PROTROMIC AND FUNCTIONAL STUDY OF *ACINETOBACTER ABUMANNII*

OUTER MEMBRANE VESICLES

APPROVED BY SUPERVISING COMMITTEE:

_____
Tao Wei, Ph.D., Chair

_____
Shatha Dallo, Ph.D.

_____
M. Neal Guentzel, Ph.D.

Accepted: _____
Dean, Graduate School

DEDICATION

This thesis is dedicated to my dear parent, Mr. Feng-Chih Tsai and Mrs. Shiu-Chen Peng thank you for keeping support through these years. Thank you to my dear wife Yi-Ling Huang and my dear brother Chung-Yu Tsai. I will never make it without your support and accompany in the United States.

PROTROMIC AND FUNCTIONAL STUDY OF *ACINETOBACTER ABUMANNII* OUTER MEMBRANE VESICLES

by

AN-YU TSAI, B.S.

THESIS
Presented to the Graduate Faculty of
The University of Texas at San Antonio
In Partial Fulfillment
Of the Requirements
For the Degree of

MASTER OF SCIENCE IN BIOLOGY

THE UNIVERSITY OF TEXAS AT SAN ANTONIO
College of Sciences
Department of Biology
August 2010

ACKNOWLEDGEMENTS

First of all, I would like to say thank you to my chair and advisor Dr. Tao Wei for directing my thesis work. He provided me many inspirational ideas and thinking in this project and also trained my presentation skills. In the one and a half years ,I learned much from him which will benefit my future. I am also grateful to my supervisor and committee member Dr. Shatha F. Dallo who helped me from the beginning of my thesis through my thesis defense. She is very knowledgeable and experienced in experimental skills and always came up with many good ideas.

I would also like to say thank you to my committee member Dr. Neal Guentzel for his time and support in my thesis work.

Special thank you go to Dr. Rodrigo.Esparzamunoz devoted his time to help me to operate the transmission electron microscope. Special thank you go also to Dr. William Haskins who helped me with proteomic analysis and to Dr. Jieh Juen Yu who kindly helped me with cytotoxicity assay.

I would like to thanks my lab mates Navya, Reshma, Koundinya, and Peter who helped and supported my thesis work. Special thanks go to my parents, my brother, my wife, and friends at UTSA who accompanied and supported me in my thesis work.

August 2010

PROTROMIC AND FUNCTIONAL STUDY OF *ACINETOBACTER ABUMANNII* OUTER MEMBRANE VESICLES

An-yu Tsai, M.S.
The University of Texas at San Antonio, 2010

Supervising Professor: Tao Wei, Ph.D.

Acinetobacter baumannii, a gram-negative aerobic bacterium, is one of the most spread bacteria which can be easily isolated from water, soil, and hospital facilities. It may cause various types of diseases such as respiratory tract infection, urinary tract infection, nosocomial pneumonia and bacteremia. It was noticed that *A. baumanii* was existed in the blood stream of military people deployed in Iraq and Afghanistan who suffered with serious injury. The treatment for the infection of *A. baumannii* is limited because it resists to resist nearly 90% of antibiotics. Therefore, to better treat the infection of *A. baumannii*,the mechanism of infection and virulence factors involved in pathogenecity needed to be studied. Similar to other gram-negative bacteria, outer membrane vesicles (OMVs) secreted by *A. baumannii* consisted of virulence factors. In my study, the *A. baumannii* OMVs were isolated and observed under transmission electron microscopy. The proteomic analysis indicated that *A. baumannii* OMVs consisted of various virulence factors such as OmpA. To understand the function of the *A. baumannii* OMVs, OMVs were incubated with macrophage cells. Results showed the macrophage cells were lysed when incubated with OMVs compared to cells in the control group which were intact. The quantification of cytotoxicity caused by OMVs showed that the mortality rate reached 80% when 0.02 ug/ml OMVs were added.

In my study, the OMVs were isolated and observed. The virulence factors were identified by proteomic analysis and OMVs are toxic to macrophage cells in vitro.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

A. baumannii can be pathogenic

A. baumannii, a Gram-negative, obligate aerobic and nonmotile bacterial species, has emerged as a nosocomial pathogen. The distribution of *A. baumannii* is quite ubiquitous and it can be generally isolated from soil, water, sewage, and also in healthcare settings (Baumann et al., 1968; Juni, 1978). Importantly, it is often associated with a wide spectrum of infectious diseases ranging from nosocomial, community-acquired infections to those acquired following war ((CDC), 2004; Davis et al., 2005; Davis, 2005; Scott et al., 2004; Zapor., 2005) or natural disasters(Falagas and Kopterides, 2006; Joly-Guillou, 2005). It causes mild-to-severe illness of various types, including post-surgical urinary tract and respiratory tract infections, nosocomial pneumonia, and bacteremia, some of which can be fatal with mortality rates as high as 75% (Chastre, 2000). Among military personnel who were deployed to Iraq and Afghanistan and suffered traumatic injuries, multidrug-resistant *Acinetobacter* has been reported to cause deep wound infections, osteomyelitis, respiratory infections, and bacteremia((CDC), 2004; Davis et al., 2005; Hawley et al., 2007; Hujer et al., 2006; Tien et al., 2007).

Acinetobacter infections impose severe health problems to service persons with battle wounds.

Recent studies showed that the majority of the battle casualties from ongoing campaigns in Iraq and Afghanistan had wounds that were infected with multidrug-resistant *A. baumannii*((CDC),

2004; Davis et al., 2005; Tien et al., 2007; Zapor., 2005). It has also been reported that bloodstream infections of *A. baumannii* occurred in patients who stayed at military medical facilities where service personnel injured in Afghanistan and the Iraq/Kuwait region were treated (Scott et al., 2004). Furthermore, individuals who have experienced war trauma are more likely to develop skin and soft-tissue infection (SSTI) with *A. baumannii*(Sebeny et al., 2008). In this retrospective study of inpatients admitted to a naval hospital ship, 14% of the inpatients were identified with *A. baumannii*-associated SSTI. Patients with SSTI, exhibited cellulitis with overlying vesicles and progressed to necrotizing infection with hemorrhagic and non-hemorrhagic bullae when untreated. Significantly, *A. baumannii* isolated from the SSTI patients were multidrug resistant (Sebeny et al., 2008). Clearly, this study demonstrates that *A. baumannii*-associated SSTI is emerging as a threat to patients who have experienced war trauma. The connection of *A. baumannii* with SSTI has been reinforced by a study conducted in *A. baumannii* SSTI rat models (Russo et al., 2008).

Options for treating such infections are severely limited.

Treatment of *Acinetobacter* infections has become increasingly difficult. First, the bacterium is capable of developing extensive antimicrobial resistance (Rice, 2006; Urban, 2003). *A. baumannii-calcoaceticus* complex is resistant to nearly all current antibiotics, presenting a formidable challenge for treatment of the infected war wounds (Murray and Hospenthal). Second, bacterial cells can attach to medical devices and the surface of host cells (Lee et al., 2006) and form biofilms(Vidal, 1996) which are multi-cellular communities of bacterial cells(Costerton, 1978) that contribute to drug resistance(Stewart, 2001) and chronic infections(Costerton et al.,

1999). Antimicrobial wound dressing has been used historically to prevent and treat wound infections. However, the primary topical application of antiseptic agents has raised a concern about the creation and promotion of bacterial resistance, superinfections, and nosocomial infections (Lozier, 1993). The genes and proteins involved in bacterial resistance to silver products have been identified (Silver et al., 2006). Antimicrobial resistance is on the rise, due to both antimicrobial pressure from the administration of antibiotics and health care–related transmission of drug-resistant strains. Multidrug-resistant *Acinetobacter* infections most frequently afflict war trauma patients, with an exceedingly high crude mortality rate. Such multidrug-resistance severely limits treatment options (Maragakis and Perl, 2008) which have led to increasing concerns about the potential for large scale epidemics from this bacterium. To address these problems, we propose to develop *novel prophylactic and therapeutic strategies against A. baumannii infections*.

Biofilms further complicate treatment.

As multi-drug resistance presents challenges to treatment, biofilms are believed to aggravate it when bacteria build the multi-cellular communities (Fig. 1)(Costerton, 1978) that contribute to drug resistance(Stewart, 2001) and chronic infections in humans(Costerton et al., 1999). *A. baumannii* is known to form biofilms in static cultures (Tomaras et al., 2003; Vidal, 1996). While the dynamic biofilm development of *A. baumannii* remains to be explored, studies with *Pseudomonas aeruginosa* have provided useful information. Biofilm formation, in general, must start with attachment of free-moving (planktonic) cells to an abiotic or biotic surface (Stage 1 in Fig. 1). When touching a surface, the planktonic cells initiate reversible attachment within a few

minutes upon living in a liquid environment (Sauer et al., 2002). Then, the cells commit to irreversible attachment in 2 h (Stage 2). The attached (sessile) cells become organized for early biofilm maturation in 1 day (Stages 3-4) and into sophisticated structures in 6 days (Stage 4). Finally, some sessile cells disperse in 9-12 days (Stage 5) (Sauer et al., 2002). Biofilm development is apparently a dynamic process (Costerton et al., 1995; O'Toole et al., 2000; Sauer et al., 2002; Stoodley et al., 2002) and the phase between irreversible attachment and early maturation is an opportune time window to investigate biofilm physiology and the lifestyle of *A. baumannii* and to control biofilm formation.

