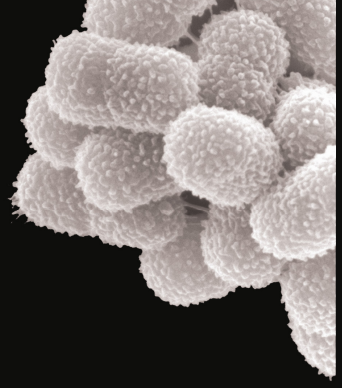

Chapter 8

General discussion and summary

General discussion

During the last decades, *A. baumannii* has emerged as an important nosocomial pathogen responsible for outbreaks of infection worldwide. The spectrum of *A. baumannii*-host interactions is complex; ranging from a quiescent colonizer of the human skin to a dangerous invader of the bloodstream causing bacteremia and septic shock. The outcome of its interaction with the host depends on the balance between the virulence factors displayed by the bacterium and the condition of the host and the quality of its immune response (Figure 1).

The studies presented in this thesis aimed to gain further insight into the bacterial and host factors associated with the pathogenesis of *A. baumannii* to seek an explanation for the clinical success of *A. baumannii*.

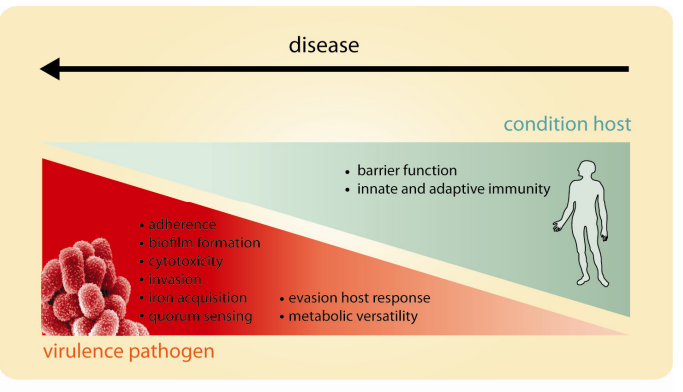

Figure 1. The pathogen-host balance. The outcome of colonization or infection by *A. baumannii* depends both on the virulence of the pathogen (in red) and on the condition of the host (in green).

Adherence to and biofilm formation on abiotic and biotic surfaces

As adherence to host cells is the first step in colonization and infection **(chapter 1)**, we asked the question whether adherence to biotic surfaces differed between *A. baumannii* and less virulent *Acinetobacter* species. Results showed that strains of *A. junii*, which is a common skin colonizer, adhered equally well to human bronchial epithelial cells as *A. baumannii* strains **(chapter 2)**. However, within each species there was a wide variation in quantitative adherence, expressed as the percentage infected epithelial cells **(chapter 2)**. Strain variation within *A. baumannii* was previously also shown by Lee et al, who quantified the adherence of a large set of *A. baumannii* strains to human bronchial

epithelial cells [1]. They did not observe a difference in the percentage of infected cells between outbreak-associated strains and strains not associated with outbreaks. However, we found a correlation between the clonal lineage and the percentage of infected cells, with strains of EU clone II being more adherent than strains belonging to EU clone I [1]. Together, these data indicate that adherence to human mucosal cells is a general feature of *Acinetobacter* strains. In the next paragraph, the mechanisms that are thought to play a role in adherence of *Acinetobacter* to human cells are discussed.

Once bacteria have attached to an abiotic or biotic surface, they can multiply and form a biofilm, which protects them against environmental stresses, such as desiccation and exposure to antibiotics. The ability to form a biofilm may contribute to the survival of bacteria on environmental surfaces and medical devices like catheters and respiratory tubes **(chapter 1)**. In this thesis, we first investigated whether *A. baumannii* strains can form biofilms on an abiotic surface. A wide variation in biofilm formation among *A. baumannii* strains was found **(chapter 2)**. Although epidemic strains did not form larger biofilms than sporadic strains, it appeared that strains of EU clone II formed larger biofilms than strains of clone I. The finding that strains of EU clone II seemed to adhere better to epithelial cells [1] and form larger biofilms than strains of clone I raises the question whether there is a difference in clinical impact between these clones. Up to date however, both EU clone I and II strains are involved in outbreaks worldwide [2–5] and, although there are temporal and geographical shifts in their occurrence, there is, to our knowledge, no clear distinction in clinical-epidemiological behaviour. Therefore, differences in adherence to human cells [1] and biofilm formation on plastic cannot be directly linked to the clinical-epidemiological significance of the clones.

