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Review

of doctoral thesis **Akshay Maniyeri Suresh'a. MA**

„Regulation of cel envelope homeostasis by Lap proteins in *Escherichia coli*"

Akshay Maniyeri Suresh's doctoral dissertation was written under the supervision of Professor Satish Raina, PhD, in the Laboratory of Bacterial Genetics at the Faculty of Chemistry, Gdańsk University of Technology. His supervisor is a leading expert in the biogenesis of lipopolysaccharide (LPS) and phospholipids (PL) of the cell envelopes of Gram-negative bacteria. Professor S. Raina's and his research team's works revealed connections in the synthesis of these two components of the outer membrane and cell membrane of these bacteria, particularly in the role of the LapB and LapC proteins in these processes. PhD student has decided to take on and continue this research. The aim of his doctoral dissertation is to obtain results that would deepen our knowledge on the regulation of LPS biosynthesis in *E. coli*, including, above all, a more comprehensive understanding of the role of the LapC and LapD proteins in the complex regulating this phenomenon.

The structure of Akshay Maniyeri Suresh's doctoral dissertation is typical for experimental PhD theses. It comprises the following sections: Introduction (theoretical part), Aim and Scope of the Work with Justification, Materials and Methods, Results, Discussion, Conclusions, and a Reference List. A list of abbreviations, abstracts in Polish

and English, and lists of figures and tables are also enclosed. The dissertation also includes four multi-authored publications, all co-authored with the doctoral candidate, published in the *International Journal of Molecular Sciences*, elaborating on his research results.

In a well-developed introduction, the PhD candidate provides an overview of the cell envelope of Gram-negative bacteria. Particular attention is paid to the outer membrane (OM), including its asymmetric structure and its function as a permeability barrier. The role of outer membrane proteins and their biogenesis using the BAM (β-Barrel Assembly Machine) complex are also discussed. The importance of lipoproteins in the integrity of the OM and its other properties is also described. The process of phospholipid biosynthesis is characterized, as well as their translocation from the cell membrane to the outer membrane via the Lol system. A large section of the Introduction is devoted to LPS, overviewing its three regions in terms of chemistry and biological significance: lipid A (LA), the core region, and the O-antigen. The biosynthesis of the Kdo-LA region according to the Reatz pathway is presented in detail, along with the role of FtsH and LapB proteins in regulating the biosynthesis of this region and its connections to PL biosynthesis. It has been established that the 3-hydroxymyristic acid residue attached to the ACP (Acyl Carrier Protein) protein is a common substrate for both the PL biosynthetic pathways and the Kdo-LA region of LPS. The author also underlines the importance of the LapC protein in regulation of the biosynthesis of the Kdo-LA region. The next section of the Introduction presents further stages of LPS formation, including the role of the MsbA flipase, which transports LPS across the cell membrane into the periplasmic space of the bacterial cell, and the Lpt (LPS transport) proteins system, which transmits LPS through the periplasmic space between the cell membrane and the outer membrane of Gram-negative bacteria. At the end of the review of LPS the author presents a characterization of the glycoforms of the *E. coli* core region and a brief overview of the biological significance of LPS as an endotoxin.

Phospholipids of Gram-negative bacteria are also extensively described, including their location in the cell membrane and the inner portion of the asymmetric outer membrane. The author presents the PL biosynthetic pathway, starting with its precursors, glycerol-3-phosphate and fatty acids. The Mla pathway – responsible for

the removal of misplaced PLs in the outer membrane and their translocation to the cell membrane – is also described in detail. The structure of *E. coli* peptidoglycan, the stages of its biosynthesis, and its translocation to the periplasm are also briefly discussed. The introduction concludes with a chapter on the σ^E regulon and its role in the biosynthesis of LPS, PLs, as well as other components of bacterial cell envelopes.

In my opinion, the Introduction, as the theoretical part of the doctoral dissertation, has been prepared appropriately. It provides an overview of data on the bacterial cell wall components whose biogenesis is the subject of the doctoral student's research. The information provided in this part of the PhD thesis is supplemented by figures illustrating the investigated processes and phenomena. This section of the dissertation demonstrates the PhD candidate's excellent theoretical preparation for the experimental part.

The aim of Akshay Maniyeri Suresh's doctoral thesis is to deepen our understanding of the regulation of *E. coli* LPS biosynthesis, particularly to better grasp the role of the LapC and LapD proteins interacting with LapB, a key protein in controlling homeostasis, i.e., the balance between the biosynthesis of LPS, PL and other cell wall components in Gram-negative bacteria. This research goal is further substantiated in the next chapter of the dissertation. PhD student noted, among others, that the mechanism underlying the modulatory action of LapB on FtsH and the way it influences LPS production is not fully understood. Furthermore, the network of proteins acting in the environment of LapB is likely more extensive than assumed so far. Therefore, it was crucial to investigate the role of LapD, given its interactions with other proteins, as indicated by co-purification and genetic studies.

In order to demonstrate the functional network in which LapD operates, the extragenic suppressor analysis was applied. Additionally, a multicopy suppressor was used to identify genes necessary to overcome phenotypic defects in bacteria with a knockout of the *lapD* gene. Simultaneously, the consequences of the lack of LapC protein function in the LPS biosynthesis regulatory system were examined. All research tasks were properly justified, demonstrating the complexity of the problems related to LPS and PL biosynthesis and a logical perspective to their explanation. Various research approaches applied in this doctoral dissertation are presented in two diagrams at the

end of the research justification section. The planned experiments created the basis for research tasks of the whole doctoral dissertation.

