B&S 200	LIC12306	Efflux pump	CYT	CYT>OM	IM	OM
B&S 201	LIC12466	Carbamoyl-phosphate synthase (glutamine-hydrolyzing), small subunit	CYT	CYT>OM>EC	CYT	OM
B&S 202	LIC11147	Flavin reductase related protein	UNK	EC>OM>CYT	EC	OM
B&S 203	LIC12535	ribC:Riboflavin synthase alpha chain	CYT	CYT>OM	EC	OM
B&S 204	LIC12231	Hypothetical lipoprotein	UNK	IM>OM	PER	OM
B&S 205	LIC11788	Hydrolase or acyltransferase	UNK	CYT>OM	CYT	OM
B&S 206	LIC11376	Endoflagellar biosynthesis protein	CYTM	IM>OM	IM	OM
B&S 207	LIC20002	5,10-methylenetetrahydrofolate reductase	CYT	CYT>OM	CYT	OM
B&S 208	LIC13192	Sensor histidine kinase and response regulator of a two component complex	CYTM	IM>OM	IM	OM
B&S 209	LIC20241	Alanine racemase	CYT	CYT>OM	CYT	OM
B&S 210	LIC13271	argJ:Glutamate N-acetyltransferase	CYT	CYT>OM>EC	UNK	OM
B&S 211	LIC10293	Glycerophosphodiester phosphodiesterase	CYT	CYT>OM	CYT	OM
B&S 212	LIC11723	hypothetical lipoprotein	UNK	PER>OM>CYT	PER	OM
B&S 213	LIC11173	Conserved hypothetical protein	CYT	CYT>OM	UNK	OM
B&S 214	LIC12217	petE:Plastocyanin	CYT	IM>EC>OM	IM	OM

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B1	LIC11268	Hypothetical protein	OM	OM	OM	OM3
B2	LIC20151	TonB-dependant outer membrane receptor	OM	OM	OM	OM3
B3	LIC11345	TonB dependent receptor	OM	OM	OM	OM3
B4	LIC13418	Hypothetical protein, part (C-term)	OM	OM>EC	EC	OM2
B5	LIC11327	Endoflagellar basal body P-ring protein	PER	OM	OM	OM2
B6	LIC13313	hypothetical protein	CYT	OM>CYT	OM	OM2
B7	LIC20031	Short chain dehydrogenase	CYT	OM	OM	OM2
B8	LIC11739	Peptidase inhibitor homologue	UNK (CYTM>OM)	OM	OM	OM2
B9	LIC12730	NHL repeat protein	UNK	OM	OM	OM2
B10	LIC10772	hypothetical protein	UNK	OM	OM	OM2
B11	LIC11366	Hypothetical protein	OM	OM	IM	OM2
B12	LIC11271	hypothetical protein	CYTM	OM>CYT>IM	OM	OM2
B13	LIC12498	hypothetical protein	CYT	OM	OM	OM2
B14	LIC11211	Conserved hypothetical protein	OM	OM	IM	OM2
B15	LIC10302	TPR-repeat-containing protein	UNK (CYTM>PER=EC)	OM>CYT>PER	OM	OM2
B16	LIC12258	OMA87-related protein	OM	OM	IM	OM2
B17	LIC13417	hypothetical protein, part (N-term)	OM	OM>EC	PER	OM2
B18	LIC11623	Outer membrane protein related to Oma87	OM	OM	IM	OM2
B19	LIC12225	Conserved hypothetical protein	UNK	OM	OM	OM2
B20	LIC13078	hypothetical protein	UNK	OM	OM	OM2
B21	LIC13078	hypothetical protein	UNK	OM	OM	OM2
B22	LIC11112	Trypsin-like serine protease	UNK (CYTM>PER=EC)	OM	OM	OM2
B23	LIC12002	sdhA:p, succinate dehydrogenase flavoprotein subunit	CYTM	PER>EC>OM	OM	OM1

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B24	LIC11013	Substrate binding protein of an ABC transporter complex	PER	CYT>PER	OM	OM1
B25	LIC20044	HSP90 molecular chaperone	CYT	OM	CYT	OM1
B26	LIC20096	Conserved hypothetical protein	CYT	OM>EC>CYT	CYT	OM1
B27	LIC20026	Short chain dehydrogenase	CYT	OM	UNK	OM1
B28	LIC12708	Hypothetical protein	UNK	OM	PER	OM1
B29	LIC11820	cysK-3:Cysteine synthase	CYT	OM	CYT	OM1
B30	LIC11718	Signal peptide peptidase (protease IV)	UNK (CYTM>CYT)	OM	CYT	OM1
B31	LIC12477	Membrane carboxypeptidase/penicillin-binding protein 1	CYTM	OM	CYT	OM1
B32	LIC10446	Phosphomannomutase	CYT	OM	CYT	OM1
B33	LIC12368	Transcriptional regulator	CYT	OM	IM	OM1
B34	LIC12500	Methyl-accepting chemotaxis protein	UNK (CYTM>OM)	OM	IM	OM1
B35	LIC11799	glpK:p, glycerol kinase [Leptospira interrogans serovar Copenhageni str. Fiocruz L1-130].	CYT	OM	CYT	OM1
B36	LIC10282	Zn-dependent oxidoreductase	CYT	OM>PER	UNK	OM1
B37	LIC11024	Glycosyltransferase	CYTM	OM	IM	OM1
B38	LIC20114	LipL45-related protein	CYT	OM>CYT	CYT	OM1
B39	LIC10900	Adenylate/guanylate cyclase, part (N-term)	CYT	OM	IM	OM1
B40	LIC10623	Endoflagellar motor protein	CYTM	OM>CYT>PER	IM	OM1
B41	LIC11707	Fructose-bisphosphatase	CYT	OM>EC	CYT	OM1
B42	LIC10157	Preprotein translocase, YidC subunit	CYTM	OM	IM	OM1
B43	LIC13384	ATP-binding protein of an ABC transporter complex	CYTM	OM>CYT>IM	CYT	OM1
B44	LIC11371	Endoflagellar biogenesis protein	UNK	OM	IM	OM1
B45	LIC11360	Conserved hypothetical lipoprotein	CYT	CYT	OM	OM1
B46	LIC20010	Delta-aminolevulinic acid dehydratase	CYT	OM	CYT	OM1
B47	LIC10534	Penicillin binding protein 1	UNK (CYTM>OM)	OM	IM	OM1

