Characterization of natural vesicles secreted by Vibrio cholerae for OmpU expression and modulation of host cellular responses by OmpU present in the vesicle

Alok Kumar

A dissertation submitted for the partial fulfilment of BS-MS dual degree in Science

Indian Institute of Science Education and Research Mohali

April, 2014

Certificate of Examination

This is to certify that the dissertation titled '*Characterization of natural vesicles secreted by Vibrio cholerae for OmpU expression and modulation of host cellular responses by OmpU present in the vesicle'* submitted by Mr. Alok Kumar (Reg No. MS09013) for the partial fulfilment of BS-MS dual degree Programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Kaushik Chattopadhyay Dr. Rachna Chaba Dr. Arunika Mukhopadhaya

(Supervisor)

Dated: 25April, 2014

DECLARATION

The work presented in this dissertation has been carried out by me under the guidance of Dr. Arunika Mukhopadhaya at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Alok Kumar

(Candidate)

Dated: 25 April, 2014

In my capacity as the supervisor of the candidate's project work, I certify that above statements by the candidate are true to the best of my knowledge.

Dr. Arunika Mukhopadhaya

(Supervisor)

ACKNOWLEDGEMENT

I express my deep sense of gratitude to Dr. Arunika Mukhopadhaya for her advice, expert guidance, valuable suggestions, discussions and constant encouragement during the entire course of this work and preparation of the thesis. I consider myself extremely fortunate to be a student under her guidance.

I express my sincere thanks to the Master's thesis committee members for their valuable suggestions and comments during the committee meeting.

I convey my regards to Shelly and Sanica for encouraging me during the entire research work. I have learnt and gained almost all the experimental skills from their knowledge and experience.

I am thankful to my other lab members Junaid, Aakanksha, Krishna, Deepinder, Dr. Ranjai and Achyut who accompanied me throughout this research work. I am thankful to Bhupinder who helped me in during this research.

I thank each of my classmates as well as neighbouring lab members who provided a wonderful and friendly atmosphere to carry out research during the entire period. It is my pleasure to thank each and every member of the Department of Biological sciences who have helped me in various ways during the course of investigation and research.

I express my sincere gratitude to each of my family members for their encouragement and moral support throughout the course of study.

I would also like to acknowledge the 'Department of Science and Technology, India' for providing the INSPIRE fellowship.

LIST OF FIGURES

CONTENT

Chapter 3-Result and discussion

CHAPTER 1

INTRODUCTION

1.1 Porins

Porins are a class of membrane proteins which do not exhibit hydrophobic stretches in their amino acid sequences and thus, form hollow β-barrel structures with a hydrophobic outer surface. Porins form water filled pores in similar way as specific channels and allows the transmembrane diffusion of small solutes. However, Specific channels contain specific ligand binding sites within the channels. Passing rates of solutes through porin channels are likely to be affected strongly by minor differences in the size, hydrophobicity, shape, or charge of the solute molecule.

The exclusion limit of the outer membrane for hydrophilic compounds is determined largely by the size of the channel, and it has been found that most porins demonstrate little chemical (as opposed to ion or charge) selectivity for different substrates. [1-4] Porin channels can be either cation or anion selective, but studies revealed that this selectivity is weak for small ions like K^+ and Cl^-.[5] .

Although the primary function of the bacterial outer membrane porins is to form diffusion channels in the bacterial outer membrane [6,7], they also have been found to serve other functions [8-12]. For example, many gram-negative bacteria porins have been involved in the survival mechanisms of the bacteria against bactericidal agents, adhesion/colonization process, and modulation of the host immunity during bacterial infections.

OmpU is one of the major outer membrane porins of *Vibrio cholerae*[13-17] which is the causal organism for the disease cholera. The *toxR* gene product of *V. cholerae* is a transmembrane DNA-binding protein that activates the expression of multiple virulence genes [18-19]. Expression of OmpU gene is regulated by ToxR regulon. It is because of their potential role in pathogenesis, characterization of OmpU protein was of considerable importance.

ToxR positively regulates OmpU in *V. cholerae*. OmpU provides bile resistance in *V. cholerae* and also develop resistance of the organism against the antibacterial peptides [20-21]. Studies have indicated that OmpU protein is also involved in the adhesion process of the bacteria during *V. cholerae* infection [22]. *V. cholerae* porin OmpU could be involved in the pathogenesis process of the organism. *V. cholerae* OmpU has been biochemically

characterized previously for its 'porin'-property [23-24]. However, particularly at the molecular level this protein has not yet been studied in terms of finding its ability to produce cellular responses that would be helpful to study their involvement for the host-pathogen interaction mechanisms.

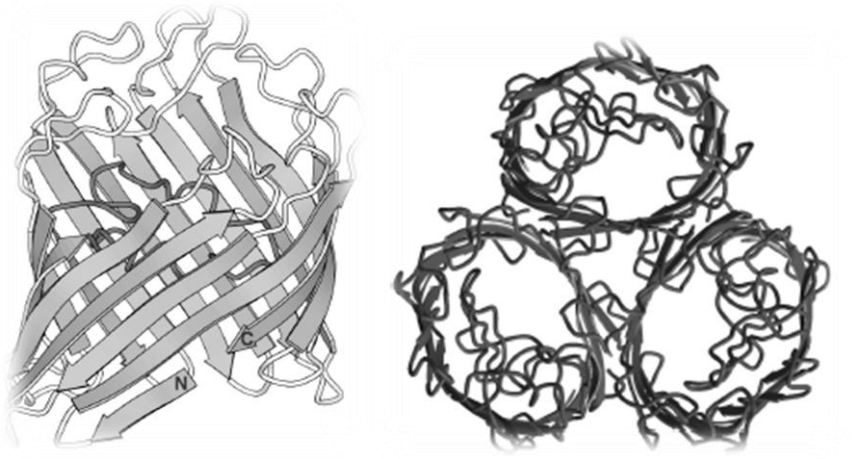

Fig. 1-General structure of porins (Nakae, *et* al T(1976) Biophys. Res. Commun.)

1.2 Structure of Porins

These membrane proteins contain several β-barrel structures. The local distribution of residues, both hydrophobic and hydrophilic, is conserved among the trimeric non-specific porins derived from different gram-negative bacteria. The presence of a ring of aromatic amino acids located near the merging area of the monomers provides stability to the protein within the lipid bilayer [25-27].

1.3 Pore function

The channel ensures the free passage of small hydrophilic molecules [28-29].An internal eyelet region formed by a long loop, bent into the channel with its carboxy-negative cluster facing the positive charges from Arg/Lys residues derived from L-sheets belonging to the L-barrel wall is an important property of these channels. This special organisation produces an electrostatic field in the lumen that regulates the diffusion of the molecule through the constriction area. The well-documented functional parameters of the channel could be determined by using several techniques including construction of artificial membranes, planar lipid bilayers, patch clamp and `liposome swelling' approaches [30]. Electrophysiological studies provided extensive information concerning

the conductance, size, selectivity and voltage gating of the channel characteristics, and yield a functional model of the pore protein inserted in the outer membrane

1.4 Regulation of porin expression

Bacteria must monitor their environment constantly through phenotypic modifications that requires an adaptive response. Gram-negative bacteria adapt their outer membrane permeability by modulating the expression of porins in the case of limited nutrient and changes in the osmolarity of medium.

1.5 *Vibrio cholerae* OmpU differentially affected by bile

OmpU as mentioned above is a pore-forming protein of the outer membrane of *Vibrio cholerae*, which is a pathogen that colonizes the intestine and produces cholera. Expression of the *ompU* genes is regulated by ToxR. It has been shown recently that bile stimulates the ToxR-mediated transcription of *ompU* and also *ompU*-expressing strains are more resistant to bile as well as anionic detergents. Studies have shown that the OmpU-mediated outer membrane permeability was not affected by external bile. The insensitivity of OmpU to bile may be due to its small pore size and may provide an explanation for the resistance of OmpU-producing cells to bile *in vivo* [31].

1.6 Outer Membrane Vesicles

Outer membrane vesicles (OMV) are the natural product secreted from outer membrane of gram negative bacteria and appear as spheroid particles having heterogeneous size (~10 to 300 nm in diameter). These vesicles are formed when the portion of the outer membrane with periplasmic content is "blebbed" off to form round vesicles.[32-33] These contain some virulence factors which include proteins, toxins, enzymes and non-protein antigen like lipopolysaccharide (LPS). Purified vesicles transport virulence factors by interacting with both prokaryotic and eukaryotic cells.

Many gram-negative bacteria produces external membrane vesicles (MVs) during their normal growth.[7-12] and thus, can be important during the initial phases of infection, as they concentrate several virulence factors and convey them to the host tissue. [34-35].

Gram-negative bacteria envelope consists of two membranes (the inner membrane and outer membrane: the peptidoglycan layer and the periplasm respectively [36]. Lipid and

protein composition are different in these membranes. In most gram-negative bacteria, the outer leaflet of the outer membrane (OM) contains mainly of lipopolysaccharide (LPS), while both the leaflets of the inner membrane contains of phospholipids. A viscous, ~13-nm periplasmic space that is an oxidizing environment lacking energy source such as ATP or NADPH exists between the two membranes and makes up 7–40% of the total cell volume [37-39].

