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Wrocław, 29.02.2024

Review of the doctoral dissertation of Adrianna Aleksandrowicz, M.Sc. entitled:

"The role of *sanA* in *Salmonella* pathogenicity"

made under the supervision of dr hab. Krzysztof Grzymajło

and dr Rafał Kolenda (assistant supervisor)

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Formal evaluation of the doctoral thesis.

The thesis submitted for review is based on two original multi-authored papers of which the doctoral student is the first author, indicating her leading role in creating them. This is corroborated by the statements of the Ph.D. student and the supervisor as to the extent of the work carried out by Mrs Adrianna Aleksandrowicz. The results on which the evaluation is based have been published in a journal listed in the List of journals of the Ministry of Science and Higher Education: Frontiers in Microbiology (IF_{2023}=5.2) and as a preprint published in bioRxiv platform (https://doi.org/10.1101/2024.01.05.574334).

The information on the financing of the research from the PRELUDIUM BIS project within the framework of the Polish National Science Centre for Research is included in the thesis. The documentation of the doctoral thesis consists of the following chapters: **Structure of the thesis**, which explains what elements of the research process are included in the articles, **List of Abbreviations**, **Abstract in English** and **Polish**, **Introduction**, in which the doctoral student introduces the reader to the topics of her research. This is followed by: **Research Objectives**,

which are clearly formulated, and **Hypotheses**. Hypotheses presented in such an elaborate and detailed form make me wonder whether they were actually created before the realisation of the dissertation or already during its course.

Next, the doctoral student attaches copies of the **Manuscripts**. The **Foreword to the manuscript** section provides a succinct and factual summary of the content of each publication and makes it easy to read. The subsequent **Summary** and **Future Prospects** and **Conclusions** sections showed the Ph.D. student's analytical mind and great awareness of the importance of her results and how her work can be developed to understand the biological function of the SanA protein. The doctoral candidate excelled in explaining the significance of the results obtained. She embedded them very well in the actual state of the art while emphasising their originality. An extensive **Bibliography**, and **Supplementary Material** end the thesis.

Substantive evaluation of the doctoral thesis.

The dissertation aimed to investigate the role of SanA in *Salmonella* pathogenicity. I reckon that the topic covered in this thesis is important and relevant not only for basic research but also for therapeutic issues. *Salmonella* infections are a major public health problem. Tens of millions of cases of salmonellosis are reported each year, and the disease is often fatal; for example, the mortality rate from typhoid fever can be as high as 7%. Given the ever-increasing threat of antibiotic resistance and the great interest of researchers in the search for alternative methods of bacterial control, the topic of the doctoral dissertation submitted for review is, in my opinion, very timely.

In the Introduction, the author introduces the reader to the subject of the research undertaken, starting with a discussion of the taxonomy and serological classification of *Salmonella* and presenting the problem of the increasing antibiotic resistance of these strains. She then describes in detail the mechanism of pathogenesis and the virulence factors. Much attention is given to the description of Salmonella Pathogenicity Islands 1 and 2 (SPI-1, SPI-2), as well as to the structural structure of the cell envelope including the role of outer membrane proteins (OMPs), peptidoglycan and inner membrane proteins in the process of *Salmonella* pathogenicity. The introduction ends with a review of the state of knowledge regarding the SanA protein, which is the subject of the research of this thesis. As the doctoral student herself points out, knowledge of the biological role of the SanA protein comes mainly from studies in *Escherichia coli*. Knowledge of the function of this protein in *Salmonella* is scarce. This

dissertation aimed to increase knowledge in this area and emphasises the importance of understanding *Salmonella* pathogenicity by highlighting the interplay between bacterial genetics, membrane physiology, antibiotic resistance, and host-pathogen interactions. *My only comment as a reviewer is that in Figure 2 (Introduction) there is no mention of elements that have an effect on the inhibition of SPI-1 activity such as FimZ, LeuO, Lon etc.*

The objectives of the research were clear. To achieve them, the Ph.D. student used appropriately selected molecular biology techniques, techniques of the microbiological assessment of antibiotic resistance, analyses to characterise the physicochemical properties of the membrane, cell line culture techniques, and *in vitro* tests to assess the degree of infectivity and survival of the bacteria.

In the first paper (Frontiers in Microbiology), the PhD student first performed a comprehensive bioinformatic analysis to investigate the SanA in S. Typhimurium 4/74. She then generated a SanA deletion mutant and complemented it *in trans* to assess its biological function. Using high-throughput phenotypic profiling of 240 xenobiotics she analysed the resistance and susceptibility of the newly generated mutant. Cytochrome c binding, hexadecane adhesion, Nile Red and ethidium bromide uptake assays were used to analyse membrane properties and permeability. Primary bone marrow macrophages were used for intracellular replication analysis.