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B48	LIC11515	Serine phosphatase RsbU, regulator of sigma subunit	CYT	OM	CYT	OM1
B49	LIC20143	Trypsin-like serine protease	UNK (CYTM>PER)	OM	CYT	OM1
B50	LIC11970	Hydrolase or acyltransferase	CYTM	OM	CYT	OM1
B51	LIC10640	Hypothetical protein	CYT	OM	CYT	OM1
B52	LIC12620	Membrane carboxypeptidase/Penicillin-binding protein	CYTM	OM	CYT	OM1
B53	LIC11912	hypothetical protein	UNK	OM>IM>PER	IM	OM1
B54	LIC12845	rplQ:50S Ribosomal protein L17	CYTM	OM	IM	OM1
B55	LIC12744	nuoD:NADH dehydrogenase (ubiquinone), D chain	UNK (CYT>CYTM)	OM	IM	OM1
B56	LIC10294	GlcG related protein	CYT	OM	CYT	OM1
B57	LIC13394	Methyl-accepting chemotaxis protein	CYTM	OM	IM	OM1
B58	LIC13065	Conserved hypothetical protein	UNK	OM	CYT	OM1
B59	LIC11627	Glycosyltransferase	CYT	OM	CYT	OM1
B60	LIC12086	Signal peptidase I-related protein	CYTM	OM>CYT	CYT	OM1
B61	LIC10311	gcvT:Aminomethyltransferase	CYT	OM	CYT	OM1
B62	LIC12957	Oxidoreductase	CYT	OM	CYT	OM1
B63	LIC10105	Conserved hypothetical protein	UNK	OM	IM	OM1
B64	LIC11486	Conserved hypothetical protein	UNK	OM>EC	IM	OM1
B65	LIC13145	gpsA:Glycerol-3-phosphate dehydrogenase (NAD(P)+)	CYT	OM	UNK	OM1
B66	LIC10207	SCO1/SenC/PrrC family protein	UNK	OM	IM	OM1
B67	LIC11105	Methylmalonyl-CoA mutase	CYTM	OM>CYT	OM	OM1
B68	LIC11455	Transcription-repair coupling factor	CYT	OM>CYT	CYT	OM1
B69	LIC11051	Leucine-rich repeat containing protein	EC	OM>EC	EC	OM1
B70	LIC10518	ATP-binding protein of an ABC transporter complex	CYTM	OM>CYT	CYT	OM1

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B71	LIC12353	hypothetical lipoprotein	CYT	CYT	OM	OM1
B72	LIC10145	Hypothetical protein	CYT	OM	IM	OM1
B73	LIC11784	hypothetical protein	CYTM	OM>CYT	IM	OM1
B74	LIC20066	Ribonuclease III	CYT	OM	CYT	OM1
B75	LIC13482	Metalloendopeptidase	UNK	OM	IM	OM1
B76	LIC11766C	Phosphomannomutase	CYT	OM>CYT	CYT	OM1
B77	LIC12133	Conserved hypothetical protein	UNK	OM>CYT	CYT	OM1
B78	LIC12539	Conserved hypothetical protein	EC	OM>EC	IM	OM1
B79	LIC10347	Arylesterase	CYT	OM	IM	OM1
B80	LIC20098	XerD related protein	CYT	OM	CYT	OM1
B81	LIC12100	Hypothetical protein	CYTM	OM	IM	OM1
B82	LIC11874	Hypothetical lipoprotein	CYT	OM	IM	OM1
B83	LIC13346	hypothetical protein	CYT	OM	EC	OM1
B84	LIC20212	Hypothetical protein	UNK	OM>EC	EC	OM1
B85	LIC20246	Sensor histidine kinase	CYTM	OM>CYT	CYT	OM1
B86	LIC11988	hypothetical protein	UNK	OM	IM	OM1
B87	LIC12127	Glycosyltransferase	UNK (CYTM>CYT)	OM	CYT	OM1
B88	LIC10799	Enoyl-CoA hydratase/carnithine racemase	CYT	OM	UNK	OM1
B89	LIC12227	Conserved hypothetical protein	UNK	EC	OM	OM1
B90	LIC11156	Na+/H+ antiporter	CYTM	IM	OM	OM1
B91	LIC11682	Hypothetical protein	CYTM	OM>CYT	PER	OM1
B92	LIC13412	Bifunctional glycerol-3-phosphate dehydrogenase/glycerol-3-phosphate acyltransferase	CYTM	OM>CYT	CYT	OM1
B93	LIC10539	hypothetical protein	UNK	OM	IM	OM1
B94	LIC12605	Phage-related protein	CYT	OM	CYT	OM1
B95	LIC10043	Methyl-accepting chemotaxis protein	CYTM	OM	IM	OM1