1.7 Natural OM Vesicles

All gram-negative bacteria were found to naturally release OM vesicles. Analysis of native OM vesicle reveals that the vesicles contains a substantial fraction of cellular material. For example, vesicles isolated from typical laboratory cultures of dividing *Pseudomonas aeruginosa* and *Escherichia coli* cells account for ~1% of the OM material in the culture[40-42]. In contrast, *Neisseria meningitidis* secretes large numbers of vesicles, constituting 8 to 12% of radiolabeled protein and endotoxin in the logphase cultures [43]. Not only are OM vesicles secreted by free-living cells, these are also abundant in naturally occurring biofilms [44]. Intracellular pathogens such as *Legionella pneumophila*, *Salmonella* spp, and *Francisella* spp. secrete OM vesicles in both intraphagosomal and extraphagosomal compartments [45-48]. In the case of *Flavobacterium*, vesicles are secreted late in the growth phase [49].

1.8 Triggering OM bulging

The first step towards creating an outer membrane vesicles (OMV) is an outward bulging of the OM. This event implies that buds form in areas where proteins linking the OM to the peptidoglycan layer are absent and this is possible if the peptidoglycan is disrupted, such as with antibiotics or autolysins, thus, may result in the secreting of OMVs containing peptidoglycan fragments and portions of OM-peptidoglycan bridging proteins. Although this model accounts for the increased vesiculation that happen during envelope disruption, it is not likely the main mode of vesiculation. Vesiculation can be increased over 100-fold without disturbing the membrane integrity [50]. Other envelope events could also trigger OMV budding. For example, OM-peptidoglycan links could be moved or targeted for destruction. The linking protein OmpA is depleted but is present in OMVs and Lpp is excluded [51-54].

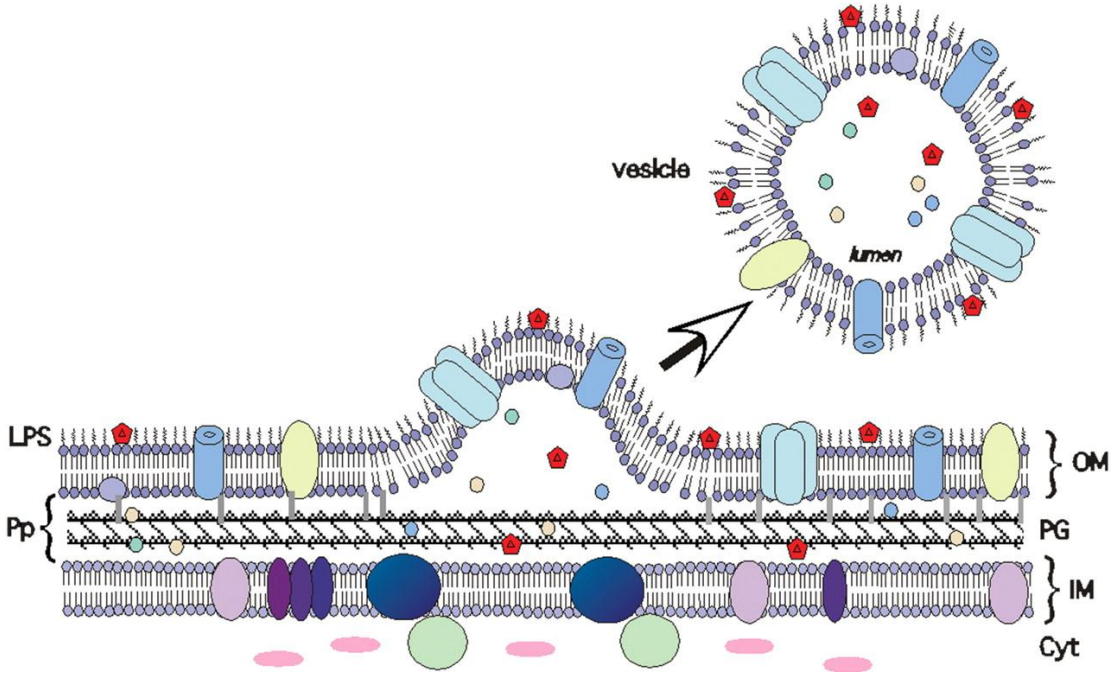

Fig. 2-Model of vesicle biogenesis. OM vesicles are proteoliposomes which contains OM phospholipids, LPS, a subset of OM proteins, and periplasmic proteins. Proteins such as LT (red) that attach to the external surface of the bacteria are associated with the outer membrane of vesicles. Vesicles bud at sites where the links between the peptidoglycan and OM are absent, infrequent or broken. (Meta J. Kuehn *et al Genes Dev.* 2005 19: 2645-2655)

1.9 Role of OM vesicles in interbacterial interactions

The interaction of bacteria with co-colonizing bacteria can be hostile. Bacteria destroy co-infectors to stop competition for limited nutrients or to provide nutrients for themselves. Vesicles facilitate bacterial communities where interspecies interactions are promoted in order to fight lethal environmental conditions. OM vesicles from one species have been observed to help in the survival of a whole mixed bacterial infective population by actively destroying host defences.

Vesiculating bacteria are capable of eliminating competing bacterial strains, thus having a survival advantage in mixed-population infections. OM vesicle secretion could be an advantage for growth in a mixed bacterial population where nutrition is limited. Further, β-lactamase was found to be packaged into *P. aeruginosa* vesicles which were secreted by strains that expressed β-lactamase[55]. OM vesicles containing β-lactamase can save the

vesicle-producing strain from a co-colonizing, β-lactam-producing species. It has also been observed that some OM vesicles contain scavenging proteases [56-57]. xylanase, and cellulose[58].

1.10 Vesicle-Associated Toxins

Native OM vesicles from a variety of gram-negative pathogens secret toxic materials. In many cases, vesicle associated toxins have been found to deliver active toxins to host cells. Enterotoxigenic *E. coli* (ETEC) secrete heat-labile enterotoxin (LT) that associates with LPS in the particulate part of the cell culture supernatant [59-62]. Secreted ETEC vesicles specifically bind, enter, and deliver active toxin into epithelial and Y1 adrenal cells. OM vesicles from *Actinobacillus actinomycetemcomitans* exhibit cytotoxicity [63-65],and *Xenorhabdus nematophilus* and *Photorhabdus luminescens* OM vesicles were cytotoxic for insect larvae as well as in tissue culture [66] .NarE is a vesicle-associated protein of *Neisseria meningitidis* vesicles that exhibits ADP-ribosyltransferase and NAD-glycohydrolase activities.Toxic vesicles produced by extra intestinal *E. coli* (ExPEC) contain a hemolysin; an RTX toxin, which gets surface bounded and a cytolethal distending toxin, which has a lipid binding domain to bind the OM [67].

1.11 Nontoxin Virulence Factors in OM Vesicles

OM vesicles are a mechanism by which bacteria can produce many periplasmic and membrane components associated with the toxic of the bacterium. Often, OM components of vesicles include adhesins that allow vesicles to interact with host cells. In addition, OM vesicles can contain proteases and signaling molecules. For example, *Pseudomonas* OM vesicles contain virulence-associated enzymes, antimicrobial quinolines, and quorum-sensing molecules [68-73].Also, the *P. aeruginosa* CiF protein is preferentially produced into vesicles and CiF-containing vesicles decrease the level of apical lung epithelial expression of the cystic fibrosis transmembrane conductance regulator protein (CFTR), the central protein in the genetic disease of cystic fibrosis.

1.12 Vesicle interactions with host cells

Protease and toxin-containing OM vesicles interact with host cells and thereby act as toxic factor delivery vehicles. Their interaction can occur via membrane fusion event or

via adhesin-receptor-mediated attachment. In some cases, adherence is followed by vesicle uptake, even by nonphagocytic cell types. The theory that vesicle binding contributes to infection by enabling the delivery of toxic bacterial cargo to host cells, often by the internalization of the whole content of the vesicle. The consequence of such a delivery process includes signalling, innate immunity and adaptive immune behaviour in the host cells. Adherence of vesicles to cultured host cells has been observed for OM vesicles secreted by a variety of pathogens. Protease and toxin-containing OM vesicles from *E. coli*, *Shigella*, *Actinobacillus*, and *Borrelia* strains interact with bacterial as well as mammalian cells. Vesicle-associated aminopeptidase increases the tendency of *P. aeruginosa* vesicles to associate with both primary and cultured human lung epithelial cells, which may occur due to receptor binding or receptor-uncovering property of this enzyme [74].

Vesicles and bacteria can use same host cell receptors. *B. burgdorferi* vesicles adhere to human umbilical vein endothelial cells (HUVECs) in such a way that it competes with whole *Borrelia* cells [75].

1.13 Vesicle Entry into Cells

In many cases, after OM vesicles adhere, vesicles can be internalized into host cells. Adhesins can act as virulence factor of OM vesicles and allow vesicles to enter using the receptor-mediated endocytic pathway which is also used by the soluble toxin. Toxin-mediated vesicle adherence and uptake can occur only if the toxin-vesicle interaction does not interfere with the toxin-receptor interaction.

1.14 Apoptosis

Apoptosis, or programmed cell death, is one of the many cellular responses generated during pathogen infection in host. Various pathogenic molecule possess the capability of inducing apoptosis of host cell. Programmed cell death contains a series of biochemical events that lead to a variety of morphological changes in cells such as blebbing, cell shrinkage, including loss of asymmetry of cellular membrane, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Cells die in a regulated and controlled fashion in response to a variety of stimuli during apoptosis. This makes apoptosis different from another form of cell death called necrosis in which uncontrolled

cell death leads to inflammatory responses, lysis of cells, and to serious health problems. The nuclear fragments and organelles are packaged in membrane-bound vesicles ingested by surrounding cells [76-79].