As there was no association between biofilm formation on plastic and clinical significance among *A. baumannii* strains, we next investigated the capacity of other *Acinetobacter* species to form a biofilm. It was found that some strains of *A. pittii* and *A. nosocomialis* and of the less virulent species *A. calcoaceticus* and *A. junii* were also able to form biofilm on plastic. As for *A. baumannii*, intraspecies variation was considerable among these species. This led us to conclude that biofilm formation on plastic is strain- and not species-specific and does not explain the success of *A. baumannii* in the susceptible host. The poor correlation between the adherence to bronchial epithelial cells and biofilm formation on plastic **(chapter 2)** may be explained by the fact that different mechanisms are involved in these processes as will be described in the next section.

Biofilm formation on plastic and adherence to human cells are widely used systems to study interactions of bacteria with abiotic and biotic surfaces, respectively. However, these systems do not adequately reflect the real-life association of bacteria with its host. Moreover, it is known that biofilm formation is strongly influenced by

environmental conditions [6], such as the physical properties of the surface and nutrient availability. The colonized skin is thought to constitute an important reservoir for *A. baumannii* during outbreaks and endemic episodes [7,8]. In **chapter 5** and **7**, adherence and biofilm formation of *Acinetobacter* was investigated on this biologically relevant surface using a 3D human epidermal skin equivalent that mimics the native skin to a high degree [9,10]. The *A. baumannii* type strain ATCC19606^T and the *A. junii* type strain RUH2228^T both colonized the epidermis and persisted up to 72 h, but did not invade the epidermis **(chapter 7)**. Moreover, strains of *A. pittii* (SH024) and *A. nosocomialis* (RUH2624) also survived and persisted on the skin equivalent, whereas a strain of the environmental species *A. calcoaceticus* (RUH2202) did not **(chapter 5)**. Although both *A. baumannii* ATCC19606^T and *A. junii* RUH2228^T were able to form a large biofilm on plastic **(chapter 2)**, only *A. baumannii* ATCC19606^T formed a biofilm on the human skin equivalent, as demonstrated using the polysaccharide stain Alcian blue-PAS **(chapter 7)**. Together, these data demonstrate that the ability of *Acinetobacter* to form biofilms depends on the substrate with which the bacteria are interacting, as was also found by Gaddy et al [11].

Mechanisms underlying adherence and biofilm formation

The differences in adherence and biofilm formation among *Acinetobacter* strains triggered us to study the possible mechanisms underlying these colonization processes. It was previously shown that the ability of *A. baumannii* ATCC19606^T to form pili and to adhere to and form biofilms on abiotic surfaces depends on the expression of *csuE*, which is part of the CsuA/BABCDE chaperone-usher pili assembly system [12]. The involvement of these CsuA/BABCDE-mediated pili in adherence to human epithelial cells was investigated **(chapter 3)**. Compared to *A. baumannii* ATCC19606^T, the isogenic *csuE*-mutant did not show impaired adherence to human bronchial epithelial cells. Strikingly, the mutant could adhere even better to these cells than the parental strain. These results were later confirmed by Gaddy et al using human alveolar epithelial cells [13]. This indicates that the csuE-mediated pili are not required for adherence to human cells, although they are important attributes for the *A. baumannii* type strain to form microcolonies and biofilm on inanimate surfaces.

As part of a multicenter study, the genome, metabolome and virulence of a range of *Acinetobacter* species that differ in success in the clinical setting was assessed **(chapter 5)**. It was found that the *csu* operon was present in pathogenic species of *Acinetobacter* (six *A. baumannii* strains, *A. pittii* and *A. nosocomialis*) but not in non-pathogenic species, suggesting that this operon is a putative virulence factor. CsuE-mediated adherence to indwelling medical devices like lines and respiratory tubes, may allow for multiplication of

the organism on these abiotic surfaces, which contributes to an increased risk of infection for the patient.

Using scanning electron microscopy, we showed that ATCC19606^T expressed short fimbrial-like structures as well as thick cell extensions connecting bacteria **(chapter 3)**. The *csuE*-mutant appeared to densely express short fimbriae on its surface but no thick cell extensions. These thick cell extensions resemble the polysaccharide poly-β-1-6-N-acetylglucosamine (PNAG) described recently by Choi et al, that was associated with *A. baumannii* biofilm formation on abiotic surfaces under shaking conditions [14]. As the *csuE*-mutant expressed more short fimbrial-like extensions on its surface than the parental strain, we hypothesized that these CsuA/BABCDE-independent short fimbrial-like extensions play a role in adherence to biotic surfaces as was shown previously for the *acuA*-encoded short fimbriae of *A. baylyi* strain ADP1 [15]. To test this hypothesis, the surface morphology of additional *A. baumannii* strains that differed in their ability to adhere to human cells was examined **(chapter 2)**. There was a wide variation in the presence of cell surface extensions between these strains and we did not find a clear association between the presence of either the thick cell extensions or the short fimbriae and biofilm formation or adherence to human cells. To investigate the involvement of cell extensions in these colonization processes in more detail the presence of cell extensions should be examined at different growth conditions as it has been shown that pilus assembly is influenced e.g. by the presence of a substratum [16]. Moreover, biochemical approaches are necessary to elucidate the structure of these extensions.