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B96	LIC12499	hypothetical protein	UNK	OM	IM	OM1
B97	LIC11675	Glycerol-3-phosphate dehydrogenase	CYT	OM	EC	OM1
B98	LIC11037	Trypsin-like serine protease	UNK (CYT>PER)	OM	PER	OM1
B99	LIC12610	hypothetical protein	UNK	OM>CYT	CYT	OM1
B100	LIC20103	hypothetical lipoprotein	UNK	OM>CYT	IM	OM1
B101	LIC10054	RlpA-like lipoprotein	CYT	OM>PER>CYT	PER	OM1
B102	LIC12506	Adenylate/guanylate cyclase	CYT	OM	CYT	OM1
B103	LIC11536	Cell wall-associated hydrolase	CYT	OM	PER	OM1
B104	LIC20043	BatD	CYT	OM	IM	OM1
B105	LIC20127	Precorrin-3B C(17)-methyltransferase	CYT	OM	CYT	OM1
B106	LIC12450	tyrA:Bifunctional prephenate dehydrogenase/chorismate mutase	UNK	OM>EC	PER	OM1
B107	LIC11946	Conserved hypothetical protein	CYTM	OM	UNK	OM1
B108	LIC20029	hypothetical protein	UNK	OM>PER	IM	OM1
B109	LIC12756	hypothetical protein	CYT	OM	IM	OM1
B110	LIC10387	LipL45-related protein	CYT	OM>CYT	IM	OM1
B111	LIC20074	Long-chain-fatty-acid--CoA ligase	CYT	OM	IM	OM1
B112	LIC13237	Lysine decarboxylase-related protein	CYT	OM	CYT	OM1
B113	LIC10251	ATPase involved in DNA repair	UNK (CYT>EC)	OM	IM	OM1
B114	LIC10114	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase	CYT	OM	CYT	OM1
B115	LIC11708	Sensor histidine kinase of a two component response regulator	CYTM	OM	CYT	OM1
B116	LIC20082	Methylase/methyltransferase	CYT	OM>CYT	CYT	OM1
B117	LIC13477	Hypothetical protein	UNK	OM	IM	OM1
B118	LIC20057	Protein kinase	CYTM	OM	CYT	OM1

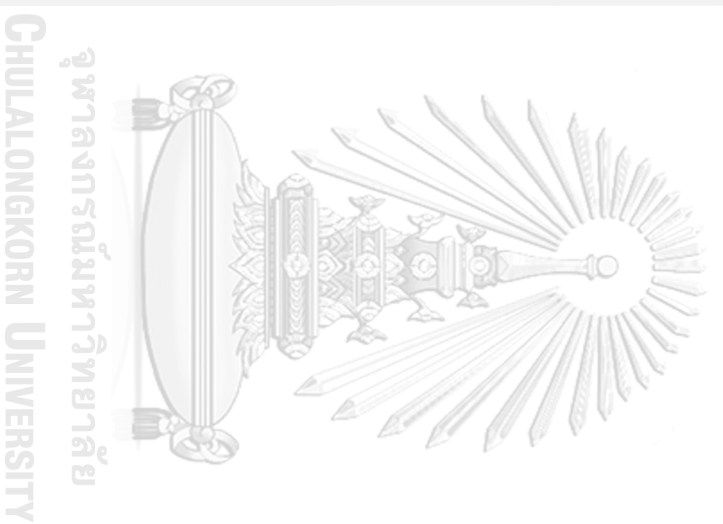
Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B119	LIC11591	Exodeoxyribonuclease VII, large subunit	CYT	OM>CYT	CYT	OM1
B120	LIC13272	Hypothetical protein	UNK	OM	IM	OM1
B121	LIC20017	Protoporphyrinogen oxidase	CYTM	OM	CYT	OM1
B122	LIC13125	Hypothetical lipoprotein	UNK	OM>EC	CYT	OM1
B123	LIC12307	TolC related protein	UNK (CYT>EC)	CYT>OM	OM	OM1
B124	LIC11732	ATP-dependent DNA helicase	CYT	OM	CYT	OM1
B125	LIC13489	ParA-like protein	CYTM	OM	UNK	OM1
B126	LIC20146	Hypothetical protein	CYT	OM	IM	OM1
B127	LIC10668	Mrr family restriction endonuclease	CYT	OM	CYT	OM1
B128	LIC11790	Aspartate/tyrosine/aromatic aminotransferase	CYT	OM	CYT	OM1
B129	LIC13229	Conserved hypothetical protein	UNK	OM	IM	OM1
B130	LIC13457	Hypothetical protein	CYTM	OM>CYT>IM	IM	OM1
B131	LIC12070	AntiSigma factor antagonist	UNK (CYT>EC)	OM	EC	OM1
B132	LIC10902	LipL45-related protein	UNK	OM	IM	OM1
B133	LIC12772	proB:Glutamate 5-kinase	CYT	OM>CYT	CYT	OM1
B134	LIC10184	hypothetical protein	CYT	OM	CYT	OM1
B135	LIC11111	Trypsin-like serine protease	UNK	OM	IM	OM1
B136	LIC13366	Strictosidine synthase	CYTM	OM	IM	OM1
B137	LIC12638	Conserved hypothetical protein	CYT	OM>CYT>EC	CYT	OM1
B138	LIC10797	hypothetical lipoprotein	UNK	PER>CYT	OM	OM1
B139	LIC11577	ype II secretory pathway component, protein K	UNK	OM	IM	OM1
B140	LIC10731	Penicillin binding protein	CYTM	OM>PER>CYT	CYT	OM1
B141	LIC10594	2-dehydropantoate 2-reductase	UNK	OM>CYT	UNK	OM1
B142	LIC11671	hypothetical lipoprotein	UNK	OM>EC>PER	EC	OM1
B143	LIC11199	Alpha-beta hydrolase	EC	OM	CYT	OM1
B144	LIC13361	Conserved hypothetical protein	UNK (CYT>EC)	OM	CYT	OM1
B145	LIC20273	Response regulator	CYT	OM	CYT	OM1