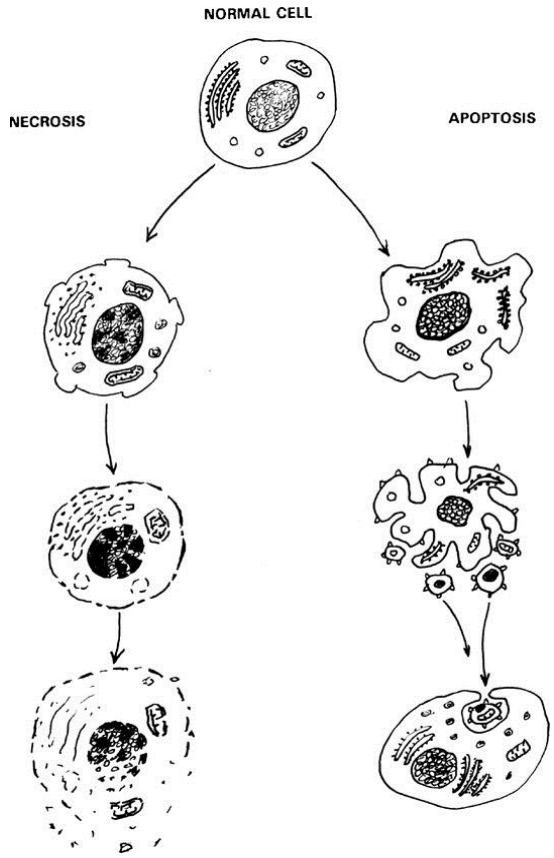

Fig. 3-Cell death - necrosis and apoptosis. Necrotic cells are swollen, loss of plasma membrane integrity and leakage of cytosol into extracellular space. Apoptotic cells are develop blebs and shrunken. Membrane integrity is not lost until late. Nuclear chromatin undergoes fragmentation and condensation. The cytoplasm becomes divided to form apoptotic bodies containing organelles and/or nuclear debris. (Meer et al, Lasers in Medical Science Vol. 25)

In normal live cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane. However, in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposes PS to the external cellular environment. In leukocyte apoptosis, PS on the outer surface of the cell marks the cell for recognition and phagocytosis by macrophages. The human anticoagulant, annexin V, is a 35–36 kDa Ca^{2+}-dependent phospholipid-binding protein that has high affinity for PS. Annexin V

labeled with a fluorophore or biotin can identify apoptotic cells by binding to PS exposed on the outer leaflet.

Propidium iodide (PI) is impermeant to live cells and apoptotic cells, but stains dead cell (red fluorescence) by binding tightly to the nucleic acids in the cell. After staining a cell population with Florescein isothiocyanate (FITC) annexin V and PI in the provided binding buffer, apoptotic cells show green fluorescence whereas dead cells show red fluorescence, and live cells show little or no fluorescence. These populations can easily be distinguished using a flow cytometer with the 488 nm line of an argon-ion laser for excitation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Protocol for preparation of Luria Agar Plate

- 2.5 g Luria broth (LB) was weighed in 250 ml flask.
- 1.5 g agar was added to 2.5 g LB in 100 ml water.
- Above media was subjected to autoclaving.
- Following autoclaving, media flask was checked for temperature to come down to around 55-56°C. Then it was poured into plates under sterile condition inside a laminar hood.
- Plates were left to solidify.
- Solidified plates were covered by parafilm and stored at 4°C for further use.

2.2 Bacterial Culture

- Glycerol stock of *V. cholerae* (EI Tor O1) was streaked on Luria agar plate inside a laminar hood.
- The plate was incubated at 37°C overnight.
- Further the plate was checked for the appearance of isolated single colonies.
- One of the obtained isolated colonies was picked and was inoculated (by tip of pipette) in 3 ml brain heart infusion (BHI) broth in 15 ml tube.
- The inoculated media was incubated for 12 h at 37°C with constant shaking.
- OD was measured continuously until OD_{600} reached 0.4.
- If the OD was greater than 0.4, then we diluted the clture with blank BHI media so that OD values were below 0.4.

2.3 Growth Kinetics

- 100 ml BHI media was first inoculated with 1 ml *V. cholerae* glycerol stock and incubated overnight.
- Followed by incubation 3 ml seed culture was inoculated in 15 ml media and incubated overnight.
- After 12 h, 100 ml media was inoculated with seed culture such that initial OD was 0.01.
- Reading was taken after every 30 minutes.

2.4 Isolation of Outer membrane vesicles [80]

- 1 L media (LB, BHI, and BHI+ Deoxycholate) was inoculated by 1% of *V. cholerae* seed culture.
- Inoculated media was incubated at 37°C for 11 h with constant shaking.
- After incubation, cells were pelleted at 3220 xg for 25 minutes at 4^{o}C.
- Further the supernatant was either stored or processed immediately.
- Supernatant was stored overnight at 4^{o}C with 0.2% sodium azide, 5 µg/ml ampicillin and 50 mM EDTA.
- Further, the supernatant was centrifuged at 18,500 xg at 4^{o}C for about 30 minutes
- Pellet was discarded and supernatant was subjected to ultra-centrifugation at 150,000 xg at 4^{o}C for 2 h.
- Pellet so obtained was washed three times with 10 ml of 20 mM Tris buffer (pH 7.6).
- Finally the pellet was resuspended in 500 µl of 20 mM Tris buffer (pH 7.6).
- Vesicle concentration was measured using bradford reagent at 595 nm. That gave an estimation of the protein content in the vesicle.
- Vesicle preparation was checked with SDS-PAGE followed by Western Blot

2.5 Isolation of outer membrane

- 1 L media was first inoculated with 1% of *V. cholerae* seed culture.
- Cells were then pelleted at 4000 xg at 4°C for about 25 minutes.
- Pellet was resuspended in 20 ml HEPES (4-(2-hydroxyethyl)- piperazine ethanesulfonic acid) (pH 7).
- Resuspended pellet was sonicated at 25 amplitude for 15 minutes (30 sec pulses with 10 sec break).
- Sonicated pellet was centrifuged at 5000 xg at 4°C for 15 minutes.
- Then the supernatant was ultra-centrifuged at 105,000 xg at 4°C for 1 h.
- Pellet obtained was resuspended in 4 ml of 0.5% N-lauryl sarcosine.
- Treated pellet was ultra-centrifuged at 105,000 xg at 4°C for 1 h.
- Finally, the pellet was resuspended in HEPES (pH 7).
- Outer membrane protein concentration was measured using bradford reagent at 595 nm.

2.6 Purification and refolding of recombinant OmpU[81]

(Purification is done following the method of Khan J. *et al*, 2011)

- *E. coli* origami cells harbouring pET-14b/OmpU plasmid were culture in liquid LB media containing 50 μg ampicillin at 37˚C till optical density 0.6 and then induced by IPTG (Isopropyl β-D-1-thiogalactopyranoside**)** for 3 hrs at 37˚C.
- Cells were harvested and resuspended in bacterial lysis buffer (GE-Bioscience).
- Cells were lysed by sonication.
- Cell lysate was subjected to centrifugation at 18500 xg for 30 minute to isolate the inclusion body.
- Inclusion body was solubilised in 8M urea and purified by NI-NTA affinity chromatography under denaturing condition.
- Ni resin bound protein was eluted in 8M urea in PBS containing 300mM imidazole. Refolding of the recombinant *V. cholerae* OmpU was carried out by rapid dilution of the denatured protein into the refolding buffer [PBS, containing 10% glycerol, 0.5% Lauryldimethylamine N-oxide (LDAO) (Sigma)] in 1:10 ratio at 25 ˚C with constant stirring and incubated overnight at 4 °C.
- This refolding protein further purified by gel filtration chromatography using sephacryl S-200 column and analyzed by SDS-PAGE comassive staining has shown 95% purity of recombinant OmpU.

2.7 Cell Culture and experiment

Human monocytic cell line, THP-1 (NCCS Pune) was cultured and maintained in RPMI 1640 (Invitrogen,Gibco) supplemented with 10% fetal bovine serum (FBS; Invitrogen). For each experiment cells were plated as $1x10^6$ cells/ml in 10 ml of media in a 10 cm dish. Cells were treated with 10 μg/ml vesicles or 10 μg/ml purified recombinant OmpU and incubated for different time points.

2.8 Whole cell lysate preparation

- Treated cells were collected in a tube.
- Cells were centrifuged at 1500 xg for 5 minutes at 4^oC.
- After centrifugation, supernatant was discarded.

- Cells were washed twice using 1 ml of 1x Phosphate Buffer Saline (PBS) at 4°C at 3000 xg.
- Washed cells were resuspended in 100 µl lysis buffer containing 10 µl protease inhibitor.
- Cells were lysed by sonication at 10 amplitude using two pulse of 5 seconds.
- Lysed cells were centrifuged at 12000 xg for 30 min for each time points at 4 ^{o}C.
- Finally, supernatant was collected and stored at -80 ^{o}C.

2.9 Isolation of mitochondria

Mitochondria were isolated from vesicle-treated or purified OmpU-treated cells using Mitochondria Isolation kit II (Sigma Adrich, Saint Louis, MO, USA) following manufacturer's protocol. Briefly,

- Treated cells were collected in a tube.
- Cells were harvested at 2500 xg for 5 minutes at 4 ^{o}C.
- Cells were washed twice with ice cold 1x PBS at 3000 xg for 5 minutes at 4 ^{o}C.
- After that, cells were resuspended in 200 µl of lysis buffer.
- The resuspended cells were subjected to temeperature shock 5 times by placing it on ice for 1 min with 1 min interval.
- 2 volume of Extraction Buffer A was added in the resuspended cells and mixed by inversion.
- Then, it was centrifuged at 600 xg, for 10 min at 4 ^{o}C.
- Supernatant was then removed and transferred to a new microcentrifuge tube.
- Further the supernatant was centrifuged at 11000 xg for 10 min at 4^{o}C.
- Supernatant and pellet were collected separately.
- The supernatant was cytoplasm fraction.
- The pellet which was mitochondria was resuspended in 50 µl CellLytic M Reagent.
- Finally, both were stored at -80 ^{o}C.