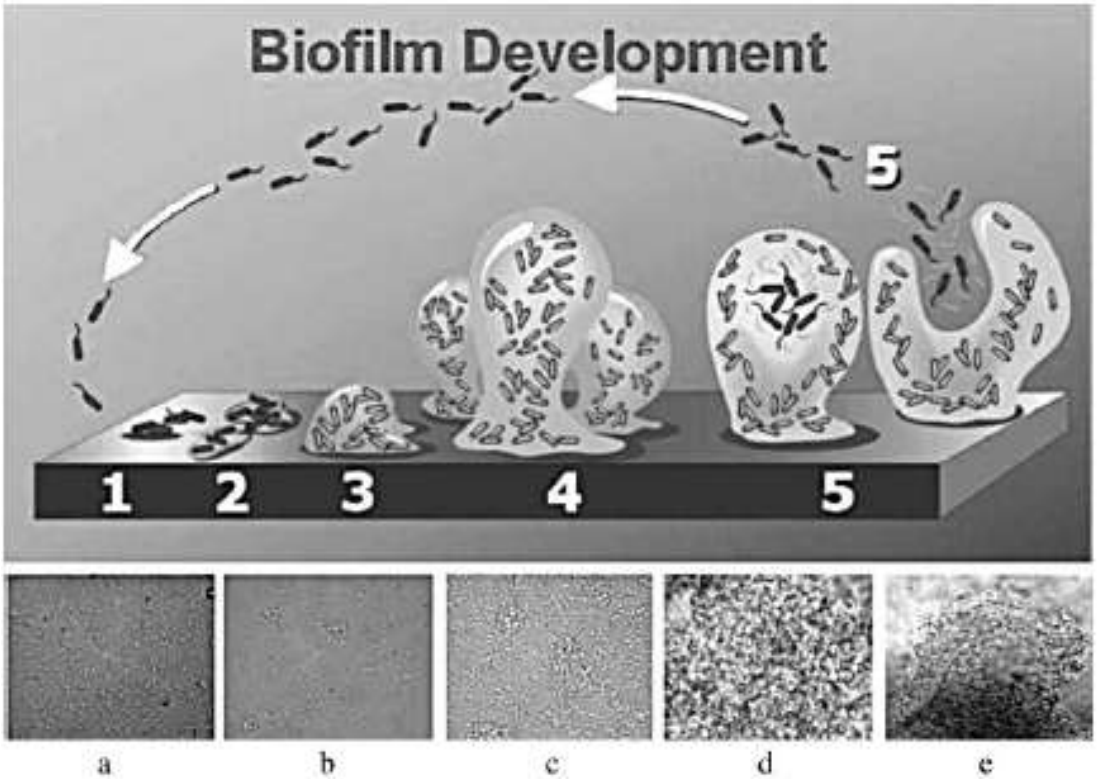

Fig. 1 Diagram showing the development of a biofilm as a five-stage process. Stage 1: Initial attachment of cells to the surface. Stage 2: production of extracellular polymeric substances resulting in more firm adherence, "irreversible" attachment. Stage 3: early development of biofilm architecture. Stage 4: maturation of biofilm architecture. Stage 5: dispersion of single cells from the biofilm. The bottom *panels* (a-e) show each of the five stages of development represented by a photomicrograph of *P. aeruginosa* when grown under continuous-flow conditions on a glass substratum (Sauer et al., 2002; Stoodley et al.,)

Understanding molecular mechanisms of biofilm formation may provide important information for developing strategies to prevent infection.

Some protein targets for *A. baumannii* biofilm formation have already been identified. Beta-lactamase production appears related to cell adhesion (Sechi, 2004). Mutants with impaired pili-like structures are deficient in attachment and biofilm formation. The genes encoding the pili display high similarity to the genes found in *P. aeruginosa*(Tomaras et al., 2003). Adherence of *A. baumannii* to human bronchial epithelial cells has been also reported (Lee et al., 2006). A homologue to the staphylococcal biofilm-associated protein (Bap) has been identified in a bloodstream isolate of *A. baumannii(Loehfelm et al., 2008)* and is likely to be bacterial cell surface adhesins. A mutant with a disrupted coding sequence of *bap* is unable to sustain biofilm thickness and volume (Loehfelm et al., 2008). The observations suggest involvement of Bap in intercellular adhesion within the mature biofilm. Additionally, the biofilm-forming clinical strain of *A. baumannii* exhibits accumulation of some outer membrane proteins (Omps) (Siroy et al., 2006). During the transition from planktonic to biofilm growth, bacteria experience considerable changes (Davey and O'toole, 2000). Information about the transition has been mostly acquired from studies with *E. coli* and *P. aeruginosa*, while the understanding with *A. baumannii* is incomplete. Yet, this knowledge may guide us in investigations of *A. baumannii.* Distinct protein expression patterns were found at different stages of biofilm development in *P. aeruginosa*, as shown in Fig. 1(Southey-Pillig et al., 2005). Distinguished patterns were identified in two functional groups: one including metabolic and housekeeping proteins and another consisting of virulence, antibiotic resistance, and quorum-sensing-related proteins. Interestingly, the latter were expressed in a biofilm stage-specific fashion, suggesting that antibiotic resistance and

virulence may contribute to the biofilm development (Southey-Pillig et al., 2005). These biofilm-specific proteins can be classified into the following functional categories: (1) general metabolism proteins, (2) sugar and amino acid transporters, and (3) regulatory proteins. Taken together, these results demonstrate distinct proteomic profiles of biofilm andplanktonic cells, suggesting that these proteins can be targets for intervention.

Outer membrane vesicles are virulence factor.

As Gram-negative bacteria during all phases of growth produce outer membrane vesicles (OMVs) (Beveridge, 1999; Mayrand, 1989), a type of virulence factor and stress response, OMVs may play a role in the pathogenesis. OMVs seem to be generated from living cells. OMVs appear as spheroid particles with a heterogeneous size ranging from 10 to 300 nm in diameter as revealed by electron microscopy. OMVs appear to bud from OM bulges with subsequent fission (Beveridge, 1999; Chatterjee and Das, 1967; Grenier and Mayrand, 1987; Kadurugamuwa and Beveridge, 1995; Kolling and Matthews, 1999; Li et al., 1996; Mayrand, 1989; Patrick et al., 1996). In fact, it may be the temporary and localized reductions in the density of Omp-peptidoglycan and Omp-peptidoglycan-inner membrane protein associations within the envelope structure invoked by cell growth and division that lead to releasing of OMVs(Deatherage et al., 2009). Released from growing cultures of *Pseudomonas aeruginosa* and *Escherichia coli* cells, OMVs constitute approximately 1% of the OM materials in the cultures (Bauman and Kuehn, 2006; Gankema et al., 1980; WENSINK and WITHOLT, 1981). OMVs do not appear to result from cell death, for OMV production does not concur with cell lysis, but OMVs package newly synthesized proteins (McBroom et al., 2006; Mug-Opstelten, 1978; Zhou et al., 1998).

Understandably, reflecting the OM composition, OMVs constitute OM proteins (Omps), lipopolysacharides (LPS), glycerophospholipids, and enclosed periplasmic components (Gankema et al., 1980; Hoekstra et al., 1976; Horstman and Kuehn, 2000; Kadurugamuwa and Beveridge, 1995). *A. baumannii* OMVs contain potent cytotoxic molecules and outer membrane protein A (OmpA) (Kwon et al., 2009).

These OMV-associated proteins may mediate OMV adherence and invasion to host cells. The OMV-associated aminopeptidase of *P. aeruginosa* was found to enhance the OMV attachment to both primary and cultured human lung epithelial cells (Bauman and Kuehn, 2009). The OMVs that carry proteases and toxins from *E. coli*, *Shigella*, *Actinobacillus*, and *Borrelia* strains were found to interact with bacterial and mammalian cells (Gankema et al., 1980; Kadurugamuwa and Beveridge, 1998; Kato et al., 2002; Saunders et al., 1999; Shoberg and Thomas, 1993). After adhesion, OMVs can enter into host cells. The entry may exploit binding of the OMV-associated proteins to the host receptor following the receptor-mediated endocytic pathway; for instance, OMVs of enterotoxigenic *E. coli* (ETEC) are internalized into epithelial cells through lipid raft-mediated endocytosis governed by G_{M1} on the host cells (Kesty et al., 2004). Additionally, other major OMV-associated proteins may mediate entry. OmpA of *E. coli* K1, a pathogen for neonatal meningitis, helps OMV invasion through interacting with surface receptor Ecgp on brain microvascular endothelial cells (Prasadarao et al., 1996; Prasadarao, 2002). These observations have led to a premise that bacterial OMVs are associated with active virulence factors and serve as natural vehicles transporting virulence factors and other materials directly into host cells (Ellis and Kuehn, 2010). In line with this thought, *P. aeruginosa* OMVs deliver multiple enzymes and virulence factors, such as beta-lactamase, alkaline phosphatase and

hemolytic phospholipase, into the host cytoplasm via fusion of OMV with lipid rafts in the host plasma membrane (Bomberger et al., 2009)

Many systems are implicated in bacteria pathogenesis. Since the pathogenesis of *Acinetobacter baumannii* is poorly understood, we investigated the role of outer membrane vesicles in biofilm formation hence adherence to host cells. We propose that outer membrane vesicles (OMVs) play an important role in forming biofilm and infect other organism. This hypothesis is well-grounded. *A. baumannii* is found to produce OMVs (Kwon et al., 2009) (our data 2010). OMVs are associated with active virulence factors and serve as natural vehicles transporting virulence factors and other materials directly into host cells (Ellis and Kuehn, 2010). For example, *Pseudomonas aeruginosa* OMVs deliver multiple virulence factors, such as beta-lactamase, alkaline phosphatase and hemolytic phospholipase, into the host cytoplasm via fusion of OMV with lipid rafts in the host plasma membrane (Bomberger et al., 2009). Similarly, *A. baumannii* OMVs contain potent cytotoxic molecules and outer membrane protein A (OmpA) as described in (Kwon et al., 2009) and in our data. in fact, *Helicobacter pylori* OMVs contribute to biofilm formation (Yonezawa et al., 2009). .The proteomics of outer membrane vesicles isolated from clinical A. baumannii was analysed by MS/MS and partial proteins conveyed OMVs were identified such as OmpA-a ligand binding related protein, Chaperonin GroEl- a protein folding related protein and many putative outer membrane proteins (Kwon et al., 2009). In our hypothesis, we propose that the outer membrane vesicles produced by *A. baumannii* might have similar effect on biofilm formation as other gram negative bacteria. Many proteins are involved in biofilm formation. A biosymthetic cytoplasmic protein elongation factor Tu has been implicated in attachment of bacteria to host cells. Since EF-Tu is present in A. baumannii outer membrane vesicle (present data) its role in biofilm formation was investigated.