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B146	LIC11028	Tol transport system component	UNK (CYT>OM)	OM	EC	OM1
B147	LIC11261	Cell division protein FtsI/penicillin-binding protein	UNK (CYTM>OM)	OM	PER	OM1
B148	LIC11430	Hydrolase or acyltransferase	CYT	CYT>OM>PER	OM	OM1
B149	LIC10385	hypothetical protein	CYTM	OM>IM	IM	OM1
B150	LIC20092	Protease	CYT	OM>CYT	UNK	OM1
B151	LIC11741	Zn-dependent protease	CYT	OM	CYT	OM1
B152	LIC11220	ABC-type transport system involved in Fe-S cluster assembly, permease component	CYT	OM>EC	CYT	OM1
B153	LIC11918	hypothetical protein	UNK	OM	UNK	OM1
B154	LIC12719	hypothetical protein	CYT	OM>CYT	CYT	OM1
B155	LIC12901	Leucine-rich repeat protein	EC	OM>EC>CYT	CYT	OM1
B156	LIC13339	motB-2:Endoflagellar motor protein	CYTM	OM>CYT	IM	OM1
B157	LIC11598	Peptidyl -tRNA hydrolase	CYT	OM>CYT>EC	CYT	OM1
B158	LIC13341	hypothetical protein	UNK	OM	CYT	OM1
B159	LIC10046	NAD(P)(+) transhydrogenase (AB-specific), alpha subunit	CYTM	PER>CYT>OM	CYT	OM
B160	LIC11630	Long-chain-fatty-acid--CoA ligase	CYT	CYT>OM	CYT	OM
B161	LIC11674	alkylglycerone-phosphate synthase	CYT	CYT>OM	CYT	OM
B162	LIC11653	Hypothetical protein	UNK	PER>OM>CYT	PER	OM
B163	LIC12529	Substrate binding protein of an ABC transporter complex	PER	CYT>PER>OM	PER	OM
B164	LIC20028	NADH dehydrogenase	CYTM	CYT>OM	CYT	OM
B165	LIC10845	Conserved hypothetical protein	UNK	PER>OM	IM	OM
B166	LIC11240	Delta subunit of the H(+)-transporting two-sector ATPase, F1 sector	CYT	CYT>OM	CYT	OM
B167	LIC11580	Hypothetical protein	CYT	CYT>OM	IM	OM
B168	LIC20192	Alpha/beta hydrolase	EC	CYT>OM	UNK	OM

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B169	LIC10037	GMC-family oxidoreductase	UNK	PER>OM	PER	OM
B170	LIC13398	Hydrolase	CYTM	IM>OM>CYT	IM	OM
B171	LIC11826	Hypothetical protein	CYT	CYT>OM	CYT	OM
B172	LIC20167	Hypothetical protein	UNK	CYT>OM	CYT	OM
B173	LIC12801	Hydrolase or acyltransferase	CYT	PER>OM	CYT	OM
B174	LIC10234	Hypothetical protein	CYT	CYT>OM	CYT	OM
B175	LIC12071	hypothetical protein	UNK	IM>OM	IM	OM
B176	LIC12392	Amidase	CYT	CYT>OM	CYT	OM
B177	LIC10526	Heat-inducible transcriptional repressor, HrcA	CYT	CYT>OM	CYT	OM
B178	LIC11786	Homoserine kinase	CYT	CYT>OM	CYT	OM
B179	LIC10629	Glycerol kinase	CYT	IM>CYT>OM	UNK	OM
B180	LIC11827	Glycosyltransferase	CYT	CYT>OM	CYT	OM
B181	LIC12451	pheA:Bifunctional prephenate dehydratase/chorismate mutase	CYT	CYT>OM	CYT	OM
B182	LIC12671	Mechanosensitive ion channel	CYTM	IM>OM	IM	OM
B183	LIC11804	ATP-binding protein of an ABC transporter complex	CYTM	CYT>OM	CYT	OM
B184	LIC11691	Methyl-accepting chemotaxis protein	CYTM	IM>OM	IM	OM
B185	LIC10444	Glucose-1-phosphate thymidylyltransferase	CYT	CYT>OM	CYT	OM
B186	LIC11990	TPR-repeat-containing protein	UNK	EC>OM	CYT	OM
B187	LIC10103	Conserved hypothetical protein	UNK	CYT>OM>IM	CYT	OM
B188	LIC12710	Hypothetical protein	UNK	CYT>OM	CYT	OM
B189	LIC13012	Conserved hypothetical protein	CYTM	IM>OM	IM	OM
B190	LIC12992	Bifunctional permease/carbonic anhydrase	CYTM	IM>OM	UNK	OM
B191	LIC10978	Conserved hypothetical protein	CYT	EC>PER>CYT>OM	CYT	OM
B192	LIC10357	Tryptophan--tRNA ligase	CYT	PER>CYT>OM	IM	OM
B193	LIC11939	hypothetical protein	UNK	EC>OM	CYT	OM