Host response to *Acinetobacter*

On the other side of the pathogen-host balance (Figure 1), the host has several innate defense mechanisms to prevent infection. In **chapter 2**, we describe that human bronchial epithelial cells respond to infection with *A. baumannii* and *A. junii* strains by the production of the inflammatory cytokine IL-6 and the chemokine IL-8. Interestingly, *A. junii* strains induced higher levels of these inflammatory mediators than *A. baumannii* strains did. This difference was even more pronounced in cultured human macrophages, where *A. junii* induced higher levels of the pro-inflammatory TNFα and IL-12p40, the chemokine IL-8 and the anti-inflammatory IL-10 than *A. baumannii* strains did. Thus, strains of *A. baumannii* induced a limited TNFα, IL-12p40, IL-6, IL-8 and IL-10 response in human cells as compared to *A. junii* strains, despite the finding that they adhered equally well to these cells. Different studies have demonstrated the importance of inflammatory cytokines for clearing of *A. baumannii* in vivo [17–20]. Therefore, we hypothesized that *A. baumannii* may survive and persist in the airways of patients and cause disease at least in part by inducing a weak inflammatory response that poorly mediates the clearance of bacteria by

the host. To investigate this possibility, the persistence of and host response to different well-characterized *Acinetobacter* strains was determined using a mouse infection model **(chapter 4)**. MDR *A. baumannii* reference strains of EU clone I (RUH875), II (RUH134) and III (LUH5875) as well as a susceptible sporadic *A. baumannii* isolate (LUH8326) had a dramatic effect on the neutropenic host as they survived and multiplied in the lungs of mice and disseminated into the bloodstream. As might be expected from the rare prevalence of *A. junii* in infection, the strain of this species did not persist in the lungs of mice, which might be explained by a strong inflammatory response to this strain, as we have shown in vitro **(chapter 2)**. Surprisingly, the type strain of *A. baumannii* ATCC19606^T was also cleared from the lungs rapidly without causing disease. This type strain, an urine isolate from the 1960s of which the clinical epidemiological significance is unknown, is a widely used model strain to study the pathogenesis of *A. baumannii*. The findings of our studies challenge the relevance of this *A. baumannii* strain in virulence studies.

A striking difference in the outcome of pneumonia among the *A. baumannii* strains was observed with the clone I and II strains being most virulent (**chapter 4**). These findings emphasize that there are great differences in virulence among *A. baumannii* strains, and stress the need for rapid identification tools of high risk strains [21,22].

The mouse model studies also revealed that the host responded differently to the various *A. baumannii* strains, with higher levels of the pro-inflammatory IL-12p40 and IL-23 and the anti-inflammatory IL-10 after infection with the less virulent EU clone III strain and the susceptible sporadic isolate as compared to the clone I and II strains **(chapter 4)**. One possible explanation for the association between high levels of IL-10 and low mortality could be that this anti-inflammatory cytokine down-regulates inflammation and its unfavourable effects. In agreement with others, who described a role for IL-12p40 and IL-23 in mice survival after infection with other pathogens [23,24], we found that high levels of these cytokines in *A. baumannii* infected mice were associated with better outcomes. IL-23 is a cytokine that together with IL-1β and IL-6 in mice drives the development of an IL-17-producing T cell population [25], which plays a role in host defense against extracellular pathogens by mediating the recruitment of neutrophils and monocytes to infected tissues. However, it is uncertain whether this cytokine plays a crucial role in host defence against *A. baumannii* as IL-17 depletion did not increase mortality in *A. baumannii* infected mice [17]. Future studies will have to clarify the precise roles of these cytokines in the outcome of *A. baumannii* infection. If these cytokines also influence the impact of *A. baumannii* strains on the human host, these mediators could have predictive values or be targets for treatment.