MATERIALS AND METHODS

Bacterium strain and growth curve

The bacteria strain used in the experiment was *Acinetobacter baumannii (ATCC19606)*. The outer membrane vesicles were found to produce at the maximal levels when the bacteria reached the early stationary phase. So the cultures at such a phase were harvested for OMV isolation. Briefly, the *A. baumannii* was inoculated in two 15-ml tubes, which contained 3-ml LB-broth. One tube was placed into a shaking incubator at 37℃, 200 rpm; the other was placed into an incubator at 37℃. A 100-ul volume was taken from both cultures and the optical density was measured at the wavelength of 590-nm every two hours. The growth curve was plotted for the cultures growing under shaking and non-shaking conditions.

Isolation of outer membrane vesicles

The bacteria were inoculated into 3-ml LB in a 15-ml tube. The culture was grown at 37℃ Cincubator for 16 hours. A 100-ul volume from the bacterial culture was taken and mixed with 1.9-ml water and the optical density at the wavelength of 590-nm was measured. Once the optical density was measured, the appropriate original bacterial culture was added into 40ml fresh LB-Broth in a 150-ml flask. The flask was placed into an incubator for 16 hours at 37℃. The cell pellet was collected from the culture by centrifugation at 4℃, 10,000 rpm for 10 minutes. The supernatant was carefully removed and the cell pellet was kept in -20℃ refrigeration for the extraction of outer membrane protein in later steps. Then, the supernatant

was filtered through a 0.22-um syringe filter. The filtered supernatant was ultra-centrifuged at 33,000rpm and 4℃ for 12 hours. After ultra-centrifugation, the supernatant was discarded and the outer membrane vesicles pellet was re-suspended with PBS and stored at 4℃.

Isolation of outer membrane protein

The bacteria pellet collected from the previous experiment was re-suspended in 20-ml of water and vortexes well. The bacteria cells were sonicated for 30 seconds on ice and cooled down for 10 seconds and these steps were repeated eight times. The Sodium lauryl sarcosinate was added into the sample after sonication to a final concentration of 2%. The sample was incubated at room temperature for 60 minutes and centrifugation at 4 ℃, 10,000 rpm for 10 minutes. The supernatant was collected into clean ultra-centrifugation tubes and the outer membrane proteins were pellet at 18,000 rpm, 4℃. After discarding the supernatant, the outer membrane protein pellet was re-suspended in PBS and stored at -20℃.

Transmission electron microscope

A 10-ul volume of the OMV sample was carefully dipped on the lacey carbon film on 300-mesh copper grids. The grids were kept at room temperature for 30 minutes, and then the standing volume of the sample was carefully removed from grids by filter paper. The grids were negative stained by 1% uranyl acetate for 20 seconds and extra staining buffer was removed by using filter paper carefully. The grids were left to air dry for 1 hour after negative staining. Secondly, to examine if the OMVs were secreted from bacteria, we took a 10-ul volume of the bacterial cell suspension from the 16-hrs growing culture. The same method was used as above.

SDS-PAGE analysis

The 10% polyacrylamide gel electrophoresis was performed to analyze the proteins extracted from the outer membrane vesicles. A 20-ul volume of each sample was mixed with 6x SDS dye and heated at 95℃for 5 minutes to denature the proteins. The samples were carefully loaded into each lane, and electrophoresis was run at 80 volts until the marker reached the bottom of the gel. The gel was carefully removed and stained in coomassie blue.

Proteomics sample preparation

The sample of outer membrane vesicles was loaded into 10% SDS-PAGE, and electrophoresis was performed. Each band of gel was cut into tiny pieces (1×1mm) and placed into 1.5 ml eppendrof tubes. A100ul-volume of 25mM NH4HCO3/ 50% CAN was added to cover the gel slice. The mixture was vortexed for 10 minutes. The supernatant was discarded. These steps were repeated for a couple of times until the blue color totally disappeared. A 100 ul volume of 10-mM DTT in 25-mM NH4HCO3 was added to each tube followed by vortexing and spinning briefly. The reagents were allowed to react with gel pieces at 56℃ for 60 minutes, and the supernatant was discarded. A volume of 25 ul of 55 mM iodoacetamide was added to the gel pieces, and the mixture was vortexed and centrifuged briefly. The reaction was incubated in the dark for 30 minutes at room temperature. The gels were dehydrated with 100 ul (or enough to cover the gels) of 25-mM NH4HCO3 / 50% ACN and vortexed and centrifuged for 5 minutes until the gel pieces became colorless. Trypsin was added to just barely cover the gel pieces, and the mixture was incubated at 37℃ overnight. The gel mixture was centrifuged briefly and the

supernatant was collected into a new tube. To the gel pieces, 30-ul (enough to cover) of 50% ACN/ 0.1% formic acid was added, followed by vortexing for 20-30 minutes and spinning briefly. The supernatant was harvested into the same tube. The collected samples were then placed upon vacuum and dried until approximately 10 ul remained in the tubes.

OMV proteomics

The OMV-associated proteins were identified by capillary LC/MS/MS analysis. Briefly, the proteins were separated by SDS-PAGE. The protein-containing gel slices were subjected to *in vitro* proteolysis by trypsin and capillary LC/MS/MS at the Proteomics Core to identify proteins in each spot. Capillary LC/MS/MS was performed with a linear ion trap tandem mass spectrometer (LTQ-XLS, ThermoFisher) where the top 7 eluting ions were fragmented by CID and ETD in the same run. MS/MS spectra of these ions were searched against the IPI protein databases with probability-based database searching algorithms (SEQUEST, ThermoFisher and MASCOT, MatrixScience) and electronically validated with *de novo* sequencing and homology-based database searching algorithms (PEAKS, Bioinformatics Solutions) for protein identification.

Biofilm formation inhibition

A .baumannii was inoculated in 5-ml LB broth and grown at 37 ℃ for 18 hours. Optical density at 595-nm wavelength was measured. The culture was diluted to a final concentration of 1x10^5 in 1-ml LB broth and antibody (1: 250) was added (for the control group was only bacteria and

LB-broth). The culture was incubated in cool room (4 ℃) for 1-hour with rocking and incubated at 37 ℃ overnight. The supernatant was discarded and washed with PBS 3 times. The biofilm was stained with 0.1% crystal violate for 10-minutes and washed with PBS 3 times. Purple color biofilm can be observed upon light.

Macrophage cell killing and MTT assays

The outer membrane vesicles were mixed with DMEM medium and the mixture was added into 96-wells plate which has been seeded with murine macrophage cells J774. The plate was incubated at 37 ℃ with 5% CO2 for 1-hour and 24 hours and observations were performed.

The commercial kit (cyto 96 non-radioactive cytotoxicity assay from promega) was used to measure OMV cytotoxicity to macrophage. The concept of this kit is to measure a stable cytosolic enzyme lactate dehydrogenase (LDH) which is released when cells are lysed. The released LDH then reacts with NAD and protons are donated to NAD from LDH. The reducing NADH then reacts with MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] and formazan (red) forms. By measuring the optical density of formazan at the wavelength of 490-nm, the cytotoxcity can be determined.

The MTT assay was carried out as follows. First, the old medium was removed from the macrophage wells on the 96-well plate. The macrophage used in this experiment was J774 murine macrophage cell, which was termed the **target** cell. The fresh medium that had been mixed with outer membrane vesicles was added into each of macrophage wells. The outer

membrane vesicles are termed **effecters**. The volume of each well was brought to 100 ul. The **experiment** wells 2 contain medium, target cells and effecters. Outlined below are the control reactions (Promega.co,). 1.

- **Effecter Spontaneous LDH Release:** Add effecters which are the outer membrane vesicles at each concentration used in the experimental wells. The effecter wells only contain medium and OMVs, to assess spontaneous LDH release from the effectors. The final volume must be the same as in the experimental wells 2.
- **Target Cell Spontaneous LDH Release:** The target wells only contain target cells (macrophage) and medium, to evaluate spontaneous LDH release from the macrophage. The total volume must be the same as experiment wells. 3.
- **Target Cell Maximum LDH Release:** These wells contain medium and target cells and the Lysis Solution to assess the maximal levels of LDH released from macrophage. A 10-µl volume of the Lysis Solution (10X) per 100µl of culture medium was added, and the mixture was incubated for 45 minutes before the supernatant was harvested. 5.
- **Volume Correction Control:** These wells contain only medium and lysis solution; the purpose is to correct the volume background. 6.
- **Culture Medium Background:** These controls were used to correct phenol red and LDH activity that may be present in serum-containing culture medium.

The 96-well plate then was incubated at 37 ℃ with 5% CO2 for 4 hours. After incubation, the supernatant (40-50 ul) from each well (except for the Maximal LDH release control) was transferred into a well in a new 96-well plate and mixed with the same volume of substrate (tetrazolium salt which must avoid from light). The 96-well plate was incubated in the dark for

30-minutes at the room temperature and the reaction was terminated by adding the stop solution. The optical density was measured at 490-nm, and the formula was applied to obtain the cytotoxicity percentage.

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous}}{\text{Target Maximum} - \text{Target Spontaneous}} \times 100$$

(Promega, Co)

RESULTS

Determining growth conditions for OMV production

We determined the growth conditions at which outer membrane vesicles were produced at the high levels for *A. baumannii.* We isolated OMVs from the 8-hour shaking culture and the 12-hour non-shaking culture. We compared the yields of OMVs and found that the yield of outer membrane vesicles harvested from stagnant culture was higher than the shaking culture. The phenomenon was observed under the transmission electron microscope in the later experiment.