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B194	LIC10262	Hypothetical protein	CYT	CYT>OM	CYT	OM
B195	LIC11926	Glycosyltransferase	CYTM	IM>EC>OM	IM	OM
B196	LIC12697	Conserved hypothetical protein, part (C-term)	CYTM	CYT>IM>OM	IM	OM
B197	LIC11154	Transcriptional regulator	CYT	CYT>OM	CYT	OM
B198	LIC11302	Phosphatase	CYT	CYT>OM>IM	CYT	OM
B199	LIC13026	Transcriptional regulator, ThiJ/PfpI family protein	UNK	CYT>IM>OM	CYT	OM
B200	LIC12238	Hypothetical lipoprotein	UNK	CYT>OM	IM	OM
B201	LIC12390	Protein kinase	CYTM	CYT>OM	UNK	OM
B202	LIC10218	Conserved hypothetical lipoprotein	CYT	EC >OM	EC	OM
B203	LIC10751	50S Ribosomal protein L10	CYT	IM>OM	CYT	OM
B204	LIC13362	eriC-1:Chloride channel protein	CYTM	IM>OM	IM	OM
B205	LIC12478	Metal-dependent hydrolase	CYT	CYT>OM	CYT	OM
B206	LIC11142	ATP-dependent serine protease	UNK (CYT>EC>OM)	CYT>OM	CYT	OM
B207	LIC12616	hypothetical protein	UNK	CYT>OM	CYT	OM
B208	LIC10099	Sensor histidine kinase and response regulator protein	CYTM	IM>OM	IM	OM
B209	LIC11628	hypothetical protein	UNK	CYT>OM	CYT	OM
B210	LIC13389	DNA mismatch repair protein ATPase component	CYTM	IM>OM	IM	OM
B211	LIC11859	Substrate binding protein of an ABC transporter complex	CYT	CYT>OM	IM	OM
B212	LIC13460	Hypothetical lipoprotein	UNK	PER>CYT>OM	EC	OM
B213	LIC20007	hypothetical protein	UNK	CYT>OM>PER	IM	OM
B214	LIC10604	hypothetical protein	CYT	CYT>PER>OM	IM	OM
B215	LIC12595	Fumarylacetoacetate hydrolase family protein	CYT	CYT>OM	CYT	OM
B216	LIC13333	Hypothetical protein	UNK	CYT>OM	PER	OM
B217	LIC10036	Hypothetical protein	UNK	CYT>OM	CYT	OM

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B218	LIC12885	Crossover junction endoribonuclease	CYT	CYT>OM	CYT	OM
B219	LIC10489	Conserved hypothetical protein	UNK	EC>OM	IM	OM
B220	LIC12482	Methyl-accepting chemotaxis protein	CYTM	IM>OM	IM	OM
B221	LIC20077	polysaccharide deacetylase	UNK	PER>OM>EC	CYT	OM
B222	LIC11882	Conserved hypothetical protein	CYT	CYT>OM	UNK	OM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Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
S1	LIC10017	hypothetical protein	OM	OM	OM	OM3
S2	LIC10968	Conserved hypothetical protein	UNK	OM	OM	OM2
S3	LIC11399	Metallopeptidase	UNK (CYTM>PER)	OM	OM	OM2
S4	LIC13066	Conserved hypothetical protein	CYTM	OM	OM	OM2
S5	LIC20231	Hypothetical protein	CYTM	OM	OM	OM2
S6	LIC10713	Iron-regulated lipoprotein	OM	OM	EC	OM2
S7	LIC11026	hypothetical protein	OM	OM	IM	OM2
S8	LIC11463	Hydrolase or acyltransferase	EC	OM	IM	OM1
S9	LIC11150	Conserved hypothetical protein	UNK	OM>CYT	CYT	OM1
S10	LIC12759	Leucine-rich repeat protein	EC	OM>CYT	CYT	OM1
S11	LIC13293	Zinc dependent protease	UNK	OM	EC	OM1
S12	LIC11626	Inositol monophosphatase family protein	CYT	OM	CYT	OM1
S13	LIC11119	Conserved hypothetical protein	UNK (CYTM>EC)	OM	IM	OM1
S14	LIC12515	ATP Binding protein of an ABC transporter complex	PER	OM	IM	OM1
S15	LIC11207	Conserved hypothetical lipoprotein	UNK	EC	OM	OM1
S16	LIC13162	3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase	CYT	OM	CYT	OM1
S17	LIC11803	lpdA-3:Dihydrolipoamide dehydrogenase	EC	OM>EC	EC	OM1
S18	LIC10208	Cytochrome C oxidase subunit II	CYT	OM>EC	CYT	OM1
S19	LIC11910	Polysaccharide deacetylase	UNK	OM>PER	CYT	OM1
S20	LIC11270	hypothetical lipoprotein	UNK	OM	IM	OM1
S21	LIC12718	Enolase-phosphatase	CYT	OM>CYT	CYT	OM1
S22	LIC11435	Hypothetical protein	UNK	OM	IM	OM1
S23	LIC10464	LigB lipoprotein	UNK (EC>OM)	EC	OM	OM1
S24	LIC12331	Phosphonomutase	CYT	CYT	OM	OM1
S25	LIC10368	hypothetical lipoprotein	UNK	EC>OM	OM	OM1

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
S26	LIC12611	Conserved hypothetical protein	CYTM	OM	IM	OM1
S27	LIC12555	Conserved hypothetical protein	CYT	OM>CYT	IM	OM1
S28	LIC20225	Short chain dehydrogenase	UNK	OM>CYT	CYT	OM1
S29	LIC12253	hypothetical protein	UNK	OM	EC	OM1
S30	LIC10364	O-methyltransferase	CYT	OM>CYT	CYT	OM1
S31	LIC12337	hypothetical protein	EC	OM	EC	OM1
S32	LIC10057	Hypothetical protein	UNK	OM	CYT	OM1
S33	LIC10316	Hypothetical protein	UNK	CYT	OM	OM1
S34	LIC12114	Poly-gamma-glutamate biosynthesis	CYTM	OM	IM	OM1
S35	LIC10527	Protein-tyrosine-phosphatase	UNK	OM>CYT	CYT	OM1
S36	LIC13511	Guanylate kinase	CYT	OM	CYT	OM1
S37	LIC11853	Homoserine O-acetyltransferase	CYT	OM	CYT	OM1
S38	LIC13231	Conserved hypothetical protein	UNK	OM	CYT	OM1
S39	LIC11222	Tetratricopeptide repeat family protein	UNK	OM	EC	OM1
S40	LIC11695	Hypothetical lipoprotein	UNK	CYT	OM	OM1
S41	LIC11167	hypothetical protein	CYT	OM>CYT	CYT	OM1
S42	LIC11624	ATP-dependent DNA helicase	CYT	OM>CYT	CYT	OM1
S43	LIC12373	hypothetical protein	UNK	OM	EC	OM1
S44	LIC10655	Conserved hypothetical protein	UNK	PER>CYT	OM	OM1
S45	LIC11960	hypothetical protein	UNK	OM	EC	OM1
S46	LIC13360	Conserved hypothetical protein	UNK (CYT>EC)	OM	UNK	OM1
S47	LIC12372	ankyrin repeat-containing protein	UNK	OM>CYT	CYT	OM1
S48	LIC13270	Sensor histidine kinase of a two component response regulator	CYT	OM	IN	OM1
S49	LIC10189	hypothetical protein	UNK	OM	EC	OM1
S50	LIC12943	cspR:rRNA methylase	UNK	OM	CYT	OM1
S51	LIC11358	Conserved hypothetical protein	UNK	OM	IM	OM1