(Lysis buffer = extraction buffer + protease inhibitor + detergent. (Provided in the kit) Extraction buffer A= extraction buffer + protease inhibitor)

2.10 SDS-PAGE (Sodeum Dodecyl Sulfate-polyAcrylamide Gel Electrophoresis)

SDS-PAGE is mainly used for the visualization of protein. The following solutions were used to run and visualize proteins by using this technique

Solutions and buffers which were used to perform SDS-PAGE technique

- **Solution A (100 ml):**

  Acrylamide- 29.2 g

  Bis-acrylamide- 0.8 g

- **Solution B (1000 ml):**

  Tris-Cl (1.5 M; pH-8.8)- 181.65 g

  SDS (0.4%)- 4 g

- **Solution C (1000 ml):**

  Tris-Cl (0.5M; ph-6.8)- 60.55 g

  SDS (0.4%)- 4 g

- **Loading Dye:**

  Tris-HCl (pH-6.8)- 10mM

  EDTA-1mM

  SDS-2%

  β-mercaptoethanol-5%

  Bromophenol-blue-0.001%

  Glycerol- 10%

- **Running Buffer (1000ml):**

  0.025 M Tris- 3 g

  0.192 M Glycine- 14.4 g

  0.1% SDS- 1 g

- **Staining Solution:**

  Water- 100 ml

  Acetic acid- 10 ml

  Methanol- 40 ml

  Coomassie Blue (0.1%)- 0.1 g

- **Destaining Solution:**

  Water- 80ml

  Acetic Acid- 15 ml

  Methanol- 5 ml

- **1X PBS (1000 ml)**

  NaCl- 8 g

  Kcl- 0.2 g

  Na_2HPO_4- 1.44 g

  NaH_2PO_4- 0.24 g

  Make up to 1 litre

- After preparing the 10% stacking gel solution, the rack for gel solidification was assembled.
- Stacking gel solution was added carefully until the level became equal to the green bar holding the glass plates. H_2O was added to the top. Waited for 15–30 minutes until the gel turning solidified
- The stacking gel was overlaid with the separating gel, after removing the water.
- The comb was inserted, ensuring that there were no air bubbles.
- Waited until the gel was solidified.
  (Solidification could be easily checked by leaving some gel solution in a tube).
- Gel was placed inside the electrophorator and connected to a power supply.
  (When connecting to the power source always connect red to red, and black to black)
- It was ensured that buffer covers the gel completely.
- Marker (5 µL) followed by samples (15 µL) was loaded in to each well.
- The gel was made to run with low voltage (90V) for separating gel; and used higher voltage (110 V) for stacking gel
- The gel was made to run for approximately an hour, or until the dye front runs off the bottom of the gel.
- Gel was taken out and stained using staining solution for over night.

- Following day, the stained gel was subjected to destaining to vidualize only the protein band.

2.11 Western Blotting

Specific OmpU band was detected using western blot. It helps to identify specific proteins from a complex mixture of proteins. The technique uses three elements:

1. Separation by size
2. Transfer to a solid support
3. Marking target protein using a proper primary and secondary antibody to visualize.

- **Transfer buffer**

  14.4 g Glycine

  3 g Tris

  150 ml Methanol

  850 ml Mili-Q water

For performing western blotting,

- Samples were loaded on a SDS-PAGE and ran untill the dye front reached at the bottom. Then the gel was placed in the transfer buffer.
- PVDF membrane (Millipore) was soaked in methanol.
- Gel was placed along side of the PVDF membrane in a electro blotting aparatus.
- For the transfer of protein from the gel to the PVDF membrane 90 volts for 90 minutes was applied.
- The membrane was then kept overnight for blocking in 5% BSA dissolved in 1x Tris Buffer Saline containing 0.1% Tween-20 (TBST) to get rid of nonspecific binding of antibodies to membrane in subsequent steps.
- Next day 0.1% 1x TBST solutions were prepared and primary antibody specifically targeted against OmpU (raised in rabbit host) was added in 1:1000 dilution (for VCC 1:500, for β-actin 1:1000 and for VDAC was 1:5000).
- Primary antibody incubation was kept for 60-180 minutes and then membrane was washed thoroughly with 5 ml TBST for four times each for 15 minutes.
- Further the secondary antibody was diluted (1: 5000) in 1x TBST and incubated for 60 minutes and then membrane was washed with 5 ml of TBST for three

times. ECL (GE Health care) mix (mixture of solution A and B using proportion as provided by the manufacturer) was prepared. The membrane was incubated for 1 to 2 min.

- The result was visualized in the LASQuant 4000 chemiluminiscent detector (GE Health care).

2.12 Experiment and Staining procedure to check apoptosis

- $1x10^6$ THP-1 cells/ml was plated in 2 ml media
- Cells were treated with either vesicle (10 µg/ml) or purified OmpU (10 µg/ml) and incubated for 24 h
- Following incubation cells were washed twice with cold PBS and subjected to the staining for apoptosis using Annexin V-FITC kit (Sigma Aldrich) using manufacturer's protocol.
- Followed by washing with cold PBS cells were resuspended in 1X Binding Buffer provided in the kit at a concentration of 1×10^6 cells/ml.
- From that 100 µl of the solution (1×10^5 cells) was transferred to a 5 ml tube.
- 5 µl of FITC conjugated Annexin V and 5 µl PI were added to the cell suspension.
- The cells were gently vortexed and incubated for 15 min at RT (25°C) in the dark.
- 400 µl of 1X Binding Buffer was added to each tube.
- Cells were acquired immediately by flow cytometry (using BD FACS Calibre, BD, San Jose, USA)
- Data were acquired and analyzed using Cell Quest Pro (BD)

RESULTS AND DISCUSSIONS

3.1 Analysis of growth kinetics of *V. cholerae* in different media:

Gram-negative bacteria secrete vesicles in the late log phase of their growth cycle. To determine the late log phase of *V. cholerae* in various media, media was inoculated with the bacteria and absorbance at 600 nm was monitored every 30 min. The growth curves for two replicates of each experiment were shown below.

3.2 Growth curve of *V. cholerae* in Luria broth (broth)

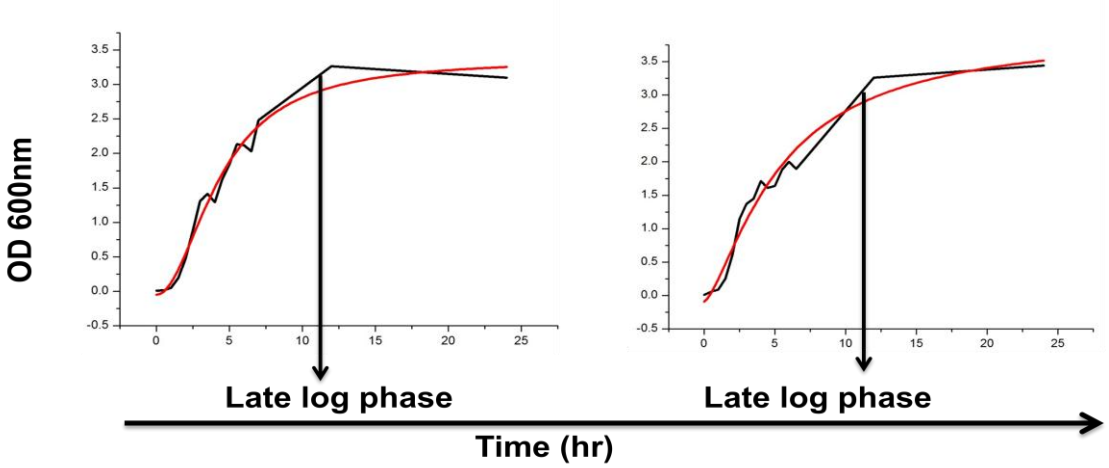

Fig. 4-*V. cholerae* growth kinetics in LB media showed that the late log phase occurs 11 h after inoculation.

3.3 Growth curve of *V. cholerae* in Brain-Heart Infusion (BHI) broth

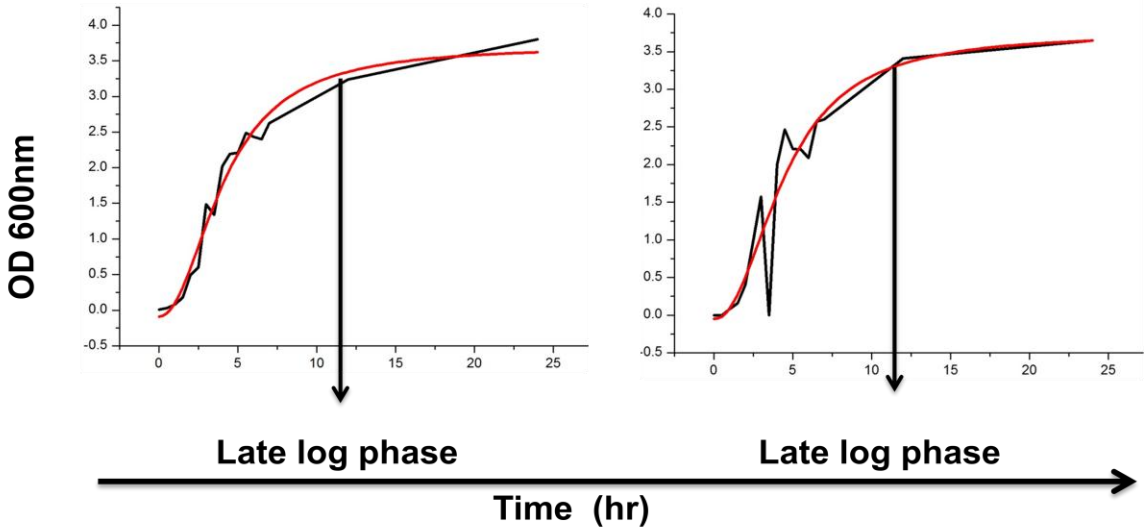

Fig. 5- *V. cholerae* growth kinetics in BHI media showed that the late log phase occurs after 11 h of inoculation.

