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Review of the doctoral thesis by **Mr Soroosh Monem, MSc**

entitled:

Formation of protein aggregates and glycation products in *Escherichia coli* and *Klebsiella pneumoniae* exposed to desiccation-rehydration stress

This review, prepared at the invitation of the Disciplinary Board for Biological Sciences at the University of Gdańsk, was written for the purpose of conferring a doctoral degree in the field of exact and natural sciences, in the discipline of biological sciences.

1. Formal Assessment of the Thesis

The doctoral thesis forms part of a research project funded by the National Science Centre (NCN), grant no. 2018/29/ZNZ6/01040, and was written in English. Prof. dr hab. Ewa Laskowska supervised the project.

The central hypothesis of this study is that protein glycation, rather than protein aggregation, is the primary driver of bacterial cell death under desiccation and rehydration stress. The study aimed to test this hypothesis and further extend it by analysing two bacterial species: *Escherichia coli* and *Klebsiella pneumoniae*.

2. Research Objectives and Tasks

The following research tasks were carried out as part of this doctoral thesis:

- Assessment of the relative contributions of glycation and protein aggregation to bacterial cell death.
- Investigation of the effects of selected osmolytes (carnosine, betaine, and trehalose) on cell survival and protein modification levels.
- Analysis of the role of lysine acetylation as a potential mechanism limiting glycation.
- Extending the study scope to include *K. pneumoniae*, accounting for population heterogeneity, including both capsulated and non-capsulated subpopulations.
- Identifying genetic alterations associated with capsule loss using next-generation sequencing (NGS) data.

- Correlation of molecular changes (advanced glycation end products [AGEs], protein aggregation, and acetylation) with physiological parameters such as cell viability, the viable but nonculturable (VBNC) state, and biofilm-forming capacity.

The subject matter of the thesis is topical and significant in modern microbiology. The research objectives have been formulated clearly and logically. Notably, the study attempts to integrate molecular and physiological processes into a coherent model of the bacterial cellular response to environmental stress.

3. Assessment of the Thesis Structure

The thesis follows a standard formal structure typical of doctoral dissertations and is comprehensive in scope. It includes abstracts in both Polish and English, an Introduction (8 pages), a chapter outlining the research objectives, Materials and Methods (12 pages), a Results section (37 pages), a Discussion (7 pages), and a Conclusion (1 page). The bibliography comprises 86 references, which is appropriate given the thesis's thematic scope. The total length of the dissertation is 86 pages.

The overall structure is clear and logically organised. The proportions of the individual chapters—particularly the extensive Results section—are well aligned with the experimental nature of the work, although the Introduction is relatively concise.

4. Introduction

The research focus of this thesis centres on anhydrobiosis in two closely related bacterial species, *E. coli* and *K. pneumoniae*, both belonging to the Enterobacteriaceae family. Water loss is a significant stressor for bacterial cells, potentially reducing their viability and leading to a transition to the VBNC state.

The introduction is substantively sound, although relatively concise. It begins with a discussion of protein aggregation in bacteria and the factors that promote this process, before transitioning rapidly to desiccation as a significant stress factor. The subsequent section addresses the formation of AGEs and the influence of osmolytes on bacterial proteostasis and protein aggregation. The mechanisms of action of betaine, carnosine, and trehalose in bacteria are then discussed, with reference to the relevant literature.

The structure of the introduction is logical, although the transitions between individual topics could, in places, be smoother and more clearly justified. It would also be beneficial to situate the discussion more broadly within the context of general mechanisms of bacterial responses to environmental stress, including diverse physiological states such as the VBNC state or dormancy. Such an approach would allow for a more comprehensive conceptual framework and better highlight the relevance of the research problem.

5. Research Methodology

The key methods employed in this study include the assessment of cell viability using the LIVE/DEAD™ BacLight™ kit in combination with a counting chamber, isolation of protein aggregates, isolation of the outer membrane (OM), Western blot analysis, determination of lipid peroxidation, measurement of AGEs, and bioinformatic analysis of genome sequencing data from *K. pneumoniae*.

The methodological approach is comprehensive, coherent, and well aligned with the stated research objectives. It is also noteworthy that some of the procedures had been previously developed and published by the research group in which the thesis was conducted, further supporting the reliability of the results.