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
S52	LIC12688	recC:Exodeoxyribonuclease V, gamma subunit	CYT	OM>CYT	CYT	OM1
S53	LIC12670	Adenylate cyclase related protein	CYTM	OM	IM	OM1
S54	LIC10592	OmpA-family protein	UNK (OM>PER)	CYT>OM	CYT	OM1
S55	LIC12233	Fructose-bisphosphate aldolase .	UNK	CYT>PER	CYT	OM
S56	LIC13470	ferredoxin--NADP reductase [Leptospira interrogans serovar Copenhageni str. Fiocruz L1-130].	CYT	CYT>OM	CYT	OM
S57	LIC11227	Conserved hypothetical protein	UNK	EC>OM>PER	PER	OM
S58	LIC10140	Chorismate synthase	CYT	CYT>OM	UNK	OM
S59	LIC11855	D-alanine--D-alanine ligase	CYT	CYT>OM	CYT	OM
S60	LIC10766	Hypothetical lipoprotein	UNK	CYT>OM>PER	CYT	OM
S61	LIC13494	Conserved hypothetical protein	UNK	EC>OM>PER	IM	OM
S62	LIC10759	Thiamine monophosphate kinase	CYT	CYT>OM	CYT	OM
S63	LIC10038	TPR-repeat-containing protein	CYT	CYT>OM>EC	CYT	OM
S64	LIC13086	Conserved hypothetical lipoprotein	UNK	EC>OM	EC	OM
S65	LIC11725	hypothetical protein	UNK	CYT>OM	CYT	OM
S66	LIC12782	2-isopropylmalate synthase	CYT	CYT>OM	CYT	OM
S67	LIC20054	Dinucleotide-binding enzyme	UNK	CYT>OM>EC	CYT	OM
S68	LIC12293	purK:Phosphoribosylaminoimidazole carboxylase, ATPase subunit	CYTM	EC>OM>CYT	EC	OM
S69	LIC12876	Protein-synthesizing GTPase complex, EF-G component	CYT	CYT>OM	CYT	OM
S70	LIC11158	Transcriptional regulator, FUR family	UNK	CYT>OM>PER	CYT	OM
S71	LIC12097	Sensor protein of a two component response regulator	CYTM	CYT>OM	CYT	OM
S72	LIC1_SPN3178	Cysteine sulfinate desulfinase or Cysteine desulfhydrase, part (N-term)	CYT	CYT>OM	CYT	OM

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
S73	LIC12965	hypothetical protein	UNK	EC>OM	IM	OM
S74	LIC11863	UDP-N-acetylmuramate--L-alanine ligase	CYT	CYT>OM	CYT	OM

extracellular (EC), periplasm (PER), inner membrane (IM), cytoplasm (CYT), unknown (UNK), OM by all 3 tools (OM3), by 2 tools (OM2), by 1 tool (OM1), or have multiple location sites with a low score at the outer membrane (OM), LIC number = the predicted coding sequences are referred to according to their genome nomenclature, LIC (*Leptospira interrogans* serovar Copenhageni strain Fiocruz L1–130).

Table 8. Hypothetical protein (OMPs) which found in both of surface biotinylation and surface shaving

Sample	LIC number	Description	PSORTb	CELLO	SOSUI-GramN	Final Prediction
B&S 1	LIC10711	Conserved hypothetical protein	OM	OM	OM	OM3
B&S 2	LIC12048	Hypothetical lipoprotein	OM	OM	OM	OM3
B&S 3	LIC12193	Conserved hypothetical protein	CYT	OM> CYT	OM	OM2
B&S 4	LIC20001	Conserved hypothetical protein	OM	OM	CYT	OM2
B&S 5	LIC10326	Hypothetical protein	UNK	OM>EC	OM	OM2
B&S 6	LIC12669	Hypothetical protein	CYTM	OM	OM	OM2
B&S 7	LIC10578	Conserved hypothetical protein	CYT	OM	OM	OM2
B&S 8	LIC11074	Conserved hypothetical protein	UNK	OM>EC	OM	OM2
B&S 9	LIC13032	Hypothetical protein	UNK	OM	OM	OM2
B&S 10	LIC20190	Conserved hypothetical protein	CYT	OM	OM	OM2
B&S 11	LIC10318	Conserved hypothetical protein	UNK	OM	OM	OM2
B&S 12	LIC11885	Conserved hypothetical lipoprotein	UNK	OM>CYT>PER	PER	OM1
B&S 13	LIC20172	Hypothetical lipoprotein	UNK	OM/ EC	IM	OM1
B&S 14	LIC13314	Conserved hypothetical protein	CYTM	OM	IM	OM1
B&S 15	LIC11009	Hypothetical protein	UNK	OM> CYT> EC	CYT	OM1
B&S 16	LIC12359	Conserved hypothetical protein	UNK	OM>EC>PER	EC	OM1
B&S 17	LIC10314	Conserved hypothetical protein	UNK	OM>EC>CYT	CYT	OM1
B&S 18	LIC11489	Hypothetical protein	UNK	OM	IM	OM1
B&S 19	LIC10486	hypothetical protein	UNK	OM	IM	OM1
B&S 20	LIC10985	hypothetical lipoprotein	CYT	OM	IM	OM1
B&S 21	LIC11935	Conserved hypothetical protein	UNK	EC	OM	OM1
B&S 22	LIC13434	hypothetical lipoprotein	UNK	OM	PER	OM1