Throughout the thesis a wide range of research materials were used (strains, plasmids, and oligonucleotides) to complete research tasks. These are summarized in tables, as are the culture media and other materials (buffers, gels, solutions, and chemical reagents).

Research methods are discussed in Chapter 3.2. and they are corresponding appropriately with research objectives. They are presented in a detailed and comprehensive way. The extensive range of methods applied in the research is worth emphasizing, including bacterial transformation and transduction, cell and outer membrane separation, LPS extraction and analysis using mass spectrometry, fatty acid isolation and analysis, protein purification and analysis using SDS-PAGE and Western blotting, as well as genetic methods (gene deletion, cloning, isolation and mapping of extragenic suppressors, and transposon mutation).

The research results were carefully compiled and presented in tables and figures. Data obtained from members of the supervisor's research team were also used, as noted in the dissertation. The most important results of the doctoral student's dissertation include among others:

1. Demonstration of coelution of the LapD protein with other proteins involved in LPS biogenesis and transport, as well as PL biosynthesis.

2. Finding that in bacteria with LapD protein synthesis deactivated (*ΔlapD*), LpxC levels decrease at higher temperatures.

3. Statement that deletion of the *waaC* and *clsA* genes in the *lapD* mutant, encoding the WaaC (links Hep to Kdo_2-LA) and ClsA (involved in cardiolipin synthesis), respectively, inhibits bacterial growth at higher temperatures. This indicates the importance of the LapD protein as one of the coordinating factors in LPS biogenesis and PL homeostasis, which translates into the stability and function of the OM in *E. coli.*

4. Demonstration of the association of the LapD protein with peptidoglycan biosynthesis.

5. Demonstration of the role of the LapD protein in the efficient translocation of LPS from the cell membrane to the outer membrane and PL biosynthesis.

6. Discovery of a functional relationship between the LapD and LapC proteins.

In summary, I would like to underline that the PhD student achieved the stated goals of the PhD thesis: to expand our understanding of the role of the LapD and LapC proteins, which interact with the LapB protein, in regulation of LPS biosynthesis in *E. coli*. The author emphasized the role of LapD and LapC proteins as integrating factors that cohere the Lap regulatory system and their importance in maintaining the homeostasis of the outer membrane structures of these Gram-negative rods. A particular achievement of Akshay Maniyeri Suresh, M.Sc., is his demonstration that the LapD protein is at the centre of a functional complex connecting the main pathways of *E. coli* cell membrane with cell wall biogenesis. It is associated with LPS biosynthesis and its transfer to the bacterial outer membrane, as well as with fatty acid biosynthesis, PL metabolism, and peptidoglycan biosynthesis. The PhD student discovered these relationships, which allowed him to propose a supplemented model of the regulation of *E. coli* cell envelope biogenesis (Fig. 42), including the synthesis of PL and LPS. In this model, the LapB/C/D complex is at the centre of this regulatory system. The LapB protein, through the FtsH protein, degrades LpxC, inhibiting LPS synthesis. This enables the use of the β-hydroxy-C14-ACP complex, as a precursor in PL synthesis, while the LapD and LapC proteins (as indicated in the figure) have influence on both coupled pathways of LPS and PL biosynthesis.

The discussion was conducted very efficiently, both in terms of a critical perspective on the outcomes of his own research and in the context of the literature in this field of studies. The detailed analysis of many results relating to various processes demonstrates the PhD student's ability to identify connections between them, as well as successfully draw conclusions and generalizations.

Chapter 15 presents the Conclusions. They are not just a list of points with short statements. Here too, PhD student shows his ability to take a broader perspective on his achievements. I want to stress that the conclusions are fully supported by the results obtained during conducted research. The work is extensively documented by numerous figures, graphs,

tables, and photographs. There are 42 figures and 13 tables, which is a very impressive amount. They certainly facilitated the study and the evaluation of the results.

The extensive literature, amounting to 320 sources, is well-selected and correctly cited.

I would like to also appreciate the doctoral student's evident diligence. Undoubtedly, completing this dissertation required excellent organization and considerable commitment. I outlined his other, research-related skills above.

Akshay Maniyeri Suresh's doctoral dissertation was funded by a grant from the National Science Centre (NCN). Additionally, as mentioned above, the doctoral student's research results have been published in a renowned scientific journal. They have already received recognition from demanding reviewers, which is worth noting.

Questions and comments:

1. The figures in the Introduction depicting *E. coli* cell envelopes do not indicate the presence of peptidoglycan in the periplasm.

2. On page 104, the PhD student correctly notes that his research results may be used in future to search for alternative ways to combat Gram-negative bacteria in the era of increasing bacterial resistance to antibiotics. Please, clarify what might be the most appropriate target for such efforts, in the context of the discovered regulatory mechanisms of LPS, peptidoglycan, and phospholipid biosynthesis.

I declare that the thesis submitted to me for review by Akshay Maniyeri Suresha entitled "Regulation of cell envelope homeostasis by Lap proteins in *Escherichia coli*" meets the requirements for doctoral dissertations in the field of exact and natural sciences, in the discipline of chemical sciences, i.e., the conditions specified in Article 187 of the Act of July 20, 2018, the Law on Higher Education and Science (Journal of Laws of 2024, item 1571, as amended). It confirms the candidate's general theoretical knowledge in the discipline and the ability to independently conduct research. Furthermore, the subject of the doctoral dissertation is an original solution to a scientific problem. Therefore, I hereby request the High Disciplinary Council for Chemical Sciences of the Gdańsk University of Technology to admit the doctoral candidate to the next stages of the doctoral process.

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