Transmission electron microscope of isolated outer membrane vesicles

The strain of *A. baumannii* was grown in LB-Broth at 37 ℃ under the stationary incubation for 16 hours and subcultured for 12 hours to reach the final concentration of 1x10^6 cells per ml. The cell pellet was collected by centrifugation. The supernatant collected from centrifugation was filtered through the 0.22-um filter. The outer membrane vesicles were harvested after ultra-centrifugation at 33,000rpm, 4 ℃ for 12 hours. The supernatant was discarded. As shown in figure. 2, the pellets attached on the bottom of the ultra-centrifuge tubes were expected to be outer membrane vesicles. For transmission electron microscopy, the OMV pellets were resuspended in PBS and placed on the grids used for the transmission electron microscope and negatively stained with uranyl acetate. The picture shows the bacterial cell that was collected from the 12-hour sub-culture **(Fig. 2A)**. The big rod shape with a black boundary is *A. baumannii* which was supported by carbon filament on the grid. Round shape circles with a

black boundary were observed in the upper right corner; they are the OMVs that appeared to bud out from the bacterial cell. Another OMV shown in the top of the bacterial cell also appeared to bud out. It shows the OMVs in the sample harvested by ultra-centrifugation from cell free supernatant **(Fig. 2B)**. The size range of the OMV is 70-150 nm.

Analysis of OMV proteins

The proteins of OMV were analyzed with the SDS- PAGE **(Fig 3.).** The lanes 1 and 2 show the proteins from supernatant collected by centrifugation without filtering through 0.22-um filter. A band with the size at 41-kDa was observed and likely to be EF-Tu based on the size. This band did not seem to majorly come from the cells, since it still existed in the supernatant after filtration through 0.22-um syringe filters (lanes 3, 4) when the bacterial cells were completely removed. It is highly likely that these proteins were carried by outer membrane vesicles because OMVs can pass through the filter. No observable protein bands were observed in the supernatant after ultra-centrifugation (Lanes 5 and 6). Lanes 7 and 8 demonstrate the proteins in OMVs that were collected from the supernatant after ultra-centrifugation. This figure also revealed that the size of the most abundant proteins are around 41-kDa, which is the size of EF-Tu; therefore, it is likely that outer membrane vesicles may carry EF-Tu.

OMV proteomic analysis

To examine the protein contents of *A. baumanni* OMVs*i*, we performed proteomic analysis. The proteins carried by OMVs was seperated by SDS-PAGE, and the whole protein-containing lanes

were cut and treated for proteomic analysis. To bettter understand the relationship between outer membrane and OMVs, we extracted outer membrane proteins for proteomic analysis. A totally of 247 proteins were identified from OMVs and classfied into 5 catogories **(Table 1)**. Metabolic proteins are the most abundant. The proteins with unknown functions are substantial in number. The number of the cytoplasmic proteins is higher than that of the outermembrane proteins. The periplasmic proteins are the least in number. **(Table 1.)**.

A. baumannii OMVs package various proteins. The OMV-associated outer membrane protein of the highest score is CsuA/B functing in pili formation. CsuA/B is also the most abundant among all the groups (emPAI 4.27). The second are the porin protiens, OmpA and a Dcap-like protein. Chaperonin GroEL is conspicuous among the OMV-associated cytoplasmic proteins. The Omp precursor for major intrinsic multiple antibiotic resistance efflux is among the OMV-associated periplasmic proteins. The low abundant proteins consist of more than 100 OMV-associated proteins; those involved in virulence include exodeoxyribonuclease phospholipase, protease, and elongation factor Tu (EF-Tu) **(Table 2.)** and in Omps (**Table 3.)**. Furthermore, there are many enzymes and chaperons associated with both outer membrane and OMVs.

Biofilm formation inhibition

The proteomics data suggests that OMVs contain proteins that are involved in biofilm formation. EF-Tu is one of them as it relates to bacterial adhesion (Granato et al., 2004).
Since EF-Tu is involved in bacterial adhesion, we tested whether blocking EF-Tu by the antibodies could disrupt biofilm formation by following the method as described previously

(Shahrooei et al., 2009) with modifications. An overnight culture of *A. baumannii* was diluted with LB and incubated with IgGs at concentrations of 1 to 5 µg/ml from pre-immune serum and *A. baumannii* EF-Tu antiserum at 4 ℃ for 2 hours. Biofilms formed on polystyrene surfaces after overnight incubation at 37 ℃. Biofilm mass decreased in the presence of the antiserum at the increased concentrations (Fig. 4). The reasons that the antiserum did not eradicate biofilms *in vitro* may stem in part from the findings that extracellular proteins are a portion of the biofilm matrix, which additionally contains polysaccharides and nucleic acids (Stoodley et al., 2002). Another may be that EF-Tu together with other proteins such as adhesins (Loehfelm et al., 2008) contributes to biofilm formation, a multi-factor process.

Macrophage cell killing assay

To understand if OMVs can cause cytotoxic effects on host cells, we carried out the macrophage killing assay. The macrophage cells were seeded in a 96-well plate and incubated with OMVs. The first observation was done after incubation for 1 hour for morphology changes. It shows the macrophage cells without OMV treatment. Each cell has a clear boundary **(Fig 5A)**. The picture shows the macrophage cells that were incubated with fresh outer membrane vesicle at 37^0C for 1 hour **(Fig 5B)**. More than 30 % of cells were lysed as observed under a light microscope. Significant alterations in the morphology of the macrophage include cell shrinkage and cell detachment. This data suggests that OMVs are cytotoxic to macrophages and leads us to hypothesize that the OMVs that produced by *A. baumannii* are a virulence factor against host cells.

To investigate whether OMVs cause cytotoxic effects on host cells, we carried out the two macrophage killing assays as described previously (Venketaraman et al., 2008) with modifications. The macrophage was seeded in a 96-well plate and incubated with OMV. First, trypan blue was added to stain the dead cells. After 0.5, 1, 2, 4, and 24 hours, macrophage morphology was examined, and dead cells were counted. OMVs took effect in one hour, and the pathologic effects appeared OMV-concentration-dependent (Fig. 4). Second, the cytotoxic effects were further quantified in the MTT assay (Fig. 5). This assay measures the level of the released cytosolic lactate dehydrogenase from the lysed macrophage. The cytotoxicity is defined as the percentage of OMV-inflicted LDH released from detergent-lysed maximal release. As shown in Fig. 5, the OMV-inflicted macrophage toxicity reached approximately 80% and appeared concentration-dependent. These data suggest that OMVs are cytotoxic to macrophage, leading to a hypothesis that *A. baumannii* produces OMVs as a virulent factor against host cells.

DISCUSSION

The aim of the study is to characterize *A. baumannii* OMVs biochemically and functionally. At first, we tried to find the best condition to isolate the maximum amount of outer membrane vesicles. The result suggested that the stationary culture after a 12-hour incubation produced more OMVs. The Transmission electron microscopy showed OMVs were released from *A. baumannii* (**Fig 2A**) and confirmed OMVs existed in the sample (**Fig 2B**). Then, SDS-PAGE was used to analyze the OMV-associated proteins, and show that specific proteins were carried in OMVs (**Fig 3.** lanes 7 and 8). The 32-kDa band may contain OmpA. The band at 41-kDa may include EF-Tu. To confirm if OMVs contain these proteins, the SDS-PAGE based proteomic analysis was performed.

The proteomic data suggested that OMVs package more than two hundred of proteins. They include 1) outer membrane proteins such as OmpA, CsuA/B, 2) metabolic proteins such as succinyl-CoA synthetase and glutamine synthetase, 3) periplamic proteins such as efflux proteins, and 4) transporter proteins **(Table 2.)**. We also compared the proteins that are associated with OMVs and the outer membrane. The result indicated that 44% of proteins are from the outer membrane. The other important discovery was that EF-Tu existed in both the outer membrane and OMVs.

We carried out some functional analyses of the OMV-associated proteins. First, since EF-Tu had been identified in outer membrane and OMVs **(Table 3)**, we would like to study if EF-Tu is involved in biofilm formation. The result indicated that biofilm was reduced in bacteria culture

incubated with the EF-Tu antibodies **(Fig 4.)**. This suggested to us that EF-Tu may have a certain connection to biofilm formation. Second, the macrophage cell killing assay was performed. The result shows that OMVs are cytotoxic to macrophage **(Fig 5)**.

In summary, *A. baumannii* OMVs was isolated and confirmed by TEM. The proteins associated with the outer membrane and OMVs were analyzed by SDS-PAGE and proteomics. The result revealed that many functional proteins were contained in OMVs, including EF-Tu. *Lactobacillus johnsonii* EF-Tu was found to adhere to the host cell surface (Granato et al., 2004). Our result suggested involvement of EF-Tu in biofilm formation. OMVs were found to cause cytotoxicity to macrophage. This suggested that OMVs are a virulent factor.

FUTURE STUDY

We performed the in vitro experiments. The future study will aim to investigate the mechanism of EF-Tu and OMVs in vivo as to how they contribute to biofilm formation, how OMVs mediate bacterial attachment and invasion of host cells.

Table 1. The summary of proteins data and number by category

No.	Catogory	Number of proteins
1	Outer membrane	17
2	Cytoplasmic	30
3	Metabolic	123
4	Periplasma	10
5	Unknown function	67
Total number		247

Table 2. Partial proteins identified from outer membrane vesicles extracted from *Acinetobacter baumannii* by using MS/MS analysis

No.	Accession No.	Protein	Function	MW (kDa)	Score
Outer membrane proteins					
1	gi|169795445	protein CsuA/B; putative secreted protein related to type I pili	Pili formation relative	18694	1885
2	gi|126642784	putative protein (DcaP-like)	Porin like protein	44818	522
3	gi|126642864	outer membrane protein A	Porin	37342	240
4	gi|37933551	CsuE	Pili formation relative	36554	139
5	gi|169632203	putative outer membrane protein	Porin like protein	47921	116
6	gi|169632592	outer membrane protein (AdeC-like)	Drug resistance relative	52910	98
Cytoplasmic proteins					
7	gi|169632653	chaperonin GroEL	Folding of protein relative	56886	490
8	gi|169632628	succinyl-CoA synthetase subunit alpha	ADP forming relative	30674	272
9	gi|126643040	malate dehydrogenase	Tri-carboxylic acid cycle	32400	148
10	gi|73918433	60 kDa chaperonin	Unknown	15201	158
11	gi|184158870	glutamine synthetase	nitrogen metabolism	52166	132
12	gi|126643149	Glutamate dehydrogenase (NAD(P)+)oxidoreductase protein	Metabolism	40128	131
13	gi|146299602	chaperonin GroEL	Folding of protein relative	57080	114
14	gi|86143882	chaperonin, 60 kDa	Folding of protein relative	57383	114
15	gi|88799071	chaperonin GroEL	Folding of protein relative	57081	109
16	gi|119503116	chaperonin, 60 kDa	Folding of protein relative	47837	109
17	gi|162286746	elongation factor Tu	Protein folding, adherence	41246	52

Table 2. Continued.

