Chapter 7

General conclusions and future perspectives

7.1. GENERAL CONCLUSIONS

Bacterial pathogens have been evolving to better resist antimicrobial therapies, presenting a significant challenge in healthcare. Among these pathogens, some are particularly dangerous due to their multidrug resistance (MDR), which includes carbapenems, a class of antibiotics often considered as a last resort. The Gram-negative bacterium *Acinetobacter baumannii* emerged as a critical pathogen in hospital settings due to its ability to colonize, resist desiccation, and form biofilms making it challenging to eradicate. These features are facilitated by numerous virulence factors, like the capsule and lipopolysaccharides (LPS), protein secretion, iron acquisition, pili, outer membrane vesicles, and others inherent to *A. baumannii*. Moreover, this bacterium exhibits a unique SOS response, which has been reported to enhance its adaptability by interacting with proteins involved in DNA repair, thereby introducing mutations, and promoting genomic variability. One of the most alarming developments is the emergence of MDR-resistant variants within *A. baumannii* isolates, particularly the Carbapenem-Resistant *A. baumannii* (CRAb) variants.

While this species did not inherently possess antibiotic-resistance genes, studies have demonstrated that these genes were acquired externally through horizontal gene transfer (HGT) mediated by mobile elements, such as plasmids, prophages and OMVs. One of the regulators of this HGT is the SOS system encoded by *A. baumannii*, which can induce the mobilization of mobile genetic elements, such as prophages. The main goal of the research described in this thesis was to comprehend the role of HGT vehicles in the evolution of the drug resistance and pathogenicity of *A. baumannii*.

To better understand the relevance of prophages within *A. baumannii*, an extensive analysis of all complete genomes submitted to NCBI was conducted. To accomplish this, a bioinformatic tool, ProphAnTool, was developed, integrating several available genomic analysis tools, to search and characterize prophages from large batches of whole genome sequences. This analysis verified previous observations regarding the widespread presence of prophages in *A. baumannii* isolates. Furthermore, these prophages were detected both in the bacterial genomes and plasmids, likely indicating the existence of phage-plasmids, and highlighting their potential for horizontal gene transfer. Among these prophages, a small group harbored a diverse set of antibiotic-resistance genes, conferring resistance to multiple antibiotics. Additionally, other virulence and fitness-related genes were highly prevalent, indicating their potential contribution to bacterial adaptation. While several defense systems were identified within these prophages, the majority of them have so far remained poorly characterized.

As the SOS response employs a unique mechanism in *A. baumannii*, the way this system influences prophage gene expression was investigated by looking at induction and virulence/fitness-related gene expressions through transcriptomics. This survival mechanism is known to be a major prophage inducer in most bacteria, including *A. baumannii*. Since this system relies upon a different set of genes, it became important to understand how it interacts with integrated prophages. Besides, since this species' SOS response is still poorly understood, the role of one of its regulators, DdrR, was also explored. This small co-regulatory protein was shown to be crucial to keep critical SOS genes, such as error-prone polymerases repressed, and to greatly influence the expression of non-DNA damage-related genes, including genes related to prophage induction, such as the phage repressor CI, Cro, and protein Q, which is

the main regulator of late gene expression in prophages. The Protein Q gene, together with most structural phage genes, was found to be overexpressed in the absence of stress. Moreover, under stress conditions, the protein Q gene was induced in all three prophages of the investigated strain, but only in the absence of the *ddrR* gene, suggesting a potential direct interaction with protein Q expression and its repressor CII. Regrettably, experimental validation of this hypothesis was not possible, due to an inaccurate phage counting method, as phage plaques were too small and, in some situations, almost impossible to count. The use of a different propagation host could improve phage counting, but unfortunately, after several attempts, no compatible host was found due to the narrow host range of these phages.

Another study detailed in this thesis involved the characterization of three new *A. baumannii* clinical isolates that were collected during an outbreak at a hospital in The Netherlands. These three isolates included a non-CRAb isolate from the index patient and two CRAb strains that emerged at a later stage during the outbreak. All three isolates were sequenced by Illumina and Nanopore technologies, resulting in closed genomes. The subsequent analyses revealed the presence of a novel MDR plasmid in two out of the three isolates. This newly identified plasmid was found to be a conjugative plasmid, and it conferred resistance to a wide variety of antibiotics, including carbapenems and the last generation of tetracyclines. Subsequently, the outer membrane vesicles of these three new isolates were characterized based on their proteomes. This analysis revealed that the OMV proteomic content was different, mainly between the non-CRAb and the two CRAb isolates. Of significance, carbapenemases encoded by the MDR-plasmid was identified in the OMV fraction of the two CRAb isolates. Therefore, the carbapenemase activity of these OMVs was characterized, demonstrating their ability to degrade carbapenems, potentially protecting the host against this class of antibiotics. Furthermore, the protective effect was demonstrated to be extensible to other species through OMV-protection experiments, wherein other susceptible species were shielded from carbapenems by the OMVs.

