A proteogenomic view on antibiotic resistance in pathogenic Enterobacter species

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Chapter 6

Summarizing discussion and future perspectives

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Species belonging to the *Enterobacter cloacae* complex are widespread in nature, which underscores their high potential to adapt to different ecological niches that range from the soil to the human body [1]. In the latter context they have emerged as nosocomial pathogens [2] that increasingly display resistance to antibiotics [3]. As shown by the studies on a highly drug resistant *Enterobacter roggenkampii* isolate with sequence type (ST) 232 that are documented in this thesis, the resistance phenotypes of representatives of the *E. cloacae* complex now even extend to carbapenems, which are important 'last resort antibiotics'. To understand how such a resistance phenotype may have emerged, what is its present status, how it compares to other related isolates, and how it may evolve in years to come was the prime objective of the here described research.

From a clinical perspective, answers to the questions how an infection presents itself and how it can best be treated have the first priority. To this end, it is relevant to isolate and identify the causative agent, and to determine its antibiotic resistance profile. The *E. cloacae* complex isolates that are described in this thesis were obtained from patients admitted to the University Medical Center Groningen (UMCG). Clearly, from the therapeutic perspective, knowing the antibiotic susceptibility profile of these isolates was more important than identifying to which species within the *E. cloacae* complex they belonged exactly. The same applied to such isolates obtained from patients implicated in bone infections, which was an unusual clinical presentation for bacteria of the *E. cloacae* complex at the time of isolation. Accordingly, the present study isolates were identified by routine culturing and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), which led to their rapid identification as '*E. cloacae*'. Immediately thereafter, the antibiotic resistance profiles of these isolates were determined, unexpectedly revealing carbapenem resistance for one isolate from a neonate, while showing antibiotic susceptibility for the isolates from bone infections. For treatment of the respective patients this information was sufficient. In contrast, for understanding the biology underlying the antibiotic resistance mechanisms and clinical presentation, this was merely the beginning.

To really understand the drivers for antibiotic resistance and clinical niche preference of bacterial isolates it is necessary to dissect their molecular architecture, from the genome to the proteome and beyond. This is a very ambitious goal that, today, can only be achieved in parts. A major limitation in understanding the behavior of infectious agents is that they can only be studied in detail after their isolation from the patient, in other words outside the patient's body. Therefore, the next best option is to analyze in detail the composition of their genome from which possible clinical behavior can be inferred. The possibilities for such genomic analyses have expanded at a very high pace over the past four years, and this development was actually followed closely in the here presented studies. For example, when studies for **Chapter 2** were performed, next generation sequencing technology had just opened the window to fast and affordable genome analyses. Yet, there were literally many gaps in the obtained sequence information, which were increasingly closed towards the end of the here described studies using third-generation nanopore-based sequencing technology. In fact, the combination of high-quality sequences obtained by short-read sequencing approaches with the long reads of lower quality obtained by nanopore sequencing can now deliver highly accurate genome sequences using hybrid *de novo* assembly approaches. This hybrid assembly is particularly important for the analysis of genomes that contain many repeated sequences, as it leads to lower numbers of contigs with, on average, higher lengths and with fewer sequencing errors than can be obtained with either method by itself [4]. Accordingly, the hybrid genome assemblies are currently the best starting points for further downstream applications, ranging from gene annotation to detailed genome comparisons, and the interpretation of mass spectrometry data to analyze the protein composition of the respective organisms, either qualitatively or quantitatively.