No.	Accession No.	Protein	Function	MW (kDa)	Score
Metabolic proteins					
18	gi|50085915	dihydrolipoamide succinyltransferase, component of 2-oxoglutarate dehydrogenase	tricarboxylic acid cycle relative	43454	127
19	gi|169634382	ketol-acid reductoisomerase	Metabolism	36830	122
20	gi|126642749	succinyl-CoA synthetase beta chain	tricarboxylic acid cycle relative	36757	118
21	gi|126642544	Aspartate aminotransferase A	Metabolism	40652	117
22	gi|50083469	F0F1 ATP synthase subunit beta	ATP proton motive force interconversion	50280	108
23	gi|184157664	Acetyl-CoA acetyltransferase	Metabolism	43124	108
24	gi|87199130	L-glutamine synthetase	Metabolism	51875	108
25	gi|21717403	acyl-CoA dehydrogenase A	Metabolism	68279	107
26	gi|126643334	dihydrolipoamide S-acetyltransferase E2 component of the pyruvate dehydrogenase complex	Metabolism	64889	104
27	gi|126641512	glutaminase-asparaginase	Metabolism	31037	101
28	gi|184157824	L-asparaginase/ Glu-tRNAGln amidotransferase subunit D	Metabolism	37834	101
29	gi|162286757	F0F1 ATP synthase subunit alpha	ATP proton motive force	55363	100
30	gi|126640175	putative UTP-glucose-1-phosphate uridylyltransferase	Metabolism	29032	100
31	gi|169795993	putative acetyl-CoA acetyltransferase (acetoacetyl-CoA thiolase)	Metabolism	40774	99
32	gi|126640439	polynucleotide phosphorylase/polyadenylase	RNA degradation relative	71168	98
Periplasmic proteins					
33	gi|50085972	major intrinsic multiple antibiotic resistance efflux outer membrane protein precursor	drug resistance relative	52747	91
34	gi|116749745	extracellular ligand-binding receptor	putative ligand binding site	48564	61
35	gi|15963975	putative cytochrome C transmembrane protein	small molecule metabolism; energy transfer	25566	41
36	gi|46202245	COG2998: ABC-type tungstate transport system, permease	ABC-type transport system	28338	41

Table 3. The list of outer membrane proteins

No.	Accession No.	Protein	Function	MW (kDa)	Score
1	gi|126642784	putative protein (DcaP-like)	Porin like protein	44818	1889
2	gi|126642864	outer membrane protein A	Porin, ligand binding relative	37342	1737
3	gi|169795445	protein CsuA/B; putative secreted protein related to type I pili	Pili formation relative	18694	750
4	gi|260554505	CsuA/B [Acinetobacter baumannii ATCC 19606]	Pili formation relative	18693	734
5	gi|70671520	outer membrane protein A	Porin, ligand binding relative	37951	429
6	gi|126643304	putative outer membrane protein	Porin like protein	24671	416
7	gi|169797605	putative outer membrane protein	Porin like protein	47833	349
8	gi|169633880	hypothetical protein ABSDF2360	Unknown function	41181	333
9	gi|72535027	putative outer membrane protein	Porin like protein	25581	312
10	gi|50085972	major intrinsic multiple antibiotic resistance efflux outer membrane protein precursor	drug resistance relative	52747	130
11	gi|134293863	chaperonin GroEL	Folding of protein relative	56775	110
12	gi|162286746	elongation factor Tu	Protein folding, adherence	41246	100
13	gi|184158870	glutamine synthetase	Cytoplasmic enzyme	52166	86
14	gi|149369942	chaperonin GroEL	Folding of protein relative	57131	83
15	gi|146299602	chaperonin GroEL	Folding of protein relative	57080	83
16	gi|51894033	chaperonin GroEL	Folding of protein relative	57862	82
17	gi|73918433	60 kDa chaperonin	Folding of protein relative	15201	81
18	gi|21229998	chaperonin GroEL	Folding of protein relative	57149	80
19	gi|50084093	30S ribosomal protein S7	Translation relative	17614	79
20	gi|126642195	oxidoreductase	Rossmann-fold NAD(P)(+)-binding proteins	23477	77
21	gi|10440998	catechol 1,2-dioxygenase	aromatic dioxygenase family	33527	77
22	gi|58040332	chaperonin GroEL	Folding of protein relative	57456	74

Table 3. Continued.

No.	Accession No.	Protein	Function	MW (kDa)	Score
22	gi|58040332	chaperonin GroEL	Folding of protein relative	57456	74
23	gi|162286757	F0F1 ATP synthase subunit alpha	ATP proton motive force	55363	72
24	gi|183598067	hypothetical protein PROSTU_01426	Unknown function	57413	73
25	gi|50083469	F0F1 ATP synthase subunit beta	ATP proton motive force interconversion	50280	71
26	gi|86143882	chaperonin, 60 kDa	Folding of protein relative	57383	69
27	gi|126642544	aspartate aminotransferase A	(PLP)-dependent aspartate aminotransferase superfamily (fold I).	40652	66
28	gi|1785912	glutamine synthetase	Metabolism of nitrogen	51436	64
29	gi|27364337	glutamine synthetase	Metabolism of nitrogen	51827	64

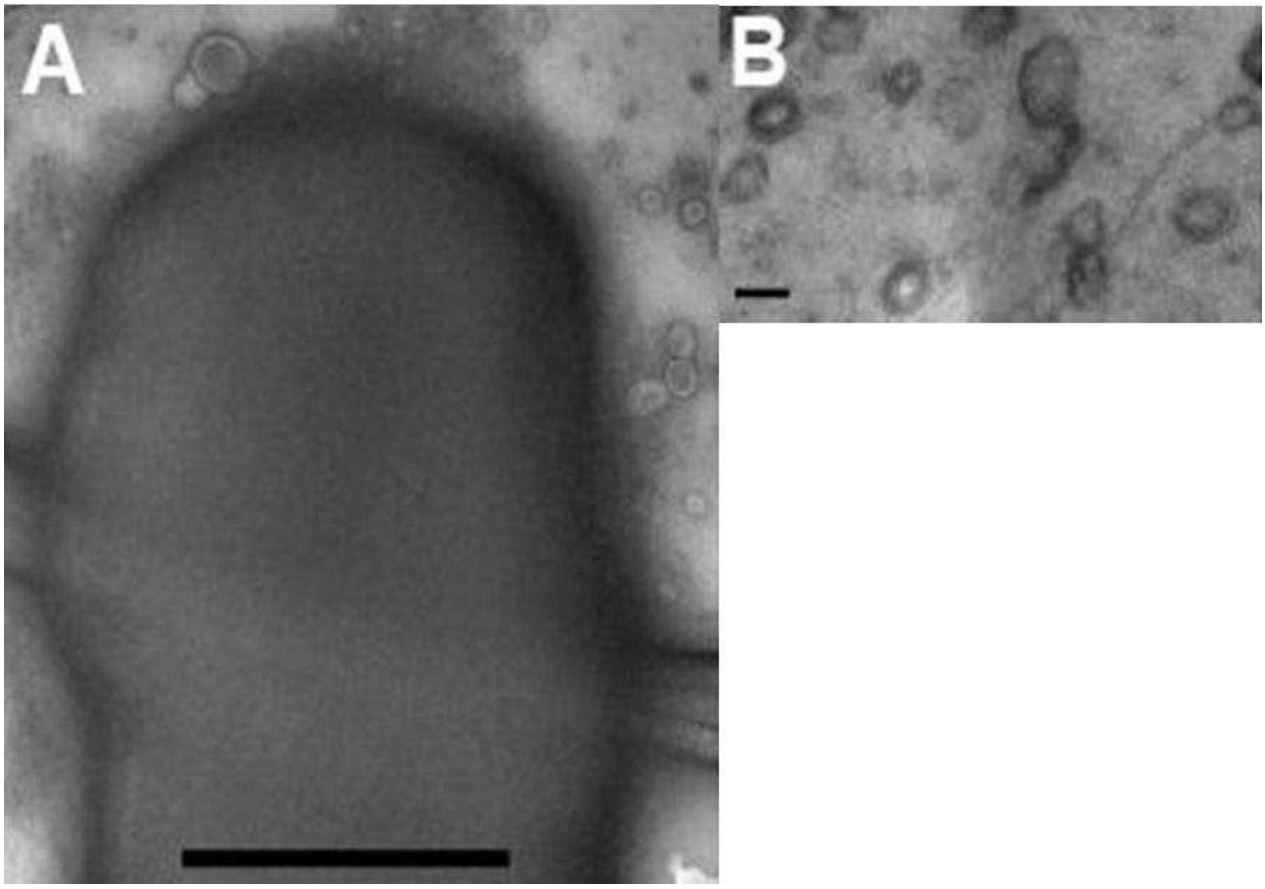
A
B

Fig 2. Transmission electron microscopy of *A. baumannii* OMVs. (A) OMV building out from a cell. Bar 500nm. (B) OMVs in the preparation. Bar 100nm