The description of the culture procedure and the establishment of stress conditions for *E. coli* is generally clear and allows the overall experimental design to be understood. Nevertheless, several key methodological details are missing, and their clarification could significantly improve the study's reproducibility. In particular, how the point of complete desiccation of the samples (~4 h) was defined is not clearly specified. The centrifugation parameters for *E. coli* are also not provided, which limits a full assessment of the procedure. Furthermore, the reported humidity (~40%) is not described in terms of how it was controlled, raising the question of whether it was maintained under defined conditions or resulted solely from ambient environmental factors. The methodology section should also include information on the number of biological replicates used. Addressing these issues would improve the transparency and reproducibility of the experimental approach.

In section III.3, cell viability was assessed using the LIVE/DEAD™ BacLight™ kit (SYTO® 9 and propidium iodide [PI]), while the VBNC population was defined as the difference between PI-excluding cells and colony-forming units (CFU). In this context, it would be appropriate to address the limitations of this approach and to consider alternative methods for identifying VBNC cells. It should be emphasised that assessing this physiological state requires particular caution in interpretation; therefore, the use of complementary methods would be advisable. Moreover, distinguishing between the resuscitation of VBNC cells and the growth of damaged cells or those present in low numbers represents a significant methodological challenge.

The change in culture conditions for *K. pneumoniae* from minimal medium (NMMM) to rich medium (LB) is justified by the lack of induction of the analysed processes under the former conditions and reflects a thoughtful approach to experimental design. At the same time, it introduces an additional variable that may affect the comparability of the results.

6. Assessment of the Research Tasks and Results

This thesis presents a comprehensive and multifaceted analysis of the relationships between bacterial cell survival, the VBNC state, protein aggregation, glycation processes (AGEs and carboxymethyllysine [CML]), and oxidative stress under conditions of desiccation and

rehydration. The scope of the research is consistent with the stated objectives and demonstrates a coherent and systematic implementation of the proposed research concept.

Among the most significant findings is the demonstration that carnosine exerts a pronounced protective effect under desiccation and rehydration stress. Specifically, it reduces cell death, increases the proportion of cells in the VBNC state, and enhances the recovery of cells capable of growth following rehydration. At the same time, carnosine decreases the formation of glycation products, including fluorescent AGEs and CML, and reduces lipid peroxidation without significantly affecting protein oxidation.

These findings point to carnosine's selective mode of action and support the hypothesis that carbonyl stress plays a significant role in the processes under investigation.

The study also demonstrates a relationship between AGE accumulation and reduced cell viability, representing a meaningful contribution to our understanding of the mechanisms underlying bacterial responses to desiccation stress. In this context, however, an important question arises: **why do only selected proteins exhibit increased glycation, despite the apparent formation of AGEs throughout the cell?** This point would be worth discussing with the doctoral candidate.

Particular emphasis should be placed on the observation of the selective susceptibility of the OmpC protein to glycation compared with other porins, such as OmpF and OmpA. This finding represents a valuable contribution at the molecular level and highlights the differential sensitivity of membrane proteins to chemical modifications.

At the same time, the absence of detectable CML in OmpA should be considered an important negative finding, reinforcing the reliability of the data and effectively ruling out the possibility of non-specific, global artefacts.

This observation raises further important questions for the PhD candidate: **what factors determine OmpC's greater susceptibility to glycation compared with OmpF and OmpA, and what are the potential functional consequences of this modification for the properties of the OM and overall bacterial cell physiology?**

The discussion of protein glycation at the molecular level, particularly the selective modification of OmpC, represents an interesting and valuable aspect of the work. The proposed mechanism linking glycation with altered porin function and reduced cell viability is plausible and well supported by structural considerations. However, this interpretation remains largely speculative, as no direct functional evidence is provided to demonstrate that OmpC glycation affects membrane permeability or contributes to the observed loss of culturability.

The study also demonstrates a **competitive relationship between acetylation and glycation**. Acetylation of lysine residues appears to inhibit glycation processes, whereas reduced acetylation is associated with increased AGE levels and decreased cell viability.

These findings suggest a potential protective role of acetylation. While this interpretation is consistent with existing literature and is presented by the author as a working hypothesis, it is not yet supported by direct experimental evidence. Moreover, this interpretation should be approached with caution. The use of acetyl phosphate as an acetyl group donor may introduce non-specific effects, influencing not only acetylation levels but also other cellular processes, including metabolism and alternative post-translational modifications. Furthermore, the prolonged in vitro incubation of samples (5 days at 37°C) may promote non-specific chemical changes, protein degradation, and the formation of experimental artefacts, potentially confounding the observed relationships.