As exemplified by the study described in Chapter 2, the high quality hybrid genome assembly of a carbapenem resistant isolate belonging to the *E. cloacae* complex allowed a detailed reconstruction of evolutionary events that may have set the stage for its horizontally acquired resistance. In particular, it allowed the identification of a mobile genomic island (MGI) that was spread among the ancestry of the investigated isolate, long before the species belonging to the *E. cloacae* complex evolved from preceding *Enterobacteriaceae*. Eventually, this MGI

became a docking platform for a range of different resistance genes, especially the clinically relevant genes specifying AmpC type cephalosporinases, one of which turned out to possess also carbapenemase activity (i.e. MIR17). This observation had several important implications. In the first place, it showed that elements that are generally regarded as belonging to the core genome of a bacterial species may actually still be mobile genomic elements. This is a fact that is often overlooked in bacterial genome investigations. Yet, the observations described in Chapter 2 show that it is important to look beyond the species barriers when discerning core and accessory genomes. Second, the results focus attention on the fact that such 'ancient MGIs' may represent hotspots for the accumulation of clinically relevant resistance properties, as exemplified by the *ampC* type cephalosporinase genes. Third, the results show that not all cephalosporinases are equal as some of them, like MIR17, may possess carbapenemase activity. Of note, this finding has been implemented in routine diagnostics at UMCG, where plates with cloxacillin are now used to assess possible carbapenemase activity of cephalosporinases. Lastly, the hybrid genome assembly has set the stage for a proteomic dissection of the carbapenem resistant *E.roggenkampii* study isolate described in Chapter 2, highlighting the high abundance of MIR17. The scientific path from clinical isolate through genome sequencing to analysis of the proteome is sketched in Figure 1.

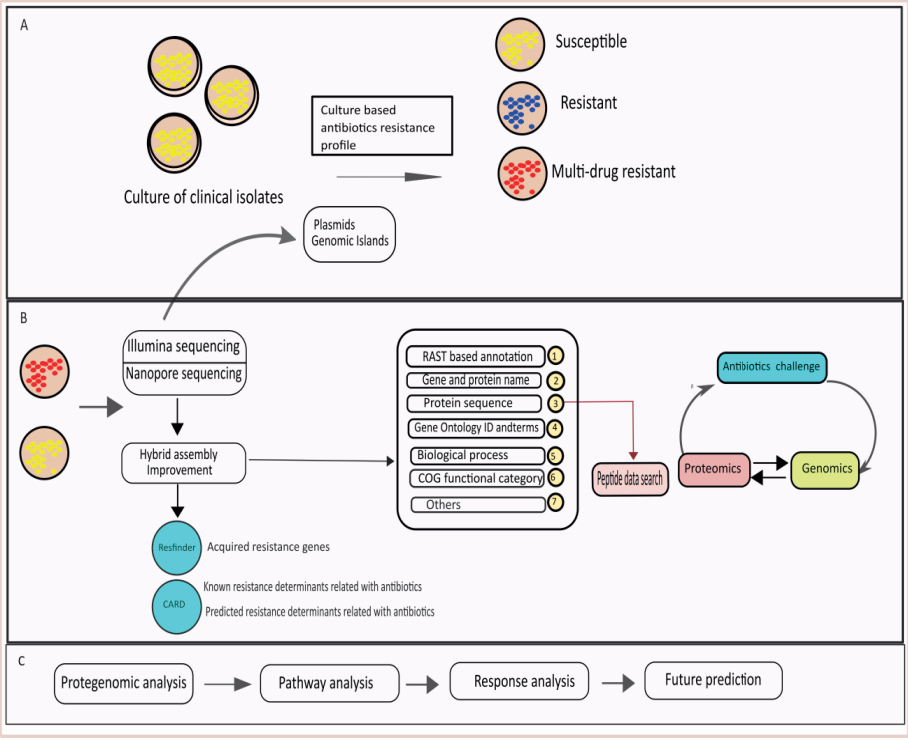

Figure 1. Overview of experiments and data analyses undertaken for the present PhD research. (A) Clinical bacterial isolates of interest were collected, cultured and tested for antibiotic resistance. (B) The isolates were subsequently characterized by DNA sequencing using two distinct methods, i.e., Illumina short-read sequencing and Oxford Nanopore Technology long-read sequencing. Upon susbsequent hybrid DNA sequence assembly using the SPAdes or Unicycler approaches, further annotation was performed to identify genomic islands, plasmids and integrons, resistance genes and resistance-related genes using suitable bio-informatics tools. Next, a table with information on the gene name, protein name, protein sequence, gene ontology (GO) and GO terms, biological processes and clusters of orthologous (COG) functional categories was prepared. The peptide sequences deduced from the whole-genome sequences were then used as a resource for peptide identification by mass spectrometry. (C) Data from the proteome and genome analyses were compared and interpreted, taking advantage of the so-called KEGG pathways, to understand the bacterial responses to an imipenem challenge, and to predict the possible future of antibiotic resistance development.