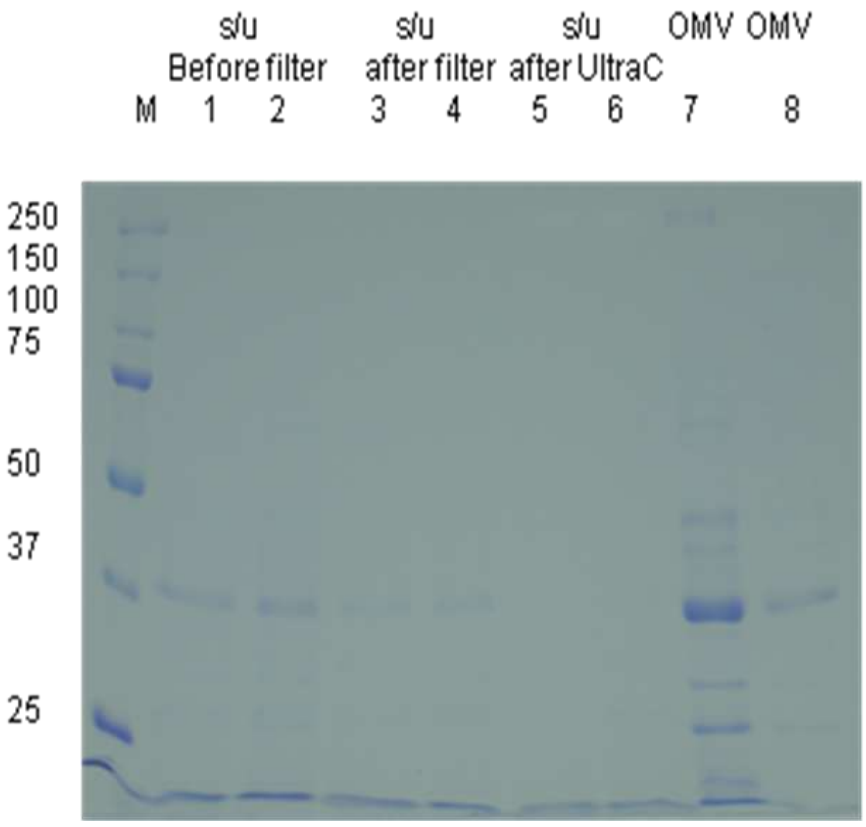
s/u
Before filter
s/u
after filter
s/u
after UltraC
OMV
OMV
M
1
2
3
4
5
6
7
8
250
150
100
75
50
37
25

Fig 3. The outer membrane proteins isolated from *A. baumannii in 10% SDS-PAGE*. Lanes 1 and 2 are the proteins in the supernatant before filtration. Lanes 3 and 4 are the proteins in the supernatant after filtration. Lanes 5and 6 are the results in the supernatant after ultra-centrifugation. Lanes 7and 8 are the proteins from OMV collected by ultra- centrifugation. The star mark at 41-KDa which reveals the possible EF-Tu exists in the OMV.

Fig 4. Biofilm formation inhibition. 1. Untreated bacteria culture, biofilm were twice than the other tubes(control), 2. Bacteria culture incubated with EF-Tu antibody.

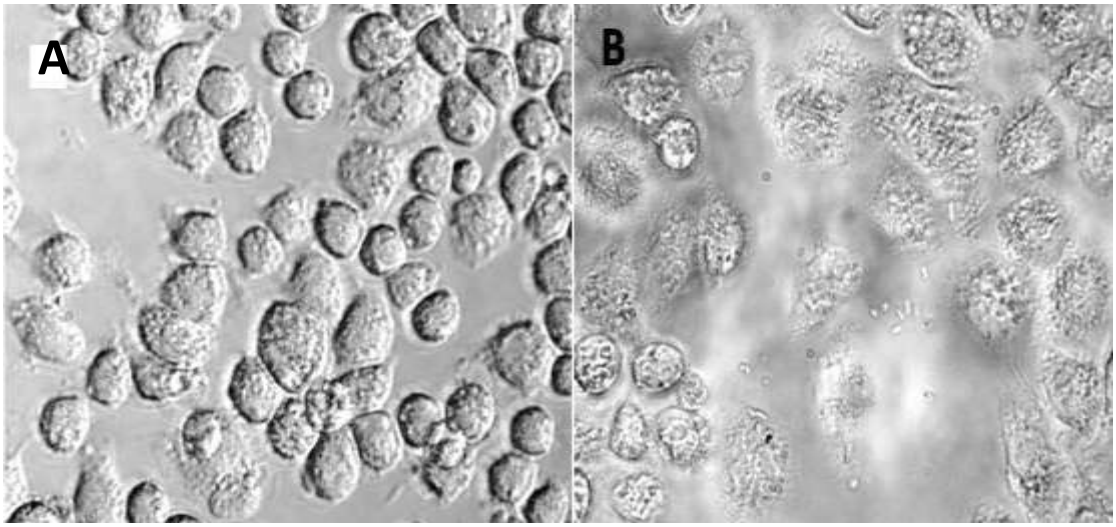
A
B

Fig 5. Macrophage cells killing assay observed by optical microscopy (A) Untreated macrophage cells incubated for 1 hour (control) **(B)** Macrophage cells incubated with outer membrane vesicles for 1 hour.

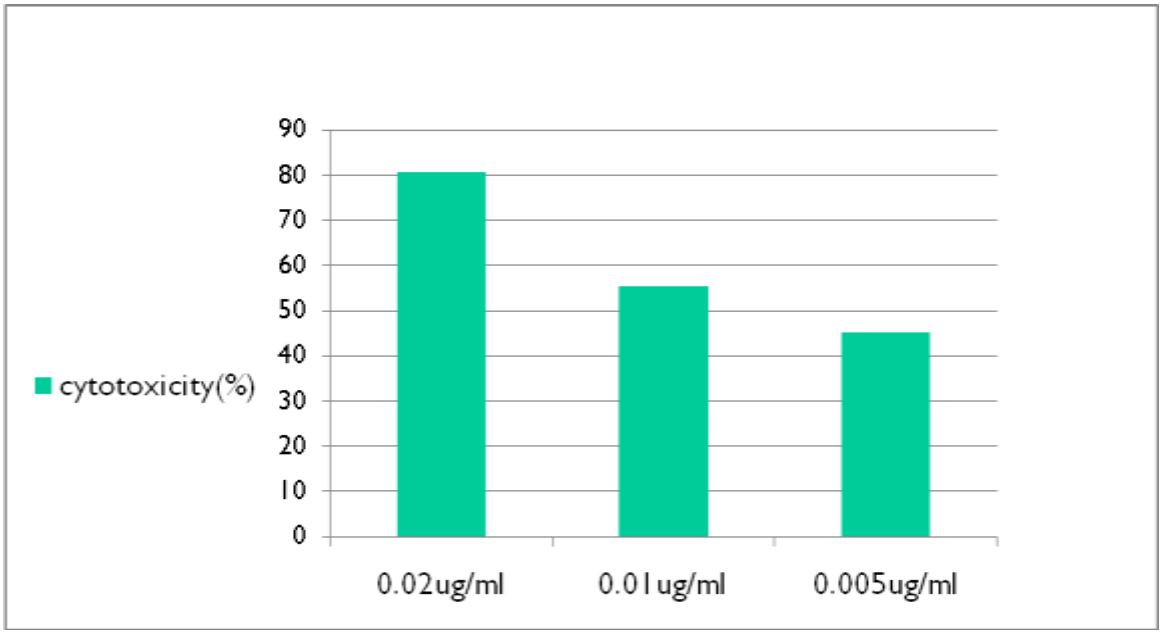
90
80
70
60
50
40
30
20
10
0
cytotoxicity(%)
0.02ug/ml
0.01ug/ml
0.005ug/ml

Fig 6. The cytotoxicity percentage caused by different volume of *A. baumannii* outer membrane vesicles.

BIBLIOGRAPHY

Alaniz, R. C., Deatherage, B. L., Lara, J. C., and Cookson, B. T. (2007). Membrane Vesicles Are Immunogenic Facsimiles of Salmonella typhimurium That Potently Activate Dendritic Cells, Prime B and T Cell Responses, and Stimulate Protective Immunity In Vivo. J Immunol *179*, 7692-7701.

Allesen-Holm, M., Barken, K. B., Yang, L., Klausen, M., Webb, J. S., Kjelleberg, S., Molin, S., Givskov, M., and Tolker-Nielsen, T. (2006). A characterization of DNA release in Pseudomonas aeruginosa cultures and biofilms. Molecular Microbiology *59*, 1114-1128.

Baensch, M., Frank, R., and Kohl, J. (1998). Conservation of the amino-terminal epitope of elongation factor Tu in eubacteria and archaea. Microbiology *144*, 2241-2246.

Bauman, S., and Kuehn, M. (2009). Pseudomonas aeruginosa vesicles associate with and are internalized by human lung epithelial cells. BMC Microbiology *9*, 26.

Bauman, S. J., and Kuehn, M. J. (2006). Purification of outer membrane vesicles from Pseudomonas aeruginosa and their activation of an IL-8 response. Microbes and Infection *8*, 2400-2408.

Baumann, P., Doudoroff, M., and Stanier, R. Y. (1968). Study of the Moraxella Group I. Genus Moraxella and the Neisseria catarrhalis Group. J Bacteriol *95*, 58-73.

Beveridge, T. J. (1999). Structures of Gram-Negative Cell Walls and Their Derived Membrane Vesicles. J Bacteriol *181*, 4725-4733.

Bomberger, J. M., MacEachran, D. P., Coutermarsh, B. A., Ye, S., O'Toole, G. A., and Stanton, B. A. (2009). Long-Distance Delivery of Bacterial Virulence Factors by *Pseudomonas aeruginosa* Outer Membrane Vesicles. PLoS Pathog *5*, e1000382.

Caldwell, H. D., Kromhout, J and Schachter, J. (1981). Purification and partial characterization of the major outer membrane protein of Chlamydia trachomatis. Infect Immunity *31*, 1161-1176.

(CDC), C. f. D. C. a. P. (2004). Acinetobacter baumannii infections among patients at military medical facilities treating injured U.S. service members, 2002-2004. MMWR Morb Mortal Wkly Rep *53*, 1063-1066.

Chastre, J., Trouillet, J.L. (2000). Problem pathogens (*Pseudomonas aeruginosa* and *Acinetobacter*). Semin Respir Infect *15*, 287-298.

Chatterjee, S. N., and Das, J. (1967). Electron Microscopic Observations on the Excretion of Cell-wall Material by Vibrio cholerae. J Gen Microbiol *49*, 1-11.

Christiane E. Bode, Werner Brabetz, and Helmut Brade (1998). Cloning and characterization of 3-deoxy- D-manno-oct-2-ulosonic acid (Kdo) transferase genes (kdtA from Acinetobacter baumannii and Acinetobacter haemolyticus. European Journal of Biochemistry *254*, 404-412.