B&S 23	LIC12936	Hypothetical protein	CYT	OM	CYT	OM1
B&S 24	LIC11320	Hypothetical protein	UNK	OM	IM	OM1
B&S 25	LIC12446	Hypothetical protein	UNK	OM>EC>CYT	IM	OM1
B&S 26	LIC11553	Conserved hypothetical protein	CYT	OM	IM	OM1
B&S 27	LIC11334	Conserved hypothetical protein	UNK	OM	CYT	OM1
B&S 28	LIC10226	Hypothetical protein	CYT	OM	CYT	OM1
B&S 29	LIC13436	hypothetical protein	UNK	OM>PER>EC	PER	OM1
B&S 30	LIC10337	Conserved hypothetical protein	UNK	OM>PER	CYT	OM1
B&S 31	LIC10547	Hypothetical lipoprotein	UNK	OM	IM	OM1
B&S 32	LIC12805	hypothetical protein	UNK	OM>CYT	IM	OM1
B&S 33	LIC11966	Hypothetical lipoprotein	UNK	CYT>EC	OM	OM1
B&S 34	LIC11224	Conserved hypothetical protein	CYTM	OM	IM	OM1
B&S 35	LIC13089	hypothetical protein	UNK	OM	EC	OM1
B&S 36	LIC12576	hypothetical protein	CYT	OM	IM	OM1
B&S 37	LIC12333	Hypothetical protein	CYTM	OM>IM	IM	OM1
B&S 38	LIC20036	hypothetical protein	UNK	OM	IM	OM1
B&S 39	LIC10231	Hypothetical protein	UNK	OM>EC>CYT	IM	OM1
B&S 40	LIC11982	hypothetical protein	CYT	OM	IM	OM1
B&S 41	LIC10151	Hypothetical protein	CYT	OM>CYT	IM	OM1
B&S 42	LIC12561	hypothetical protein	UNK	PER	OM	OM1
B&S 43	LIC12586	hypothetical protein	CYT	OM	IM	OM1
B&S 44	LIC11612	hypothetical protein	UNK	OM	PER	OM1
B&S 45	LIC10227	Conserved hypothetical protein	CYT	OM>CYT	CYT	OM1
B&S 46	LIC11711	Hypothetical lipoprotein	CYT	OM	CYT	OM1
B&S 47	LIC10815	Conserved hypothetical protein	CYTM	OM>IM	IM	OM1
B&S 48	LIC12325	hypothetical observed protein	CYT	OM	CYT	OM1
B&S 49	LIC11468	Hypothetical protein	CYT	OM	EC	OM1
B&S 50	LIC13363	Hypothetical protein	UNK	OM	EC	OM1
B&S 51	LIC11458	Conserved hypothetical protein	OM	OM	EC	OM1
B&S 52	LIC20019	Hypothetical protein	UNK	OM	CYT	OM1
B&S 53	LIC20030	hypothetical protein	UNK	CYT>OM	OM	OM1
B&S 54	LIC20109	Hypothetical protein	CYTM	CYT	OM	OM1
B&S 55	LIC10810	Hypothetical protein	UNK	OM	IM	OM1

B&S 56	LIC11027	hypothetical lipoprotein	UNK	PER>CYT	OM	OM1
B&S 57	LIC10187	Hypothetical protein	UNK	OM	EC	OM1
B&S 58	LIC10012	hypothetical protein	UNK	OM	CYT	OM1
B&S 59	LIC10175	Conserved hypothetical protein	CYT	CYT/ OM	EC	OM
B&S 60	LIC12075	hypothetical protein	CYT	CYT>OM	PER	OM
B&S 61	LIC20196	Hypothetical protein	UNK	CYT>PER>OM	CYT	OM
B&S 62	LIC11888	hypothetical protein	UNK	CYT>OM	UNK	OM
B&S 63	LIC11884	Conserved hypothetical protein	PER	CYT>OM	CYT	OM
B&S 64	LIC12231	Hypothetical lipoprotein	UNK	IM>OM	PER	OM
B&S 65	LIC11723	hypothetical lipoprotein	UNK	PER>OM>CYT	PER	OM
B&S 66	LIC11173	Conserved hypothetical protein	CYT	CYT>OM	UNK	OM

extracellular (EC), periplasm (PER), inner membrane (IM), cytoplasm (CYT), unknown (UNK), OM by all 3 tools (OM3), by 2 tools (OM2), by 1 tool (OM1), or have multiple location sites with a low score at the outer membrane (OM), LIC number = the predicted coding sequences are referred to according to their genome nomenclature, LIC (*Leptospira interrogans* serovar Copenhageni strain Fiocruz L1–130), Cell surface biotinylation method (B), Cell surface shaving (S)

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Table 9. The 6 of 510 OMPs from surface biotinylation and proteinase K, which was previously shown as a surface-exposed fibronectin-binding protein (94)

Sample	LIC number	Description	PSORTb	CELLO	SoSui GramN	Final Prediction
B&S	LIC11436	LipL45-related protein	OM	OM	IM	OM2
B&S	LIC11051	Leucine-rich repeat containing protein	EC	OM>EC	EC	OM1
S	LIC13066	Conserved hypothetical protein	CYTM	OM	OM	OM2
B&S	LIC12631	Sphingomyelinase C precursor	EC	PER>OM	EC	OM
B&S	LIC10714	Outer membrane receptor for Fe3+-dicitrate/TonB-dependent receptor	OM	OM	OM	OM3
S	LIC10713	Iron-regulated lipoprotein	OM	OM	EC	OM2

extracellular (EC), periplasm (PER), inner membrane (IM), cytoplasm (CYT), unknown (UNK), OM by 2 tools (OM2), by 1 tool (OM1), or have multiple location sites with a low score at the outer membrane (OM), LIC number = the predicted coding sequences are referred to according to their genome nomenclature, LIC (*Leptospira interrogans* serovar Copenhageni strain Fiocruz L1–130), Cell surface biotinylation method (B), Cell surface shaving (S)

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Table 10. The 37 proteins in this study have been confirmation the PSEs in other study.