A particular advantage of having a high quality genome sequence as obtained by hybrid genome assembly is that it allows detailed genome comparisons of related *Enterobacter* species belonging to the *E. cloacae* complex. Indeed, such a phylogenetic analysis was performed, revealing that the carbapenem resistant study isolate of Chapter 2 was *de facto* an *E. roggenkampii* isolate.

Importantly, the more classical proteome approach applied for the studies described in Chapter 2 provides a good overview of the 'protein landscape' of the bacterial cell, but it provides no dynamic information on how the cells respond to environmental insults, such as the administration of antibiotics. However, knowing how the cells respond to the presence of an antibiotic provides valuable information on adaptive behavior that, upon long-term antibiotic exposure, may become locked into an intrinsic resistance genotype. Therefore, the studies documented in **Chapter 3** assessed how the carbapenem resistant *E. roggenkampii* isolate responded to the presence of the carbapenem imipenem and, again, the availability of the hybrid genome assembly turned out advantageous as it allowed the identification of an additional set of 400 proteins that were initially overlooked. Of note, it was anticipated that a quantitative proteome analysis to follow the responses of a highly antibiotic resistant bacterial isolate to the presence of an antibiotic to which it is resistant might provide a glance of potential future adaptive or even intrinsic resistance. Indeed, the results showed that the presence of imipenem at a concentration below the minimal inhibitory concentration triggered various responses that allow the bacteria to minimize (i) the waste of cellular resources for cell wall biogenesis upon impaired function of penicillin binding proteins, and (ii) the potentially detrimental generation of reactive oxygen species (ROS) due to increased flux through the glycolytic and tricarboxylic acid (TCA) pathways that may occur when cell wall biogenesis is impaired. Additionally, the generation of ROS by respiration was potentially decreased by (i) reduced expression of the biosynthetic pathway for iron-sulphur cluster formation, and (ii) by upregulation of a key enzyme in the pathway for formation of the antioxidant vitamin K. These observations show that the bacteria still have many options to overcome the detrimental effects of antibiotics like imipenem, in addition to the production of MIR17 and the mutation of porins in the outer membrane as documented in Chapter 2. Interestingly, the findings presented in Chapter 3 are consistent with

results from previous studies by others, who investigated responses to ß-lactam antibiotics and showed that these antibiotics do not only cause a bactericidal weakening of the bacterial cell wall, but also elicit excessive formation of ROS which may contribute to the bacterial killing [567]. Likewise, other studies showed that overcoming ROS formation by slowing down the carbon flux through glycolysis and the TCA cycle allowed the formation of so-called bacterial L-forms, which lack a cell wall [8]. These observations focus attention on the central role of metabolic pathways in the bacterial life cycle and the possibility to target central carbon metabolism with novel, yet to be developed, antibiotics. For instance, if it would be possible to increase metabolic activity in the presence of antibiotics like imipenem, rather than letting the bacteria slow down their metabolism, it should be possible to elevate ROS production to levels that strongly enhance the bacterial killing. Alternatively, such an effect could potentially be achieved by interfering with the down-regulation of the biosynthetic pathway for iron-sulphur clusters. In the latter case, the Fur regulator might be an interesting target, as the findings described in Chapter 3 indicate that the presumed downregulation of iron-sulphur cluster biogenesis is mediated by upregulation of Fur.