3.4 Growth curve of *V. cholerae* in BHI media supplemented with 0.05% deoxycholate

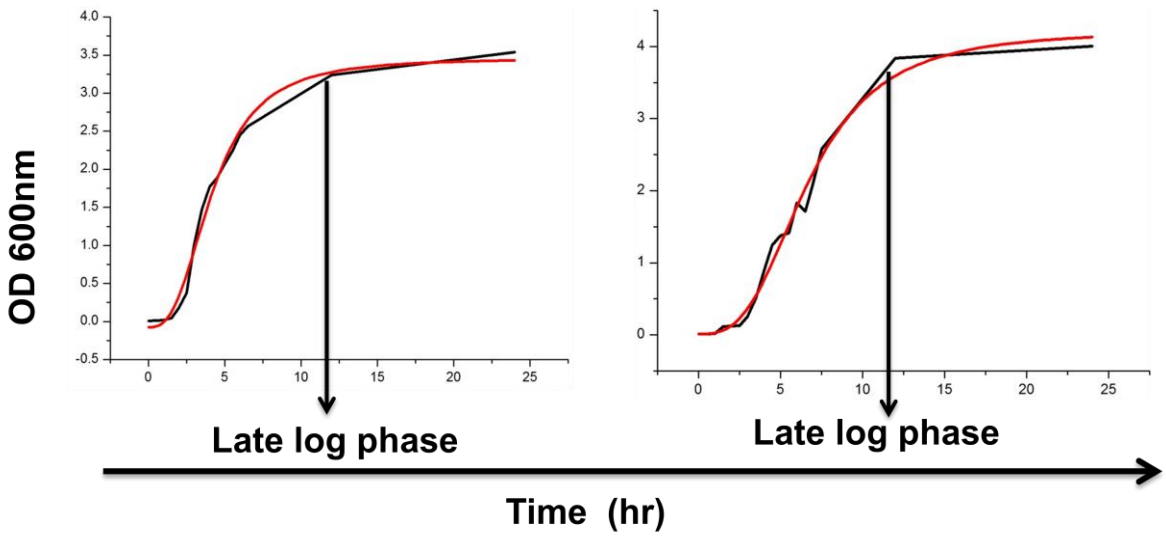

Fig. 6- Growth curve for *V. cholerae* cultured in BHI+deoxycholate indicates that late log phase occurs around 11 h

3.5 Secreted vesicles shows the presence of OmpU

For the analysis of the protein profile of naturally secreted vesicles in LB, BHI and in BHI + deoxycholate, SDS-PAGE was run. A 38 kDa band corresponding to OmpU was observed in vesicles isolated from all the types of media used.

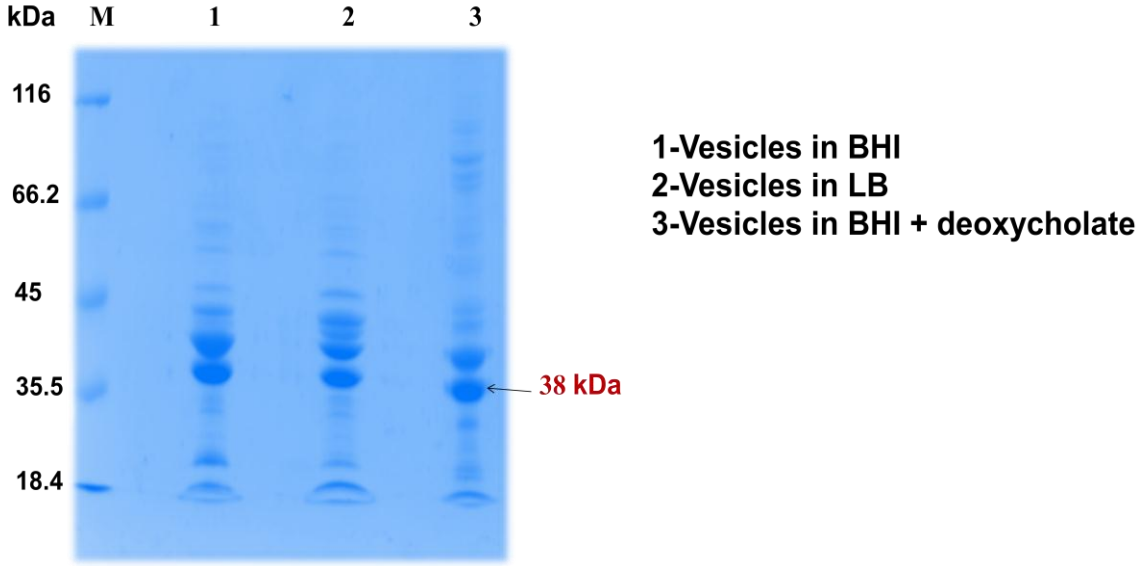

Fig. 7- SDS-PAGE analysis of vesicles secreted naturally by *V. cholera*e cultured in LB, BHI and in BHI + deoxycholate

3.6 Western blot confirms the presence of OmpU in vesicles

To confirm the presence of OmpU in naturally secreted vesicles in Luria broth (LB), Brain heart infusion (BHI) broth, and in BHI + deoxycholate, samples were analyzed for the presence of OmpU by western blotting using the primary antibody specific to *V. cholerae* OmpU. Western blotting confirmed the presence of OmpU and concentration of OmpU in vesicles did not vary with the use of different media.

Fig. 8- Western blot confirmed the presence of OmpU in naturally secreted vesicles

3.7 *Vibrio cholerae* cytolysin (VCC) is also detected in the vesicle preparation

For the analysis of *V. cholerae cytolysin* (VCC) in the naturally secreted vesicles, western blotting was performed using the anti-VCC antibody. This confirmed that *V. cholerae* cytolysin occurs in naturally secreted vesicles.

Fig. 9- Western blot confirmed the presence of VCC in vesicles from BHI media supplemented with 0.05% deoxycholate

3.8 OmpU was detected in whole cell lysates prepared from vesicle-treated target cells

THP-1 cells were treated with vesicles (10 µg/ml) and incubated for different time periods (15 m, 30 m, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h). Whole cell lysates were subjected to western blotting by using antibody specific to *V. cholerae* OmpU. β-actin was used as well as the loading control. OmpU is found in whole cell lysates of the treated cells at 15 mins and gradually increases with time. OmpU was found to be intact upto 24 h.

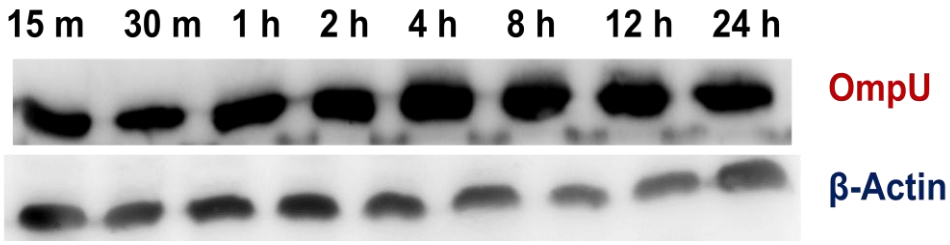

Fig. 10- Presence of OmpU was detected in the target cells up to 24 h

3.9 OmpU from vesicles translocates to the mitochondria of treated cells

THP-1 cells were treated with vesicles (10 µg/ml) and incubated for different time periods (1 h, 2 h, 4 h, 8 h, 12 h, 24 h). Mitochondria isolated from treated cells and were analyzed for the presence of OmpU by western blotting using antibody specific to *V. cholerae* OmpU. Initially, VDAC was used as the mitochondrial marker as well as the loading control. In later experiment β-actin was used as loading control. Translocation of OmpU to the mitochondria of the treated cells was observed from 1 h to 24 h.