Costerton, J. W., Geesey, G.G., Cheng, K.J. (1978). How bacteria stick. Sci Am *238*, 86-95.

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., and Lappin-Scott, H. M. (1995). Microbial biofilms. Annual Review of Microbiology *49*, 711-745.

Costerton, J. W., Stewart, P. S., and Greenberg, E. P. (1999). Bacterial Biofilms: A Common Cause of Persistent Infections. Science *284*, 1318-1322.

Dallo, F., S., Denno, J., Hong, S, and Weitao, T (2010). Adhesion of Acinetobacter baumannii to extracellular proteins detected by a live cell-protein binding assay. Ethnicity & Disease, *20*, 7-11.

Dallo, S. F., Kannan, T. R., Blaylock, M. W., and Baseman, J. B. (2002). Elongation factor Tu and E1 beta subunit of pyruvate dehydrogenase complex act as fibronectin binding proteins in Mycoplasma pneumoniae. Molecular Microbiology *46*, 1041-1051.

Davey, M. E., and O'toole, G. A. (2000). Microbial Biofilms: from Ecology to Molecular Genetics. Microbiol Mol Biol Rev *64*, 847-867.

Davis, K. A., Moran, K. A., McAllister, C. K., and Gray, P. J. (2005). Multidrug-resistant Acinetobacter extremity infections in soldiers. Emerging Infectious Diseases *11*, 1218-1224.

Davis, K. A., Moran, K.A., McAllister, C.K., Gray, P.J. (2005). Multidrug-resistant Acinetobacter extremity infections in soldiers. Emerg Infect Dis *11*, 1218-1224.

Deatherage, B. L., Lara, J. C., Bergsbaken, T., Barrett, S. L. R., Lara, S., and Cookson, B. T. (2009). Biogenesis of bacterial membrane vesicles. Molecular Microbiology *72*, 1395-1407.

Ellis, T. N., and Kuehn, M. J. (2010). Virulence and Immunomodulatory Roles of Bacterial Outer Membrane Vesicles

10.1128/MMBR.00031-09. Microbiol Mol Biol Rev *74*, 81-94.

Falagas, M. E., and Kopterides, P. (2006). Risk factors for the isolation of multi-drug-resistant Acinetobacter baumannii and Pseudomonas aeruginosa: a systematic review of the literature. Journal of Hospital Infection *64*, 7-15.

Gankema, H., Wensink, J., Guinee, P. A. M., Jansen, W. H., and Witholt, B. (1980). Some Characteristics of the Outer Membrane Material Released by Growing Enterotoxigenic Escherichia coli. Infect Immun *29*, 704-713.

Grenier, D., and Mayrand, D. (1987). Functional characterization of extracellular vesicles produced by Bacteroides gingivalis. Infect Immun *55*, 111-117.

Hawley, J. S., Murray, C. K., Griffith, M. E., McElmeel, M. L., Fulcher, L. C., Hospenthal, D. R., and Jorgensen, J. H. (2007). Susceptibility of Acinetobacter Strains Isolated from Deployed U.S. Military Personnel. Antimicrob Agents Chemother *51*, 376-378.

Hoekstra, D., van der Laan, J. W., de Leij, L., and Witholt, B. (1976). Release of outer membrane fragments from normally growing Escherichia coli. Biochimica et Biophysica Acta (BBA) - Biomembranes *455*, 889-899.

Horstman, A. L., and Kuehn, M. J. (2000). Enterotoxigenic Escherichia coli Secretes Active Heat-labile Enterotoxin via Outer Membrane Vesicles. Journal of Biological Chemistry *275*, 12489-12496.

Hujer, K. M., Hujer, A. M., Hulten, E. A., Bajaksouzian, S., Adams, J. M., Donskey, C. J., Ecker, D. J., Massire, C., Eshoo, M. W., Sampath, R., *et al.* (2006). Analysis of Antibiotic Resistance Genes in Multidrug-Resistant Acinetobacter sp. Isolates from Military and Civilian Patients Treated at the Walter Reed Army Medical Center. Antimicrob Agents Chemother *50*, 4114-4123.

Ibrahim, A., Gerner-Smidt, P., Liesack, W. (1997). Phylogenetic relationship of the twenty-one DNA groups of the genus Acinetobacter as revealed by 16S ribosomal DNA sequence analysis. Int J Syst Bacteriol *47*, 837-841.

Joly-Guillou, M.-L. (2005). Clinical impact and pathogenicity of Acinetobacter. Clinical Microbiology and Infection *11*, 868-873.

Jonak, J. (2007). Bacterial elongation factors EF-Tu, their mutants, chimeric forms, and domains: Isolation and purification. Journal of Chromatography B Analytical Tools for Proteomics *849*, 141-153.

Juni, E. (1978). Genetics and Physiology of Acinetobacter. Annual Review of Microbiology *32*, 349-371.

Kadurugamuwa, J., and Beveridge, T. (1995). Virulence factors are released from Pseudomonas aeruginosa in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. J Bacteriol *177*, 3998-4008.

Kadurugamuwa, J. L., and Beveridge, T. J. (1998). Delivery of the Non-Membrane-Permeative Antibiotic Gentamicin into Mammalian Cells by Using Shigella flexneri Membrane Vesicles. Antimicrob Agents Chemother *42*, 1476-1483.

Kato, S., Kowashi, Y., and Demuth, D. R. (2002). Outer membrane-like vesicles secreted by Actinobacillus actinomycetemcomitans are enriched in leukotoxin. Microbial Pathogenesis *32*, 1-13.

Kesty, N. C., Mason, K. M., Reedy, M., Miller, S. E., and Kuehn, M. J. (2004). Enterotoxigenic Escherichia coli vesicles target toxin delivery into mammalian cells. *23*, 4538-4549.

Kolberg, J., Hammerschmidt, S., Frank, R., Jon, J., anderov・ H., and Aase, A. (2008). The surface-associated elongation factor Tu is concealed for antibody binding on viable pneumococci and meningococci. FEMS Immunology & Medical Microbiology 53, *2*22-230.

Kolling, G. L., and Matthews, K. R. (1999). Export of Virulence Genes and Shiga Toxin by Membrane Vesicles of Escherichia coli O157:H7. Appl Environ Microbiol 65, *1*843-1848.

Kwon, S.-O., Gho, Y. S., Lee, J. C., and Kim, S. I. (2009). Proteome analysis of outer membrane vesicles from a clinical *Acinetobacter baumannii* isolate. FEMS Microbiology Letters 29*7, 1*50-156.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 22*7, 6*80-685.

Lee, J. C., Koerten, H., van den Broek, P., Beekhuizen, H., Wolterbeek, R., van den Barselaar, M., van der Reijden, T., van der Meer, J., van de Gevel, J., and Dijkshoorn, L. (2006). Adherence of Acinetobacter baumannii strains to human bronchial epithelial cells. Research in Microbiology 15*7, 3*60-366.

Li, W., Guentzel, M. N., Seshu, J., Zhong, G., Murthy, A. K., and Arulanandam, B. P. (2007). Induction of Cross-Serovar Protection against Genital Chlamydial Infection by a Targeted Multisubunit Vaccination Approach. Clin Vaccine Immunol 14, *1*537-1544.

Li, W., Murthy, A. K., Guentzel, M. N., Seshu, J., Forsthuber, T. G., Zhong, G., and Arulanandam, B. P. (2008). Antigen-Specific CD4+ T Cells Produce Sufficient IFN-{gamma} to Mediate Robust Protective Immunity against Genital Chlamydia muridarum Infection. J Immunol 18*0, 3*375-3382.

Li, Z., Clarke, A., and Beveridge, T. (1996). A major autolysin of Pseudomonas aeruginosa: subcellular distribution, potential role in cell growth and division and secretion in surface membrane vesicles. J Bacteriol 17*8, 2*479-2488.

Loehfelm, T. W., Luke, N. R., and Campagnari, A. A. (2008). Identification and Characterization of an Acinetobacter baumannii Biofilm-Associated Protein. J Bacteriol 19*0, 1*036-1044.

Lozier, S. M. (1993). Topical wound therapy (Saunders, Philadelphia).

Maragakis, L. L., and Perl, T. M. (2008). Antimicrobial Resistance: Acinetobacter baumannii: Epidemiology, Antimicrobial Resistance, and Treatment Options. Clinical Infectious Diseases 46, *1*254-1263.

Mayrand, D., Grenier, D. (1989). Biological activities of outer membrane vesicles. Can J Microbiol 35.

*M*cBroom, A. J., Johnson, A. P., Vemulapalli, S., and Kuehn, M. J. (2006). Outer Membrane Vesicle Production by Escherichia coli Is Independent of Membrane Instability. J Bacteriol 18*8, 5*385-5392.

Mug-Opstelten, D., Witholt, B. (1978). Preferential release of new outer membrane fragments by exponentially growing Escherichia coli. Biochim Biophys Acta 50*8, 2*87-295.

Murray, C. K., and Hospenthal, D. R. Treatment of multidrug resistant Acinetobacter. [Review] [35 refs]. Current Opinion in Infectious Diseases 18(6):502-6, 2005 Dec.

Murthy, A. K., Cong, Y., Murphey, C., Guentzel, M. N., Forsthuber, T. G., Zhong, G., and Arulanandam, B. P. (2006). Chlamydial Protease-Like Activity Factor Induces Protective Immunity against Genital Chlamydial Infection in Transgenic Mice That Express the Human HLA-DR4 Allele. Infect Immun 74, *6*722-6729.

O'Neill, E., Pozzi, C., Houston, P., Humphreys, H., Robinson, D. A., Loughman, A., Foster, T. J., and O'Gara, J. P. (2008). A Novel Staphylococcus aureus Biofilm Phenotype Mediated by the Fibronectin-Binding Proteins, FnBPA and FnBPB. J Bacteriol 19*0*, *3*835-3850.

O'Toole, G., Kaplan, H. B., and Kolter, R. (2000). Biofilm formation as microbial development. Annual Review of Microbiology 54, *4*9-79.

