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The PhD Thesis of Mrs. Irina Kudryakova focuses on the mechanism of biogenesis of outer-membrane derived vesicles (OMVs) from *Lysobacter sp.* XL1, development and pilot applications of a principally new liposome nanocarrier-based antimicrobial drug formulations. Almost all Gram-negative bacteria studied to date release OMVs in every stage of growth and in a range of different environmental conditions. Originating from the cell envelope, OMVs are spherical bilayered phospholipids, approximately 10 to 250 nm in diameter, composed of a proteins, LPS and phospholipids, composition and properties of which are still poorly understood. OMVs are very often served to concentrate and transfer virulence factors of protein origins (cytotoxic hemolytic toxins, proteases etc.) for focused delivery to target host cells. Such cargo proteins within OMVs are obviously protected from degradation and/or recognition by infected host. While biochemical and biological approaches have been utilized in OMV studies, little attention has been given to the characterization of individual lipid composition determining physio-chemical properties of OMVs. To understand the mechanism of generation of OMVs, the role of individual phospholipids and cargo protein (s) (e.g. encapsulated protease itself) must be clarified comprehensively. The PhD Thesis proposal and research objectives on isolation, thoroughfull morphological, biochemical and immunochemical characterization of OMVs from *Lysobacter sp.* XL1, structural analysis of cargo protein(s) at high and low resolution by X-ray crystallography and electron microscopy respectively as such as all functional assays are very reasonable and allowed to conduct a comparative study of native and recombinant L5 proteases liberated from *Lysobacter sp* XL1 and *Pseudomonas fluorescens* respectively by mean of vesicular secretion and envelope remodeling. The author utilized well established biochemical preparative and analytical methods, different microbiological techniques and assays, recombinant bacterial cell technology, as such as state of the art methods (X-ray crystallography, electron microscopy with most contemporary immunocytochemical application) to isolate and characterize OMVs and test a mechanism of their biogenesis and probe the structure and function of its cargo protein (L5 protease) at a defined level of molecular complexity of bacterial envelope of Gram-negative bacteria. In this excellent and novel study, author successfully analyzed the phospholipid and protein composition of isolated OMVs to gain insight into the biogenesis of OMVs itself as such as individual lipid and protein topogenesis within bacterial envelope and physiological roles of secreted vesicles.

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The experimental aspects of this work are of very high quality.

I would like to emphasize several very important and novel findings:

1. The vesicular secretion of L5 protease as main OMV cargo protein is associated with morphologically distinguished OMVs (Figs 1 and 3).

2. To the best of my knowledge this PhD thesis is the one of the first to describe unique characteristics of OMVs in terms of phospholipid composition. L5 protease filled OMVs (Fig. 2b) consists mostly from cardiolipin (CL) (Fig. 7), a phospholipid with unique structural and physico-chemical properties! It is known that the phospholipid composition of *Escherichia coli* OMVs previously reported by Horstman et al. appears to be similar to that of the outer membrane (OM) where these vesicles derived from (Horstman and Kuehn, J. Biol. Chem., 2000, 275, 12489) while lipid composition of OMVs (mostly phosphatidylglycerol) was quite different from *Pseudomonas aeruginosa* cellular OM made mostly from phosphatidylethanolamine (Tashiro et al., Biosci. Biotechnol. Biochem., 2011, 75, 605).

3. By taking an advantage of heterologous expression of *Lysobacter sp.*XL1 L5 protease in *Pseudomonas fluorescens* (it is very clever experiment!) (Fig. 4) PhD Candidate described a novel mechanism of generation of OMV by this bacterium (Fig. 8). The author demonstrated that cargo protein itself can induce formation of OMVs via interaction with periplasmic leaflet of the OM at specific membrane loci potentially composed from specific phospholipids. This finding has broad significance to mechanism of biogenesis of OMVs in context of functioning and remodeling of bacterial envelope of Gram-negative bacteria as self-repairing and self-organizing "organelle".