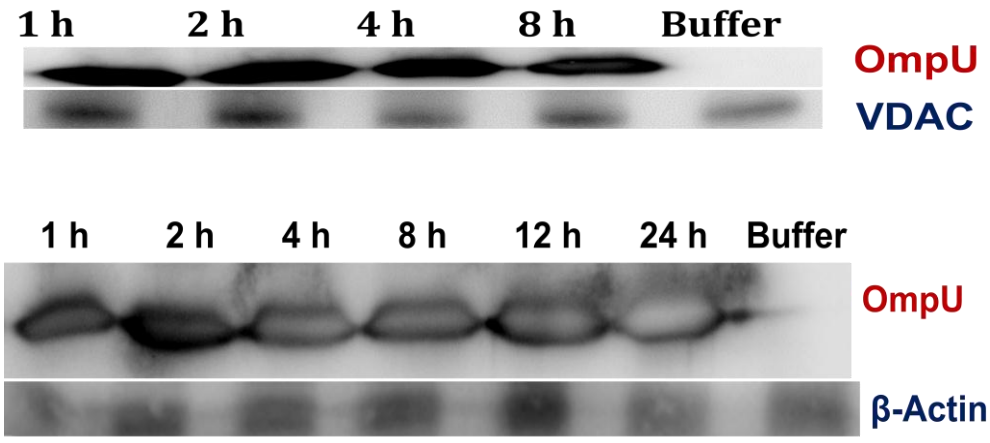

Fig. 11- OmpU translocation to mitochondria of treated cells begins within 1 hr and is observed till 24 h

3.10 Presence of *V. cholerae* cytolysin (VCC) in mitochondrial lysates indicated that entire vesicle translocates to the mitochondria

To probe whether the vesicle remains intact after OmpU translocates to the mitochondria, western blotting was performed for *V. cholerae* cytolysin (VCC). THP-1 cells was treated with vesicles (10µg/ml) and incubated for different time period (1h, 2h, 4h, 8h, 12h, 24h). The mitochondrial fractions of treated cells were analyzed for the presence of VCC by using the anti-VCC antibody. A clear translocation of VCC to the mitochondria of the treated cells was observed which was visible from 1 h up to 4 h.

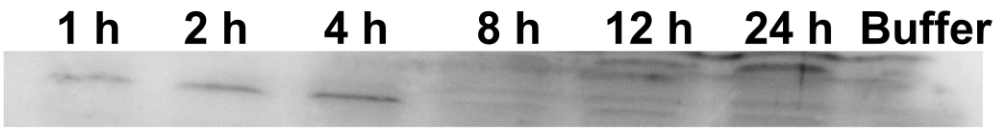

Fig. 12-VCC was detected in mitochondria of THP-1 cells up to 4 h

3.11 Purified OmpU translocates to the mitochondria of treated cells

THP-1 cells were treated with purified OmpU (10 µg/ml) and incubated for different time period (1 h, 2 h, 4 h, 8 h, 12 h, 24 h). The mitochondrial fraction from treated cells was analyzed for the presence of OmpU. β-Actin used for the marker as well as the loading control. Translocation of OmpU to the mitochondria of the treated cells was observed at 1 h upto 2 h. After 2 h, amount of OmpU in mitochondira started decreasing.

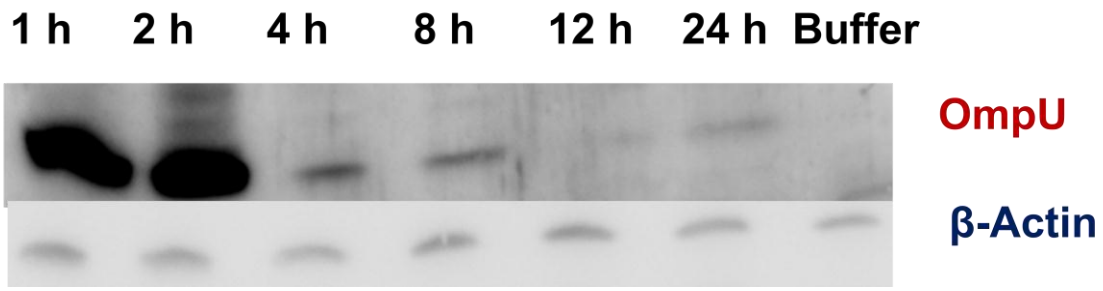

Fig. 13- Purified OmpU though translocates to the mitochondria but disappears with the increase in incubation time

3.12 Purified OmpU (10 µg/ml) and vesicles (10 µg/ml) induce similar extent of phosphatidyl-serine flipping, a characteristic feature of apoptosis

THP-1 monocytes treated and incubated with OmpU or vesicles (10 µg/ml) for 24 h were subjected to Annexin V-FITC/PI staining and further analyzed by flow cytometry. Annexin binds to phosphatidyl serine and PI stains necrotic cells. The dot plot has four quadrants; the upper left represents necrotic population which is PI positive. The upper right quadrant represents necrotic and late apoptotic population which is double positive. The lower right quadrant represents the early apoptotic population which is Annexin V positive. The extent of early apoptosis induced by 10 µg/ml of vesicles is 9% and late apoptotic changes 10%, whereas, 10 µg/ml of purified OmpU induced early apoptotic changes in 8% and late apoptotic changes in 8% and late apoptotic changes in 9% of cells.

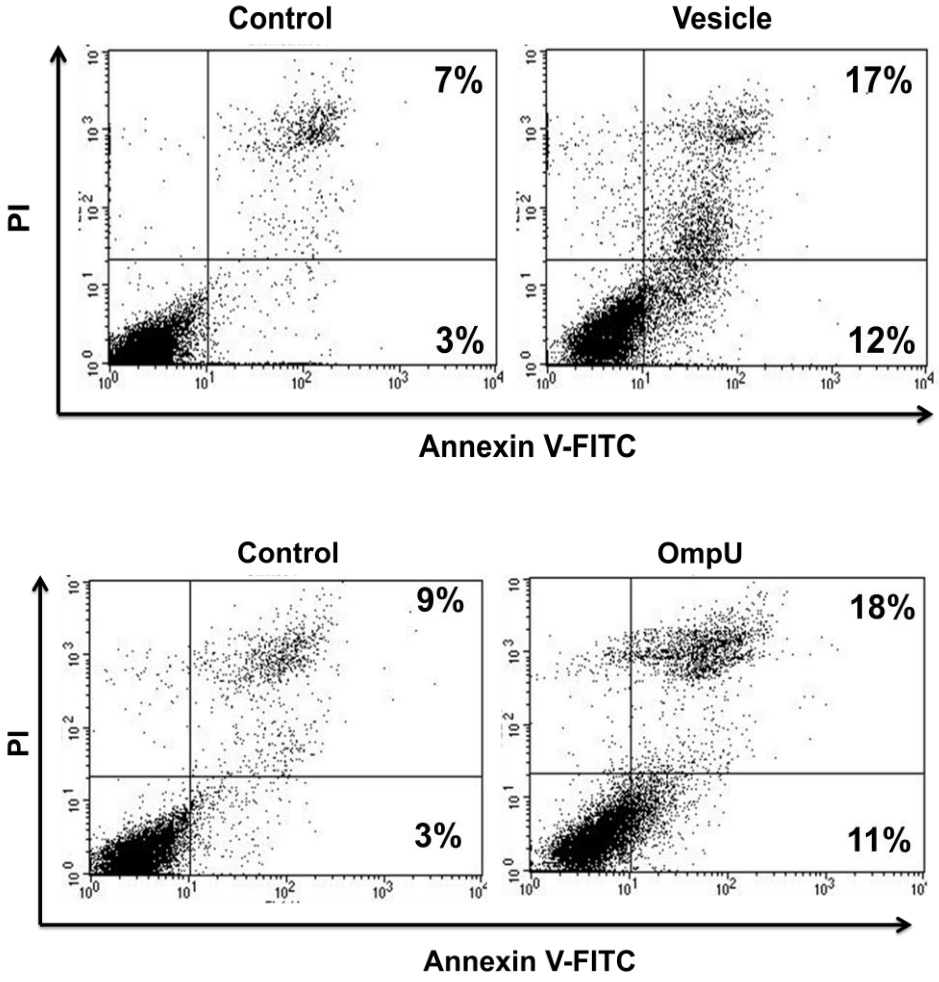

Fig.14-Resulting dot plots show that purified OmpU and vesicles induce almost equal extent of apoptosis in target cell

Discussion

The importance of *V. cholerae* porin OmpU in pathogenesis is highlighted by the fact that it confers resistance against bile acids and certain host anti-microbial peptides [31]. These functions help the bacteria for well survival in the gut. Our laboratory has been working on probing the host immune-modulatory effects of OmpU. Towards, these aim our laboratory has some observations with purified OmpU. As OmpU is an outer rmembrane protein to put the study on physiological basis, our laboratory is interested whether natural vesicle can be one of the means used by the bacteria for OmpU-mediated cell modulation. My study focussed on whether OmpU is secreted in naturally secreted vesicles and if it is secreted then can it affect host cell function.

To determine whether OmpU expression in naturally secreted vesicles differs with different types of media, *V. cholerae* was inoculated in BHI broth, LB broth and BHI supplemented with 0.05% deoxycholate. As, it was reported, the expression of OmpU increases with increase in salt concentration and bile salts [31] we added deoxycholate to the culture media to check whether OmpU expression in vesicle becomes enriched. We observed that vesicle production by bacteria is maximum in the late log phase of the bacterial growth cycle. Therefore, growth kinetics of *V. cholerae* in different media was determined (Fig. 4, Fig 5, Fig 6) and the late log phase occurred at 11 h in all the types of media. At 11 h, vesicles were isolated and protein profile was analyzed by SDS-PAGE. A 38 kDa band indicative of OmpU was observed in naturally secreted vesicles (Fig. 7) and is one of the major proteins present in vesicles as well. Further, by western blotting, the presence of OmpU was confirmed and the amount of OmpU in naturally secreted vesicles did not vary with different media used (Fig. 8).

Purified OmpU translocates to the mitochondria *(as observed in the lab)* and the next question which we wanted to answer was whether, OmpU present in naturally secreted vesicles is able to do so as well. First, we observed that OmpU from vesicles enters target cells and is present upto 24 h (Fig. 10) as revealed by western blot analysis of whole cell lysates. Further, presence of OmpU was confirmed in mitochondrial fraction of target cells upto 24 h after treatement of cells with vesicles (Fig. 11).