Lastly, a comprehensive analysis of the mobile genetic elements of the three outbreak-associated isolates was performed, as well as an extensive study of their OMV proteomes. This involved OMV isolation from the bacteria cultured in two different media, the regular and nutrient-rich Lysogeny Broth (LB) and a typical cell culture medium known as DMEM. Several mobile genetic elements (MGEs) were detected among the three isolates. These MGEs included several intact prophages, as well as different plasmids, besides the afore-mentioned MDR plasmid. However, a careful characterization revealed a low prevalence of virulence genes among these MGEs. Nevertheless, prophages were inducible as observed through TEM, confirming their mobility potential. Conversely, OMV proteomes, showed a high prevalence of virulence effectors and antibiotic-resistance proteins, that were implicated in increased host colonization and invasion, attachment and biofilm formation, survival, and other capabilities. In addition, there were no absolute differences between the different isolates 'DMEM-OMV' proteomes. Conversely, 'LB-OMV' proteomes significantly differed between the different isolates. Additionally, virulence-associated proteins were detected in higher amounts in OMVs from bacteria cultured in LB than in OMVs from bacteria cultured in DMEM. It was also concluded that the MDR plasmid was not directly responsible for proteome differences between the CRAb and non-CRAb isolates. Instead, it is hypothesized that either the plasmid or the afore-

mentioned prophages are regulating the expression of specific genes that lead to these differences.

In conclusion, the analysis of mobile elements in *A. baumannii* verified their important role in the emergence of highly virulent and multidrug-resistant variants, particularly those resistant to carbapenems. Furthermore, the investigations into the interactions between the SOS response and prophages in this pathogen are beginning to shed light on previously unexplored gene regulatory mechanisms.

7.2. FUTURE PERSPECTIVES

The work described in this thesis represents a significant step to a broader objective aiming at understanding all interactions between the mobile elements and *A. baumannii*. Despite the progress made, several mechanisms remain unknown. For example, how do prophages, plasmids, and OMVs interact all together? It is known that OMVs can carry plasmids to another host, but can they also carry prophages? In theory, it would be possible, since prophages are DNA fragments, therefore they could be incorporated into the OMVs. Moreover, can prophages influence OMV production to promote plasmid mobilization, or how are OMVs formed and how do they select their cargo? These are some of the questions that are still awaiting answers.

To tackle these questions, various approaches can be employed. For example, to search for prophage DNA within the OMVs, DNA could be extracted from OMVs and sequenced to map all DNA content. With this, it could be possible to detect, not only prophage DNA, but also other mobile genetic elements or genes, which could be critical to understanding how the outbreak described in this thesis started, and if OMVs drove this event by mobilizing the plasmid (1). It would also be interesting to show that prophage DNA mobilized through OMVs could integrate into a new host, with a prophage-cured variant, and by inserting in the prophage a selective marker, it could be possible to prove this phenomenon, which has not been described yet.

Prophages are still the mobile genetic element that have been subject to relatively few studies in *A. baumannii*, although they have been proven to have the potential to influence their host's behavior. Therefore, to identify the mechanisms that could be influencing OMV production, first different knockouts would be necessary to verify the genes that are responsible for OMV production. Then, using a prophage-cured variant, in comparison to the wild-type, for transcriptomics would provide a gene expression profile showing the genes that are potentially influencing it.

Lastly, it would be very interesting to verify whether DdrR is indeed influencing prophage induction by screening protein-protein interactions between DdrR and other phage proteins. For example, as performed with DdrR and LexA/UmuDAb proteins (2), using surface plasmon resonance it could be investigated whether DdrR interacts with the CII repressor or protein Q, which were seen to be influenced by DdrR, according to the results described in this thesis. Besides, improving phage detection would also benefit this study, so a different phage detection method could be used, for example, flow cytometry to count phage particles as shown in some studies (3,4). Understanding all these interactions would unveil the main mechanisms of prophage induction in *A. baumannii*, thereby providing important knowledge for dealing with this increasingly dangerous pathogen.

7.3. REFERENCES

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