Otto, K., Norbeck, J., Larsson, T., Karlsson, K.-A., and Hermansson, M. (2001). Adhesion of Type 1-Fimbriated Escherichia coli to Abiotic Surfaces Leads to Altered Composition of Outer Membrane Proteins. J Bacteriol 18*3*, *2*445-2453.

Patrick, S., McKenna, J. P., O Hagan, S., and Dermott, E. (1996). A comparison of the haemagglutinating and enzymic activities ofBacteroides fragiliswhole cells and outer membrane vesicles. Microbial Pathogenesis 20, *1*91-202.

Prasadarao, N., Wass, C., Weiser, J., Stins, M., Huang, S., and Kim, K. (1996). Outer membrane protein A of Escherichia coli contributes to invasion of brain microvascular endothelial cells. Infect Immun 64, *1*46-153.

Prasadarao, N. V. (2002). Identification of Escherichia coli Outer Membrane Protein A Receptor on Human Brain Microvascular Endothelial Cells. Infect Immun 70, *4*556-4563.

Rice, L. B. (2006). Challenges in identifying new antimicrobial agents effective for treating infections with Acinetobacter baumannii and Pseudomonas aeruginosa. Clin Infect Dis 43, *S*100-105.

Russo, T. A., Beanan, J. M., Olson, R., MacDonald, U., Luke, N. R., Gill, S. R., and Campagnari, A. A. (2008). Rat Pneumonia and Soft-Tissue Infection Models for the Study of Acinetobacter baumannii Biology. Infect Immun 76, *3*577-3586.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (2001). Molecular Cloning: A Laboratory Manual. 2nd Ed.,. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY,, 655 • 77.

Sanderova, H., and Jonak, J. (2005). Opposite roles of domains 2+3 of Escherichia coli EF-Tu and Bacillus stearothermophilus EF-Tu in the regulation of EF-Tu GTPase activity. Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics 175*2*, *11*-17.

Sauer, K., Camper, A. K., Ehrlich, G. D., Costerton, J. W., and Davies, D. G. (2002). Pseudomonas aeruginosa Displays Multiple Phenotypes during Development as a Biofilm. J Bacteriol 184, *11*40-1154.

Saunders, N. B., Shoemaker, D. R., Brandt, B. L., Moran, E. E., Larsen, T., and Zollinger, W. D. (1999). Immunogenicity of Intranasally Administered Meningococcal Native Outer Membrane Vesicles in Mice. Infect Immun 67, *11*3-119.

Scaronanderova, H., Hulkova, Marta., Malon, Petr., Kepkova, Marketa ., Jonak, Jiri. (2004). Thermostability of multidomain proteins: Elongation factors EF-Tu from Escherichia coli and Bacillus stearothermophilus and their chimeric forms. Protein Science 13, *89*-99.

Schooling, S. R., and Beveridge, T. J. (2006). Membrane Vesicles: an Overlooked Component of the Matrices of Biofilms. J Bacteriol 188, *59*45-5957.

Scott, P. T., K Petersen, J Fishbain, DW Craft, AJ Ewell, K Moran, DC Hack, GA Deye, S Riddell, G Christopher, et *al. (20*04). Acinetobacter baumannii infections among patients at military medical facilities treating injured U.S. service members, 2002-2004. MMWR Morb Mortal Wkly Rep 53, *10*63-1066.

Sebeny, P. J., Riddle, M. S., and Petersen, K. (2008). Acinetobacter baumannii Skin and Soft-Tissue Infection Associated with War Trauma. Clinical Infectious Diseases 47, *44*4-449.

Sechi, L. A., Karadenizli, A., Deriu, A., Zanetti, S., Kolayli, F., Balikci, E., Vahaboglu, H. (2004). PER-1 type beta-lactamase production in Acinetobacter baumannii is related to cell adhesion. Med Sci Monit 10, *BR*180-184.

Shahrooei, M., Hira, V., Stijlemans, B., Merckx, R., Hermans, P. W. M., and Van Eldere, J. (2009). Inhibition of Staphylococcus epidermidis Biofilm Formation by Rabbit Polyclonal Antibodies against the SesC Protein. Infect Immun 77, *36*70-3678.

Shanks, R. M. Q., Meehl, M. A., Brothers, K. M., Martinez, R. M., Donegan, N. P., Graber, M. L., Cheung, A. L., and O'Toole, G. A. (2008). Genetic Evidence for an Alternative Citrate-Dependent Biofilm Formation Pathway in Staphylococcus aureus That Is Dependent on Fibronectin Binding Proteins and the GraRS Two-Component Regulatory System. Infect Immun 76, *24*69-2477.

Shoberg, R. J., and Thomas, D. D. (1993). Specific adherence of Borrelia burgdorferi extracellular vesicles to human endothelial cells in culture. Infect Immun 61, *38*92-3900.

Silver, S., Phung, L. T., and Silver, G. (2006). Silver as biocides in burn and wound dressings and bacterial resistance to silver compounds. Journal of Industrial Microbiology and Biotechnology V33, *62*7-634.

Siroy, A., Cosette, P., Seyer, D., Lemaitre-Guillier, C., Vallenet, D., VanDorsselaer, A., Boyer-Mariotte, S., Jouenne, T., and De, E. (2006). Global Comparison of the Membrane Subproteomes between a Multidrug-Resistant Acinetobacter baumannii Strain and a Reference Strain. J Proteome Res 5, *33*85-3398.

Southey-Pillig, C. J., Davies, D. G., and Sauer, K. (2005). Characterization of Temporal Protein Production in Pseudomonas aeruginosa Biofilms. J Bacteriol 187, *81*14-8126.

Stewart, P. S., and Costerton, W. J. (2001). Antibiotic resistance of bacteria in biofilms. The Lancet 358, *13*5-138.

Stoodley, P., Sauer, K., Davies, D. G., and Costerton, J. W. (2002). Biofilms As Complex Differentiated Communities. Annual Review of Microbiology 56, *18*7-209.

Stuart, T. L., Mulvey, M., Simor, A.E., Tien, H.C., Battad, A., Taylor, G., Vayalumkal, J.V., Weir, C., Ofner, M., Gravel, D., Paton, S. (2007). Acinetobacter baumannii in casualties returning from Afghanistan. Can J Infect Control 22, *15*2-154.

Tashiro, Y., Sakai, R., Toyofuku, M., Sawada, I., Nakajima-Kambe, T., Uchiyama, H., and Nomura, N. (2009). Outer Membrane Machinery and Alginate Synthesis Regulators Control Membrane Vesicle Production in Pseudomonas aeruginosa. J Bacteriol 191, *75*09-7519.

Tien, H., Battad, A., Bryce, E., Fuller, J., Mulvey, M., Bernard, K., Brisebois, R., Doucet, J., Rizoli, S., Fowler, R., and Simor, A. (2007). Multi-drug resistant Acinetobacter infections in critically injured Canadian forces soldiers. BMC Infectious Diseases 7, *95*.

Tomaras, A. P., Dorsey, C. W., Edelmann, R. E., and Actis, L. A. (2003). Attachment to and biofilm formation on abiotic surfaces by Acinetobacter baumannii: involvement of a novel chaperone-usher pili assembly system. Microbiology 149, *34*73-3484.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76, *43*50-4354.

Urban, C., Segal-Maurer, S., Rahal, J.J. (2003). Considerations in control and treatment of nosocomial infections due to multidrug-resistant Acinetobacter baumannii. Clin Infect Dis 36, *12*68-1274.

Venketaraman, V., Lin, A. K., Le, A., Kachlany, S. C., Connell, N. D., and Kaplan, J. B. (2008). Both leukotoxin and poly-N-acetylglucosamine surface polysaccharide protect Aggregatibacter actinomycetemcomitans cells from macrophage killing. Microbial Pathogenesis 45, *17*3-180.

Vidal, R., Dominguez, M., Urrutia, H., Bello, H., Gonzalez, G., Garcia, A., Zemelman, R. (1996). Biofilm formation by Acinetobacter baumannii. Microbios ;: 86, *49*-58.

Wang, G., Wilson, T. J. M., Jiang, Q., and Taylor, D. E. (2001). Spontaneous Mutations That Confer Antibiotic Resistance in Helicobacter pylori. Antimicrob Agents Chemother 45, *72*7-733.

WENSINK, J., and WITHOLT, B. (1981). Outer-Membrane Vesicles Released by Normally Growing *Escherichia coli* Contain Very Little Lipoprotein. European Journal of Biochemistry 116, *33*1-335.

Witt, C. M., Raychaudhuri, S., Schaefer, B., Chakraborty, A.K., Robey, E.A. (2005). Directed migration of positively selected thymocytes visualized in real time. PLoS Biology 3, *e16*0.

Yonezawa, H., Osaki, T., Kurata, S., Fukuda, M., Kawakami, H., Ochiai, K., Hanawa, T., and Kamiya, S. (2009). Outer Membrane Vesicles of Helicobacter pylori TK1402 are Involved in Biofilm Formation. BMC Microbiology 9, *19*7.

Zapor., M. J., Moran., K.A. (2005). Infectious diseases during wartime. Curr Opin Infect Dis 18, *39*5-399.

Zhong, G., Smith, G. P., Berry, J., and Brunham, R. C. (1994). Conformational mimicry of a chlamydial neutralization epitope on filamentous phage. Journal of Biological Chemistry 269, *24*183-24188.

Zhou, L., Srisatjaluk, R., Justus, D. E., and Doyle, R. J. (1998). On the origin of membrane vesicles in Gram-negative bacteria. FEMS Microbiology Letters 163, *22*3-228.

VITA

An-Yu Tsai was born in Kaohsiung Taiwan on Feb 23, 1981, the first son of Mr. Feng-Chih Tsai and Mrs. Shiu-Chen Peng. After graduating from St. Dominic high school, he entered in Da-Yeh University and earned his bachelor's of Food Engineering degree. Later, he worked in Genetic lab of Taipei general hospital as a research assistant. He studied in University of Texas at San Antonio in 2008 and worked under Dr. Tao Wei and Dr. Dallo's supervise since 2009 to 2010 August.