Further, we wanted to probe whether vesicles are intact after entering cells or whether OmpU present in the vesicles alone enters the cells. To probe this aspect, we chose

another protein present in the vesicle. We showed that *V. cholerae* cytolysin (VCC) is present in vesicles (Fig. 9) and VCC is intact in the mitochondria of treated cells at least upto 4 h suggesting that vesicle as a whole enters the host cells and probably after 4 h the vesicle disintegrates (Fig. 12). Further the observation that OmpU stays in the mitochondria for only 4 h when cells treated with purified OmpU suggests that vesicle mediated delivery gives more stability to OmpU inside the host cell.

Further, another colleague in the lab observed that purified OmpU induces apoptosis of host cells. It induces flipping of phosphatidyl serine from inner membrane to outer membrane of host cell which is a characteristic feature of programmed for cell death. Therefore, we wanted to probe whether OmpU present in the vesicle can induce apoptosis. Results indicate that naturally secreted vesicles and purified OmpU) induce similar extent of phosphatidyl serine flipping (Fig. 14), suggesting probably OmpU alone is responsible for vesicle-mediated cell death.

In conclusion, this study confirmed that OmpU is present in naturally secreted vesicles. Our study also revealed that OmpU is able to translocate to the host cell mitochondria from the naturally secreted vesicle. We also confirmed that apoptosis is induced in vesicle-treated cells and is probably due to the presence of OmpU in the vesicle.

Bibliography

1 Benz, R., and R. E. W. Hancock. 1981. Properties of large ion permeable pores formed from protein F of Pseudomonas aeruginosa in lipid bilayer membranes. Biochim. Biophys. Acta 646:293-308.

2 Nikaido, H., and E. Y. Rosenberg. 1981. Effect of solute size on diffusion rates through the transmembrane pores of the outer membrane of Escherichia coli. J. Gen. Physiol. 77:121-135.

3 Nikaido, H., E. Y. Rosenberg, and J. Foulds. 1983. Porin channels in Escherichia coli: studies with P-lactams in intact cells. J. Bacteriol. 153:232-240.

4 Nikaido, H., and M. Vaara. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. 49:1-32.

5 Lee, Y., El Andaloussi, S. & Wood, M. J. A. Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy. *Hum. Mol. Genet.* 21, R125–R134 (2012).

6 McBroom, A., and M. J. Kuehn. 12 May 2005, posting date. Chapter 2.2.4, Outer membrane vesicles. *In* R. Curtiss III et al. (ed.), EcoSal—Escherichia coli and Salmonella: cellular and molecular biology. ASM Press, Washington, DC.

7 Chatterjee, S. N., and J. Das. 1967. Electron microscopic observations on the excretion of cell-wall material by *Vibrio cholerae*. J. Gen. Microbiol. 49:1–11.

8 Devoe, I. W., and J. E. Gilchrist. 1973. Release of endotoxin in the form of cell wall blebs during *in vitro* growth of *Neisseria meningitidis*. J. Exp. Med. 138:1156–1166.

9 Dorward, D. E., C. F. Garon, and R. C. Judd. 1989. Export and intercellular transfer of DNA via membrane blebs of *Neisseria gonorrhoeae*. J. Bacteriol. 171:2499–2505.

10 Kadurugamuwa, J. L., and T. J. Beveridge. 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.

11 Kondo, K., A. Takade, and K. Amako. 1993. Release of outer membrane vesicles from *Vibrio cholerae* and *Vibrio parahaemolyticus*. Microbiol. Immunol. 37:149–152

12 Wai, S. M., A. Takade, and K. Amako. 1995. The release of outer membrane vesicles from the strains of enterotoxigenic *Escherichia coli*. Microbiol. Immunol. 39:451–456.

13 classical strain 569B: evidence for the formation of large cation-selective ion permeable

channels by OmpU, Microbiologia 13 (1997) 321–330.

14] S.R. Chakrabarti, K. Chaudhuri, K. Sen, J. Das, Porins of Vibrio cholerae: purification and characterization of OmpU, J. Bacteriol. 178 (1996) 524–530

15 N. Nakasone, M. Iwanaga, Characterization of outer membrane protein OmpU of Vibrio cholerae O1, Infect. Immun. 66 (1998) 4726–4728

16 V. Sperandio, C. Bailey, J.A. Giron, V.J. DiRita, W.D. Silveira, A.L. Vettore, J.B. Kaper, Cloning and characterization of the gene encoding the OmpU outer membrane protein of Vibrio cholerae, Infect. Immun. 64 (1996) 5406–5409

17 V. Sperandio, J.A. Giron, W.D. Silveira, J.B. Kaper, The OmpU outer membrane protein, a potential adherence factor of Vibrio cholerae, Infect. Immun. 63 (1995) 4433–4438.

18 Wyllie A. Apoptosis. An endonuclease at last. Nature 1998; 391: 20-1.

19 Lopaczynski W, Zeisel SH. Antioxidants, programmed cell death, and cancer. Nutr Res 2001; 21: 295-307.

20 J. Mathur, M.K. Waldor, The Vibrio cholerae ToxR-regulated porin OmpU confers resistance to antimicrobial peptides, Infect. Immun. 72 (2004) 3577– 3583.

21] J.A. Wibbenmeyer, D. Provenzano, C.F. Landry, K.E. Klose, A.H. Delcour, Vibrio cholerae OmpU and OmpT porins are differentially affected by bile, Infect. Immun. 70 (2002) 121–126

22 V. Sperandio, J.A. Giron, W.D. Silveira, J.B. Kaper, The OmpU outer membrane protein, a potential adherence factor of Vibrio cholerae, Infect. Immun. 63 (1995) 4433–4438.

23 J.A. Wibbenmeyer, D. Provenzano, C.F. Landry, K.E. Klose, A.H. Delcour, Vibrio cholerae OmpU and OmpT porins are differentially affected by bile, Infect. Immun. 70 (2002) 121–126.

24 V.C. Simonet, A. Basle, K.E. Klose, A.H. Delcour, The Vibrio cholerae porins OmpU and OmpT have distinct channel properties, J. Biol. Chem. 278 (2003) 17539–17545

25 Koebnik, R., Locher, K.P. and Van Gelder, P. (2000) Structure and function of bacterial outer membrane proteins: barrels in a nutshell. Mol. Microbiol. 37, 239^253.

26 Cowan, S.W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R.A., Jansonius, J.A. and Rosenbusch, J.P. (1992) Crystal structures explain functional properties of two E. coli porins. Nature 358, 727^733.

27 Weiss, M.S. and Schulz, G.E. (1992) Protein, Structure of porin re- ¢ned at 1.8 Aî resolution. J. Mol. Biol. 227, 493^509.

29 Nikaido, H. (1996) Outer membrane. In: Escherichia coli and Salmonella, Cellular and Molecular Biology (Neidhardt, F.C., Ed.), pp. 29^ 47. ASM Press, Washington, DC.

30 Koebnik, R., Locher, K.P. and Van Gelder, P. (2000) Structure and function of bacterial outer membrane proteins: barrels in a nutshell. Mol. Microbiol. 37, 239^253.

31 *Vibrio cholerae* OmpU and OmpT Porins Are Differentially Affected by Bile Jamie A. Wibbenmeyer,1 Daniele Provenzano,2† Candice F. Landry,1 Karl E. Klose,2 and Anne H. Delcour1*

32 Beveridge, T. J. 1999. Structures of gram-negative cell walls and their derived membrane vesicles. J. Bacteriol. 181:4725–4733.

33 Mayrand, D., and D. Grenier. 1989. Biological activities of outer membrane vesicles. Can. J. Microbiol. 35:607–613.

34 Chatterjee, S. N., and J. Das. 1967. Electron microscopic observations on the excretion of cell-wall material by *Vibrio cholerae*. J. Gen. Microbiol. 49:1–11.

35 Devoe, I. W., and J. E. Gilchrist. 1973. Release of endotoxin in the form of cell wall blebs during *in vitro* growth of *Neisseria meningitidis*. J. Exp. Med. 138:1156–1166.

36 . Dorward, D. E., C. F. Garon, and R. C. Judd. 1989. Export and intercellular transfer of DNA via membrane blebs of *Neisseria gonorrhoeae*. J. Bacteriol. 171:2499–2505.

37 Kadurugamuwa, J. L., and T. J. Beveridge. 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.

38 Kondo, K., A. Takade, and K. Amako. 1993. Release of outer membranevesicles from *Vibrio cholerae* and *Vibrio parahaemolyticus*. Microbiol. Immunol.37:149–152

39 Wai, S. M., A. Takade, and K. Amako. 1995. The release of outer membranevesicles from the strains of enterotoxigenic *Escherichia coli*. Microbiol. Immunol. 39:451–456.

40 Bauman, S. J., and M. J. Kuehn. 2006. Purification of outer membrane Vvesicles from Pseudomonas aeruginosa and their activation of an IL-8 response. Microbes Infect. 8:2400–2408.

41 Gankema, H., J. Wensink, P. A. Guinee, W. H. Jansen, and B. Witholt. 1980. Some characteristics of the outer membrane material released by growing enterotoxigenic Escherichia coli. Infect. Immun. 29:704–713

42 Wensink, J., and B. Witholt. 1981. Outer-membrane vesicles released by normally growing Escherichia coli contain very little lipoprotein. Eur. J. Biochem. 116:331–

335

43 Devoe, I. W., and J. E. Gilchrist. 1973. Release of endotoxin in the form of cell wall blebs during in vitro growth of Neisseria meningitidis. J. Exp. Med. 138:1156–1167

44 Schooling, S. R., and T. J. Beveridge. 2006. Membrane vesicles: an overlooked component of the matrices of biofilms. J. Bacteriol. 188:5945–5957

45 Anthony, L. D., R. D. Burke, and F. E. Nano. 1991. Growth of Francisella spp. in rodent macrophages. Infect. Immun. 59:3291–3296.