The results obtained by the PhD student demonstrate that the absence of SanA is associated with increased resistance to vancomycin as well as different classes of antibiotics associated with the cell wall synthesis, like ceftriaxone and carbenicillin. In contrast, the same strain revealed higher susceptibility to phosphomycin. The deletion mutant also showed increased replication rates within primary macrophages, indicating its potential to evade the bactericidal effects of the antigen presenting cells. Her findings highlight the complex interaction between membrane physiology, antibiotic resistance, and *Salmonella* pathogenicity.

This research has been published and has already been carefully evaluated by reviewers appointed by the editors of Frontiers in Microbiology. However, I would like to point out significant differences in the description of the details of the experiments carried out. *The research methodology for determining the biological properties of the mutant was described in great detail (ml, quantities), in contrast to the molecular biology part. In the context of the results obtained, I would also like to ask how it was confirmed that the cells obtained from the*

tibia and femur were indeed macrophages? Were macrophage-specific markers identified? What was the efficiency of the procedure? It would also be interesting to know whether macrophages treated with the SanA mutant produced inflammatory cytokines at different levels than after stimulation with the WT strain? Why was SEM used instead of SD in the statistical analysis? How would the PhD student explain the results of determining sensitivity to sulphamonomethoxine (Figure S3) - why is the WT more sensitive than the mutant at higher concentrations, but the opposite at lower concentrations? I think there is also a mistake in the statistical significance of the umbelliferone effect.

In the second paper, published as a preprint in bioRxiv, the PhD student set out to investigate the expression of SanA during infection, to decipher its location and how it interacts with a key *Salmonella* virulence factor called Salmonella Pathogenicity Island 1 (SPI-1). She also investigated *in vitro* the effect of SanA on *Salmonella* infection using Caco-2 and iBMDM cells. Using subcellular fractionation and Western blotting, she was able to show that SanA is mainly located in the inner membrane. She then performed a transcriptional fusion of SanA with luciferase (sanARBS::luc) and analysed the environmental conditions that induce SanA expression. She also determined the expression pattern of SanA during infection of mouse bone marrow macrophages (iBMDM). Using human intestinal epithelial cells (Caco-2) and iBMDM, she showed that deletion of SanA increased bacterial invasion. She also analysed the molecular basis of this phenomenon, focusing on the relationship between SanA and SPI-1 using green fluorescent protein (GFP) and mCherry reporter systems. Flow cytometry and Western blot analyses allowed her to study this phenomenon at the level of promoter activity and protein expression, respectively. The demonstration that the inner membrane protein SanA is important for regulating Salmonella's response to environmental stress, an important aspect of both *Salmonella* invasion and survival, was the PhD student's main achievement in this work.

My first objection concerns the methodology. I am of course aware that the work is presented as a collection of original papers and not as a monograph, *but the molecular biology research methods are much less detailed than the biological one.*

There is no information in the biology section about the origin of the iBMDMs? Some information can be found in Fig. 2 and Fig. 3. How were the macrophages immortalized? What was the passage of the Caco-2 cells? The penicillin and streptomycin were used in the cell culture. I wonder if these antibiotics do not affect the expression of SanA? I think it would be

worthwhile to carry out these experiments without the addition of antibiotics. What is the origin of the anti-OmpA and anti-LepB antibodies used in the WB? Next, how does the Ph.D. student explain the lack of difference between WT and mutant at 2% yeast extract availability, while these differences occur at 0.5% (Figure 5)? Why are there no standard deviations in Figure 3 for WT and sanA+pWSK29-sanA? As previously mentioned, why was SEM used instead of SD in the statistical analysis? My final question is about the Ph.D. student's general opinion on how her results can be applied to the development of new therapeutic compounds for the prevention/treatment of salmonellosis. What further research should be done on this topic?

In conclusion, my minor objections do not change the fact that Mrs. Adrianna Aleksandrowicz delivered very interesting and valuable observations, and hence, I rate her work very highly. Therefore, I declare that Rozprawa doktorska spełnia warunki określone w art. 187 ust. Ustawy z 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz.U. 2018 poz. 1668 z późn. zm.). and I recommend admitting Adrianna Aleksandrowicz to public defense of her doctoral thesis. At the same time, I am asking the Scientific Council of the Wrocław University of Environmental and Life Sciences to consider awarding the Dissertation.

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