It should also be noted that using a model system to compare lysine acetylation and glycation provides valuable insight into the intrinsic chemical competition between these modifications; however, it primarily reflects their reactivity at the molecular level rather than regulated cellular processes. Therefore, while the results support the proposed protective role of acetylation, they do not fully establish its physiological relevance in vivo.

Another noteworthy finding is the detection of acetylated proteins in the *Δack-pta-pka* strain, which provides indirect evidence for the existence of alternative acetylation mechanisms independent of this pathway. However, further mechanistic studies are required to confirm their nature and biological significance.

Questions for the PhD candidate: **Your results suggest a competitive relationship between acetylation and glycation. How would you experimentally distinguish between enzymatic regulation and non-specific chemical modification in this system?**

A key aspect of this study is the analysis of the effects of osmolytes. The results indicate that betaine functions as an osmoprotectant, promoting cell transition into the VBNC state; however, this effect appears detrimental following rehydration. Carnosine exhibits the most comprehensive protective effect, whereas trehalose does not confer protection during drying but significantly improves survival upon rehydration.

In this context, an important interpretative issue concerns the role of protein aggregates. The data do not demonstrate a consistent correlation between the level of aggregation and cell survival. This inconsistency is particularly evident in the case of trehalose, which increases aggregation yet does not enhance survival during the drying phase. These observations suggest that protein aggregation may not constitute a direct protective mechanism, but rather reflect the level of cellular stress or play a role that is dependent on the stage of the process (i.e., drying versus rehydration).

At the same time, the author proposes a protective role of protein aggregates, supported by observations of increased aggregation under conditions associated with improved survival (e.g., in the presence of osmolytes or within the ring subpopulation of *K. pneumoniae*). While this interpretation is consistent with reports in the literature, the evidence presented here remains predominantly correlative and internally inconsistent. In particular, the case of

trehalose—where increased aggregation does not translate into improved survival during desiccation—weakens the argument for a direct protective function.

Taken together, these observations suggest that protein aggregation does not constitute a direct protective mechanism, but rather reflects the level of cellular stress or plays a role that depends on the stage of the process (i.e., drying versus rehydration). The absence of a clear negative correlation between aggregation and survival further indicates that aggregation may be tolerated under certain conditions; however, the current data do not support a direct protective function for aggregation. Instead, its role may be context-dependent, becoming functionally relevant only at specific stages, such as during rehydration. This interpretation requires further experimental validation. In particular, it would be valuable to directly assess the functional role of aggregates by modulating their formation using chemical chaperones or genetic approaches (e.g., overexpression or deletion of key chaperones such as DnaK and ClpB) and evaluating the effects on cell survival during both drying and rehydration. Such experiments would help to determine whether aggregation is causally linked to protection or merely represents a secondary consequence of cellular stress.

In this broader context, a valuable aspect of the study is its extension to *K. pneumoniae*. The results presented in Figures 13 and 14 are interesting; however, they would benefit from further clarification and discussion. In particular, the reported decrease in the percentage of dead cells following rehydration in the presence of trehalose raises interpretative concerns, as cell death is generally considered irreversible. This observation may reflect limitations of the detection method employed, especially in distinguishing between dead and VBNC cells.

It is plausible that some cells initially classified as dead were in fact in the VBNC state and regained metabolic activity upon rehydration, leading to their reclassification as viable. In this context, it would be valuable for the PhD candidate to **clarify whether this observation is interpreted as a genuine biological effect (e.g., resuscitation of VBNC cells) or as a consequence of methodological limitations.**

A significant and valuable aspect of this work is the extension of the research to *K. pneumoniae* 577-BA, including a detailed analysis of macrocolony organisation. This part of the study is coherent and well documented, effectively integrating genetic, phenotypic, and functional data. The identification of two distinct subpopulations—capsulated (central) and non-capsulated (ring-shaped)—represents an important finding. The central subpopulation is characterised by higher levels of AGEs, a greater proportion of VBNC cells, and reduced viability, whereas the ring subpopulation exhibits higher survival and a greater proportion of culturable cells. Although these results suggest a role for glycation processes, they remain correlative and do not allow for definitive conclusions regarding causality. Notably, the lack of a clear relationship between aggregation and survival in this system further weakens the hypothesis of a protective role of protein aggregates.