46 Fernandez-Moreira, E., J. H. Helbig, and M. S. Swanson. 2006. Membrane vesicles shed by Legionella pneumophila inhibit fusion of phagosomes with lysosomes. Infect. Immun. 74:3285–3295

47 Garcia-del Portillo, F., M. A. Stein, and B. B. Finlay. 1997. Release of lipopolysaccharide from intracellular compartments containing Salmonella typhimurium to vesicles of the host epithelial cell. Infect. Immun. 65:24–34.

48 Golovliov, I., V. Baranov, Z. Krocova, H. Kovarova, and A. Sjostedt. 2003. An attenuated strain of the facultative intracellular bacterium Francisella tularensis can escape the phagosome of monocytic cells. Infect. Immun. 71:5940–5950.

49 Kondo, M., K. Kawai, K. Yagyu, K. Nakayama, K. Kurohara, and S. Oshima. 2001. Changes in the cell structure of Flavobacterium psychrophilum with length of culture. Microbiol. Immunol. 45:813–818

50 McBroom, A., and M. J. Kuehn. 12 May 2005, posting date. Chapter 2.2.4, Outer membrane vesicles. *In* R. Curtiss III et al. (ed.), EcoSal—Escherichia coli and Salmonella: cellular and molecular biology. ASM Press, Washington, DC.

51 Schooling, S. R., and T. J. Beveridge. 2006. Membrane vesicles: an overlooked component of the matrices of biofilms. J. Bacteriol. 188:5945–5957.

52 Anthony, L. D., R. D. Burke, and F. E. Nano. 1991. Growth of Francisella spp. in rodent macrophages. Infect. Immun. 59:3291–3296

53 Fernandez-Moreira, E., J. H. Helbig, and M. S. Swanson. 2006. Membrane vesicles shed by Legionella pneumophila inhibit fusion of phagosomes with lysosomes. Infect. Immun. 74:3285–3295.

54 Garcia-del Portillo, F., M. A. Stein, and B. B. Finlay. 1997. Release of lipopolysaccharide from intracellular compartments containing Salmonella typhimurium to vesicles of the host epithelial cell. Infect. Immun. 65:24–34.

55 Ciofu, O., T. J. Beveridge, J. Kadurugamuwa, J. Walther-Rasmussen, and N. Hoiby. 2000. Chromosomal beta-lactamase is packaged into membrane vesicles and secreted from Pseudomonas aeruginosa. J. Antimicrob. Chemother. 45:9–13

56 Dutson, T. R., A. M. Pearson, J. F. Price, G. C. Spink, and P. J. Tarrant. 1971. Observations by electron microscopy on pig muscle inoculated and incubated with Pseudomonas fragi. Appl. Microbiol. 22:1152–1158.

57 Thompson, S. S., Y. M. Naidu, and J. J. Pestka. 1985. Ultrastructural localization of an extracellular protease in Pseudomonas fragi by using the peroxidase-antiperoxidase reaction. Appl. Environ. Microbiol. 50: 1038–1042.

58 Hoekstra D, van der Laan JW, de Leij L, Witholt B. Release of outer membrane fragments from normally growing Escherichia coli. Biochim. Biophys. Acta. 1976; 455:889–899. [PubMed: 793634] Performed the first thorough analysis of OMVs to determine their origin

59 Gankema, H., J. Wensink, P. A. Guinee, W. H. Jansen, and B. Witholt1980. Some characteristics of the outer membrane material released by growing enterotoxigenic Escherichia coli. Infect. Immun. 29:704–713.

60 Horstman, A. L., and M. J. Kuehn. 2000. Enterotoxigenic Escherichia coli secretes active heat-labile enterotoxin via outer membrane vesicles. J. Biol.Chem. 275:12489–12496.

61 Wai, S. N., A. Takade, and K. Amako. 1995. The release of outer membrane vesicles from the strains of enterotoxigenic Escherichia coli. Microbiol. Immunol. 39:451–456.

62 138. Wensink, J., H. Gankema, W. H. Jansen, P. A. Guinee, and B. Witholt. 1978. Isolation of the membranes of an enterotoxigenic strain of Escherichia coli and distribution of enterotoxin activity in different subcellular fractions. Biochim. Biophys. Acta 514:128–136.

63 Demuth, D. R., D. James, Y. Kowashi, and S. Kato. 2003. Interaction of Actinobacillus actinomycetemcomitans outer membrane vesicles with HL60 cells does not require leukotoxin. Cell. Microbiol. 5:111–121.

64 Kato, S., Y. Kowashi, and D. R. Demuth. 2002. Outer membrane-like vesicles secreted by Actinobacillus actinomycetemcomitans are enriched in leukotoxin. Microb. Pathog. 32:1–13.

65 Nowotny, A., U. H. Behling, B. Hammond, C. H. Lai, M. Listgarten, P. H. Pham, and F. Sanavi. 1982. Release of toxic microvesicles by Actinobacillus actinomycetemcomitans. Infect. Immun. 37:151–154

66 Khandelwal, P., and N. Banerjee-Bhatnagar. 2003. Insecticidal activity associated with the outer membrane vesicles of Xenorhabdus nematophilus. Appl. Environ. Microbiol. 69:2032–2037.

67 Balsalobre, C., J. M. Silvan, S. Berglund, Y. Mizunoe, B. E. Uhlin, and S. N. Wai. 2006. Release of the type I secreted alpha-haemolysin via outer membrane vesicles from Escherichia coli. Mol. Microbiol. 59:99–112

68 Wai, S. N., A. Takade, and K. Amako. 1995. The release of outer membranevesicles from the strains of enterotoxigenic Escherichia coli. Microbiol. Immunol. 39:451–456

69 Wensink, J., H. Gankema, W. H. Jansen, P. A. Guinee, and B. Witholt.1978. Isolation of the membranes of an enterotoxigenic strain of Escherichiacoli and distribution of enterotoxin activity in different subcellular fractions. Biochim. Biophys. Acta 514:128–136.

70 Demuth, D. R., D. James, Y. Kowashi, and S. Kato. 2003. Interaction ofActinobacillus actinomycetemcomitans outer membrane vesicles with HL60 cells does not require leukotoxin. Cell. Microbiol. 5:111–121.

71 Kato, S., Y. Kowashi, and D. R. Demuth. 2002. Outer membrane-like vesicles secreted by Actinobacillus actinomycetemcomitans are enriched in leukotoxin. Microb. Pathog. 32:1–13

72 Nowotny, A., U. H. Behling, B. Hammond, C. H. Lai, M. Listgarten, P. H. Pham, and F. Sanavi. 1982. Release of toxic microvesicles by Actinobacillus actinomycetemcomitans. Infect. Immun. 37:151–154.

73 Masignani, V., E. Balducci, F. Di Marcello, S. Savino, D. Serruto, D. Veggi, S. Bambini, M. Scarselli, B. Arico, M. Comanducci, J. Adu-Bobie, M. M. Giuliani, R. Rappuoli, and M. Pizza. 2003. NarE: a novel ADP-ribosyltransferase from Neisseria meningitidis. Mol. Microbiol. 50:1055–1067.

74 Berlanda Scorza, F., F. Doro, M. J. Rodriguez-Ortega, M. Stella, S. Liberatori, A. R. Taddei, L. Serino, D. Gomes Moriel, B. Nesta, M. R. Fontana, A. Spagnuolo, M. Pizza, N. Norais, and G. Grandi. 2008. Proteomics characterization of outer membrane vesicles from the extraintestinal pathogenic Escherichia coli deltatolR IHE3034 mutant. Mol. Cell. Proteomics 7:473–485

75 Kadurugamuwa, J. L., and T. J. Beveridge. 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.

76 89. Li, Z., A. J. Clarke, and T. J. Beveridge. 1998. Gram-negative bacteria produce membrane vesicles which are capable of killing other bacteria. J. Bacteriol. 180:5478–5483

77 MacEachran, D. P., S. Ye, J. M. Bomberger, D. A. Hogan, A. Swiatecka-Urban, B. A. Stanton, and G. A. O'Toole. 2007. The Pseudomonas aeruginosa secreted protein PA2934 decreases apical membrane expression of the cystic fibrosis transmembrane conductance regulator. Infect. Immun. 75: 3902–3912.

78 Mashburn, L. M., and M. Whiteley. 2005. Membrane vesicles traffic signalsand facilitate group activities in a prokaryote. Nature 437:422–425.

79 Renelli, M., V. Matias, R. Y. Lo, and T. J. Beveridge. 2004. DNA-containing membrane vesicles of Pseudomonas aeruginosa PAO1 and their genetictransformation potential. Microbiology 150:2161–2169

80 Association of cholera toxin with Vibrio cholerae outer membrane vesicles which are internalized by human intestinal epithelial cells Debashree Chatterjee, Keya Chaudhuri ⇑ D. Chatterjee, K. Chaudhuri / FEBS Letters 585 (2011) 1357–1362

81 Refolding and functional assembly of the Vibrio cholerae porin OmpU recombinantly expressed in the cytoplasm of Escherichia coli J. Khan et al. / Protein Expression and Purification 85 (2012) 204–210