Locus	Label	Annotation	Function	References
LIC10465	LigA	LigA-like protein	surface adhesin binding to Collagen I/Collagen IV/Laminin/Fibronectin/Tropoelastin	(49, 124)
LIC10973	OmpL1	outer membrane protein	surface adhesin binding to Laminin/Fibronectin	(14, 114, 115)
LIC13050	OmpL47	conserved hypothetical protein	surface adhesin binding to Collagen III/Laminin/Fibronectin/Elastin	(14)
LIC11436	MFn7	conserved hypothetical protein	surface adhesin binding to Fibronectin	(94)
LIC10714	FecA	TonB-dependent receptor	confirmed OMP	(125)
LIC20151	HbpA	TonB-dependent hemin-binding protein	Fe^{3+}-siderophores/hemin receptors	(118)
LIC11051	MFn6	conserved hypothetical protein	surface adhesin binding to Fibronectin	(94)
LIC11436	MFn7	conserved hypothetical protein, LipL45	surface adhesin binding to Fibronectin	(94)
LIC13066	MFn15	Hypothetical protein	surface-exposed OMP	(94)
LIC12238		conserved hypothetical protein	surface adhesin binding to plasminogen	(126-128)
LIC11360		putative lipoprotein	surface adhesin binding to fibrinogen	(128)
LIC12730		conserved hypothetical protein	surface adhesin binding to plasminogen	(129)
LIC10054	MPL36	RlpA-like lipoprotein	surface adhesin binding to Plasminogen	(126)
LIC11954		enolase	surface adhesin binding to plasminogen	(130)
LIC13006	LenC	Endostatin-like protein, hypo	surface adhesin binding to Fibronectin/Laminin	(121)

LIC13060	LipL36	outer membrane lipoprotein	known antigen	(131)
LIC12966	LipL41	Putative lipoprotein	known antigens	(118, 132)
LIC11885	LipL46	Putative lipoprotein, cons hypo	major antigens	(133)
LIC10465	LigA	LigA-like protein	surface adhesin binding to Collagen I/Collagen IV/Laminin/Fibronectin/Tropoelastin	(49, 124)
LIC10464	LigB	LigB-like protein	surface adhesin binding to Collagen I/Collagen IV/Collagen III/Laminin/Fibronectin/Elastin/Tropoelastin/Heparin	(49, 124, 134, 135)
LIC11003	LruA/ LipL71	lipoprotein	immunopathogenic factor	(106)
LIC10713	LruB/MFn14	Lipoprotein, Iron-regulated lipoprotein	immunopathogenic factor	(106)
LIC20172	LruC	lipoprotein	pathogenic factor	(106)
LIC10368	Lsa21	putative lipoprotein, hypo lopoprotein	surface adhesin binding to Collagen IV/Laminin/Fibronectin	(48)
LIC11360	Lsa23	putative lipoprotein, cons.hypo.lopo	surface adhesin binding to Fibrinogen	(136)
LIC12253	Lsa25	putative lipoprotein, hypo	surface adhesin binding to Laminin	(137)
LIC10314	Lsa63	putative lipoprotein, ctP synthase	surface adhesin binding to Collagen IV/Laminin	(126)
LIC10050	Lsa77	OmpA family protein	surface adhesin binding Laminin	(95)
LIC10793	Lp49	surface antigen OrfC lipoprotein	serodiagnostic antigen	(138)
LIC10973	OmpL1	outer membrane protein	surface adhesin binding to Laminin/Fibronectin	(14, 114, 115)
LIC12263	OmpL37	conserved hypothetical protein	surface adhesin binding to Laminin/Fibronectin/Elastin	(14)

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LIC13050	OmpL47	conserved hypothetical protein	surface adhesin binding to Collagen III/Laminin/Fibronectin/Elastin	(14)
LIC10592	Omp52	OmpA family protein	interaction of host cells	(139)
LIC11612	MFn1	Hypothetical protein	surface adhesin binding to Fibronectin	(94)
LIC10714	MFn2	TonB-dependent receptor	surface adhesin binding to Fibronectin	(94)
LIC12631	MFn4/Sph2	Sphingomyelinase 2	Mg(++)-dependent hemolysin	(94, 140)
LIC11345		TonB-dependent outer membrane receptor	OMP	(141)

LIC number = the predicted coding sequences are referred to according to their genome nomenclature, LIC (*Leptospira interrogans* serovar Copenhageni strain Fiocruz L1–130)

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Poster presentation; Surface biotinylation of leptospiral outer membrane proteins. The 44th National Graduate Research Conference “Graduate Research Driven Thailand 4.0”. At U-Place Hotel, Ubon Ratchathani University, Thailand, October 19-20, 2017: Poster presentation; Surface Proteomics of Leptospira interrogans Serovar Pomona to Search for Novel Vaccine Candidates. 2018 Global Vaccine and Immunization Research Forum. At Shangri La Hotel Bangkok, Thailand (GVIRF) March 20-22, 2018

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