While the high-quality genome sequence of the *E. roggenkampii* isolate unveiled the presence and sequence identity of its *ampC* gene, more classical microbiological approaches were necessary to show that the encoded cephalosporinase also had carbapenemase activity. This was a finding that could not have been predicted from the genome sequence. In turn, this underpins the fact that the genome sequence is only the blueprint of what a bacterium is actually capable of. Also, the genome sequence would not allow the prediction of the adaptive behavior of the investigated *E. roggenkampii* isolate in the presence of imipenem. Another feature in terms of antibiotic resistance behavior that could not be predicted from the genome sequence alone, at least not yet, was the fact that the studied *E. roggenkampii* isolate carried an *aadA* gene for aminoglycoside resistance on an In127 integron but, nonetheless, showed no resistance to aminoglycosides. While this observation tells us something about the evolutionary history of the investigated *E. roggenkampii* isolate, it also reinforces the need for combining genome analyses with functional assays or proteomics approaches to obtain a clearer view of what the investigated bacteria are really up to. In addition, future use of machine learning and artificial intelligence approaches may overcome the

current limitations of genomic analyses.

Chapter 4 presents the results of a combined genomics and proteomics approach to find out which features could distinguish human gut-resident isolates belonging to the *E. cloacae* complex from related isolates implicated in bone infections. First, a comparison of the genome sequences showed that the investigated isolates with different clinical presentations belonged to different *Enterobacter* species. The gut resident isolates were in fact identified as *E. roggenkampii* and *E. hormaechei* subsp*. hoffmannii*, whereas the bone isolates belonged to different *E. hormaechei* subspecies or *E. cloacae*. This provided already a strong clue for a different evolutionary history of the respective isolates, which might explain a different niche preference in the human body. Intriguingly, the subsequent proteome analysis showed that the bone isolates produced many proteins related type VI secretion systems, which are generally implicated in virulence, and proteins related to flagellar function for bacterial motility. Consistent with these proteome data, the bone isolates contained two to three type III secretion systems for flagellar biogenesis, and two to three type VI secretion systems. The genes for these systems were all integrated between tRNA-encoding genes, suggestive of acquisition by horizontal gene transfer. On the other hand, the carbapenem-resistant *E. roggenkampii* isolate more prominently expressed proteins related to antibiotic resistance and resistance to heavy metals. Together, these observations at the genome and proteome levels support the view that the investigated isolates, all belonging to the *E. cloacae* complex, have evolved in different directions to optimally adapt to different niches with different selective pressures. In the context of this evolution, they have acquired different molecular functions that have increased their fitness in the respective niches.

Related to bacterial evolution and the horizontal transfer of resistance genes, **Chapter 5** focuses attention on two plasmids encoding the OXA-427 carbapenemase, which were isolated from a *Klebsiella pneumoniae* and an *E. cloacae* complex strain, respectively. These two IncA/C2 plasmids share the same backbone, but in the *K. pneumoniae* isolate this plasmid is cointegrated into an IncFIB plasmid, which has resulted in a 321-kb megaplasmid. Of note, this megaplasmid carries multiple multi-resistance regions. The presence of the different resistance genes within the megaplasmid is definitely a worrisome

observation, because this may significantly facilitate their dissemination to other bacteria that may already possess other antibiotic resistances. The identified megaplasmid could, thus, turn into a vehicle for the emergence of pan-resistant *Enterobacteriaceae*.

In conclusion, the present PhD thesis reports on the identification and characterization of bacterial isolates belonging to the *E. cloacae* complex, which were remarkable in terms of their antibiotic resistance profile or clinical presentation. In the vast majority of cases, such observations are essentially applied to treat the patients infected by these bacteria, whereas the complex underlying mechanisms that have led to their drug resistance or niche preference remain covered. Only with the advent of rapid multi-omics technology and its implementation at the interface of clinical and fundamental scientific research, it has become possible to open the molecular window that permits unprecedented views into the past, presence and future of the isolated pathogens. This is an exciting development as the collected insights may ultimately help us to develop better strategies and drugs to prevent or treat dangerous nosocomial infections.

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