The role of capsule loss in the ring-shaped subpopulation is also noteworthy. While it may confer short-term adaptive advantages, such as enhanced biofilm formation, it appears to

increase susceptibility to environmental stress over time. **This raises an important question: is the increased biofilm-forming capacity a direct consequence of capsule loss, or does it reflect broader regulatory changes within the cells?**

Particular attention should be given to the identification of an IS5 insertion in the *wbaP* gene as the mechanism underlying capsule loss and phenotypic heterogeneity. The author appropriately situates these findings within the broader context of phenotypic plasticity in *K. pneumoniae*. The observed association between capsule loss and traits such as biofilm formation and persistence suggests broader phenotypic consequences of this transition. Overall, the results highlight the important role of population heterogeneity in promoting bacterial survival under stress conditions. However, further experimental evidence is required to disentangle causal relationships from correlative observations, particularly regarding the roles of glycation, protein aggregation, and capsule loss.

In summary, the results presented are valuable and make a significant contribution to our understanding of the mechanisms by which bacteria survive environmental stress.

Discussion

The discussion is structured around the investigated phenomena and the hypotheses addressed, namely: protein glycation in bacteria; the relationship between acetylation and glycation; the protective functions of osmolytes; protective protein aggregation in bacteria; and macrocolonies of *K. pneumoniae* 577-BA. This organisation provides a clear framework for interpreting results that are extensive and, at times, difficult to integrate. Overall, this part of the dissertation is evaluated very positively.

Other Comments

The thesis contains minor editorial and proofreading errors, which do not affect its substantive value but require correction:

- Incorrect reference in the text — on p. 43, 'Figure 15C' should be replaced with 'Figure 15B';
- Minor terminological inaccuracies (e.g., the use of "trehalose" instead of "carnosine" on p. 24 in the sentence: "These results suggested that trehalose may stabilise protein structures or prevent their misfolding");
- In Table 1, the name "*Salmonella typhimurium*" should be written correctly as "*Salmonella* Typhimurium" (serotype name, without italics and with a capital letter).

Assessment of the Literature Cited

The PhD candidate cites 86 references, the majority of which are publications from the last 10 years, including numerous works from the last 5 years, which demonstrates the relevance and sound selection of the literature. The cited works also include publications by the

research team in which the thesis was carried out, including an article co-authored by the PhD candidate: *Łupkowska A., Monem S., Dębski J., Stojowska-Swędrzyńska K., Kuczyńska-Wiśnik D., Laskowska E., Protein aggregation and glycation in Escherichia coli exposed to desiccation-rehydration stress, Microbiological Research 270 (2023): 127335.*

It is worth noting, incidentally, that it would be advisable to supplement the thesis with brief information regarding the PhD candidate's academic output, including his contribution to publications and conference activity, which would allow for a more comprehensive assessment of his academic development.

Summary of the Review

This doctoral thesis is a valuable academic study that addresses a topical and significant research problem concerning the mechanisms by which bacteria respond to desiccation and rehydration stress. The PhD candidate has demonstrated the ability to plan and conduct complex experimental studies and to analyse the results. Particular mention should be made of the comprehensive approach to the subject under investigation, covering both molecular (protein aggregation and glycation) and physiological (cell survival, VBNC state, population heterogeneity) aspects.

The results obtained provide new insights into the role of osmolytes, the potential significance of protein acetylation, and the complex relationships between aggregation, glycation, and bacterial survival. Another valuable aspect of the work is the extension of the research to *K. pneumoniae* and the analysis of subpopulation heterogeneity.

At the same time, some conclusions, particularly those concerning the causal role of glycation in cell death and the protective function of protein aggregates, are indirect and require further experimental verification.

The doctoral thesis by **Mr Soroosh Monem**, MA, meets the requirements for doctoral theses, represents a significant contribution to research into bacterial physiology under stress conditions, and satisfies the criteria set out in Article 187(1) and (2) of the Act of 20 July 2018 on Higher Education and Science (Journal of Laws 2018, item 1668, as amended), and I therefore request that **PhD candidate Soroosh Monem be admitted to the subsequent stages of the doctoral procedure.**

Signature

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