4. The PhD Candidate demonstrate that this potential OMV forming ability of L5 protease can be due by unique tertiary and quaternary structure-forming features and abilities of this protein (Fig. 9). Like most amyloidogenic proteins L5 protease polymerizes and form fibers (Fig. 11a) with overall cross β-strand structure (Fig. 11 b). PhD Candidate suggested that this conformational duality of protease L5 may provide "do not eat me" mechanism which may prevent a destructive action of this serine protease acting as glycyl-glycine endopeptidase and N-acetylmuramoyl-L-alanine amidase (Table 3) on its own peptidoglycan (PG) in the periplasm of *Lysobacter sp. XL1*. This unique ability of L5 protease can be considered although a new protein quality control system: promoting protein insolubility and aggregation instead of overcommitted proteolysis of aggregation-prone polypeptides in the periplasm of Gram-negative bacteria.

5. Bacteriolytic effect of protease L5-filled OMVs on wide spectra of Gram-negative bacteria and Gram-positive bacteria (Table 2) demonstrate that Gram-negative bacteria may utilize their own OMVs to achieve intra- and interspecies antagonistic interaction and competition.

6. The using of liposomes made from OMV derived phospholipids (e.g. CL) loaded with protease opens a new avenue to novel antimicrobial drug strategies (Table 4).

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It is important to conclude that author answered both experimentally and theoretically the questions which were asked in Experimental Aims of PhD Thesis. I would like to raise only several questions which are related to these Aims specifically and future directions of this project in general.

1. What is lipid composition of OMVs liberated by *Pseudomonas fluorescence* heterologously expressed L5 protease from *Lysobacter sp.* XL1?

2. Whether PbgA protein (a specific CL-translocase recently identified in Gram-negative bacteria as a complex that may bridge the envelope for regulated CL delivery from inner membrane to OM, Dalebroux et al., Cell Host Microbe 2015, 17:441) is involved in delivery of CL to periplasmic leaflet of OM?

3. PhD Candidate attracted unique chemical and structural properties rather than collective physical properties of CL to explain its potential contribution to mechanism of generation of OMVs and change in the rigidity of OM. However, CL is non-bilayer lipid, owing to a high spontaneous curvature of their monolayers towards water. Introducing CL into periplasmic leaflet of OM increases the monolayer curvature but as the monolayers are held flat in the bilayer, the stored curvature stress increases and therefore could serve as a driving force for generation of OMV. This mechanism can be one of the major and alternative factor promoting specific targeting and localization of proteins at membrane loci enriched with CL as high-intrinsic-curvature lipid. Protease L5 can be one of these curvature-sensing proteins (in the accordance with Mukhopadhyay et al., Biophys J. 2008 95: 1034)

Further investigations dissecting the effects of individual lipid and cargo protein properties are likely to be illuminating in elucidating the key factors for regulating the OMV biogenesis.

1. Whether localization of L5 protease coincide with CL-rich domains within envelope of *Lysobacter sp.* XL? To visualize, localize and merge a L5 protease with CL microdomains, L5 protease tagged at N-terminus with tetracysteine motif MCCPGCC and subsequent combined staining of the *Lysobacter sp.* XL cells with a membrane-permeant fluorescein derivative FlAsH-EDT2 fluorescing in the green and CL-specific NAO dye (10-N -nonyl-3,6-bis (dimethylamino)acridine) fluorescing in the red can be utilized in experiments dedicated to testing a model proposed in Fig. 8.

2. It is widely accepted that β-sheet rich amyloid fibers bind to dyes such as thioflavin T (ThT) and Congo red (CR) which can be therefore used to study the conversion of soluble amyloidogenic monomeric proteins to an ordered amyloid fiber *in vitro* or visualization of these structures in vivo. Thus, aggregation rate of L5 protease can be measured *in vitro* by monitoring changes in yield of ThT fluorescence in the presence or absence of CL–containing liposomes while CR as an amyloid-binding dye can be used to detect the presence of fibers in periplasm of EDTA treated *Lysobacter sp.* XL1 cells (in the accordance with Reichhardt et al., PLoS One. 2015, 10:e0140388)

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It is important to conclude that PhD Candidate answered both experimentally and theoretically all questions which were asked in Experimental Aims of PhD Thesis.

In summary Mrs. Irina Kudryakova designed and performed crucial experiments that resulted in major advances towards our understanding of mechanism of OMV biogenesis in Gram-negative bacteria. The results also have important implications for development of improved antimicrobial agents and novel diagnostic tests.

Because of all above I strongly recommend the granting of Doctor of Philosophy (PhD) Degree to Mrs. Irina Kudryakova.

Sincerely yours,

MBogdanov

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