Mechanisms underlying host innate immune response during *Acinetobacter* infection

Several mechanisms can play a role in the observed inflammatory responses induced by the different *Acinetobacter* strains. The lipid A part of the LPS of *A. baumannii* was suggested to have a high cytokine-inducing capacity [26]. In line with this, it was shown that LPS of *A. baumannii* strain RUH2037 and of *A. nosocomialis* strains induced a pro-inflammatory cytokine response in mice [18] and human cells in vitro [27], respectively. Using three-dimensional human skin equivalents, we demonstrated that *A. baumannii* ATCC19606^T and *A. junii* RUH2228^T both induced a very weak inflammatory response **(chapter 7)**. This observation may be related to the barrier function of the stratum corneum but also by the low expression of TLR4, which serves as a LPS pattern recognition receptor, in keratinocytes, as also reported by others [28]. These studies underline the role of LPS and TLR4 in *Acinetobacter* signaling.

It has been demonstrated for *Pseudomonas aeruginosa* that adaptive and dynamic changes can occur in the chemical composition of lipid A resulting in different potencies to activate the host innate immunity via binding to TLR4 [29]. Unpublished results showed that there are structural differences in the lipid A part of the LPS of several *Acinetobacter* species (personal communication with L. Dijkshoorn). Future studies investigating the host response to the LPS of different *Acinetobacter* strains are necessary to determine to what extent lipid A diversity contributes to the differences in host response among species.

It has been suggested that the coupling of pili to host cell receptors induces inflammation through the production of inflammatory mediators [30]. We found that *A. baumannii* type strain ATCC19606^T and its *csuE*-mutant induced similar levels of inflammatory cytokines IL-6 and IL-8 in bronchial epithelial cells **(chapter 3)**, suggesting that CsuA/BABCDE-mediated pili are not involved in induction of inflammatory responses in human bronchial epithelial cells interacting with this bacterial pathogen.

Membrane vesicles

For several Gram-negative bacterial species outer membrane vesicles (OMVs) were shown to transfer virulence factors into host cells, thereby inducing an immune response [31]. *A. baumannii* ATCC19606^T has been shown to excrete membrane vesicles in vitro that were proposed to play a role in the delivery of virulence factors, including the cytotoxic OmpA, to host cells [32]. These studies used extensive purification methods for the isolation of OMV, including (ultra)-filtration and centrifugation. A disadvantage of this harsh isolation procedure could be the presence of inner membrane and cytoplasmic proteins in OMV resulting from random capture by membrane fragments. Since it is not known whether these OMVs represent the naturally occurring OMVs or are artefacts [33], we examined

the formation and structure of membrane vesicles by *A. baumannii* in different growth phases without the use of extensive purification methods **(chapter 6)**. Our results showed that *A. baumannii* ATCC19606^T forms structurally different MVs depending on its growth phase: (i) small OMVs and (ii) large OMVs, both formed during early growth phases; and (iii) vesicles containing both inner and outer membrane (IOMVs) formed during late stationary growth phases. Stationary phase or dying bacteria appeared to form the most MVs. Moreover, it was found that sub-lethal concentrations of the antibiotic ceftazidime, which interferes with peptidoglycan synthesis [34] and structure, enhanced the formation of OMVs at distal and septal sites of the bacterial cells. It is conceivable that these OMVs carry LPS on their surface. Therefore, the possibility that inadequate dosing of antibiotics, such as penicillins and cephalosporins, has serious adverse effects in patients suffering from *A. baumannii* infection should be considered [35].

Jin et al demonstrated that *A. baumannii* ATCC19606^T secreted MVs during pneumonia in a mouse model of infection [32]. Moreover, it was reported that *A. baumannii* present in alveolar macrophages in a patient with fatal *A. baumannii* pneumonia secreted multiple pleomorphic vesicles [36], suggesting the possibility of secretion of different types of vesicles during in vivo infection. We argue that these differences in structure may have implications for their function. Obviously, future studies are needed to identify the virulence attributes present in the various membrane vesicles formed by *A. baumannii* and the interaction of these vesicles with the host.

Metabolic versatility

In addition to its ability to adhere to surfaces and form a biofilm, the capacity to utilize a wide range of different carbon sources and to replicate at room temperature and up to 44^{o}C [37,38] are likely to contribute to the persistence of *A. baumannii* in the hospital environment and the human host. In this connection, the multicenter study **(chapter 5)** identified a diverse repertoire of core metabolic genes in *A. baumannii*. Moreover, using phenotype microarrays, it was shown that *A. baumannii* ATCC19606^T was able to utilize nitrogen sources more effectively and was more tolerant to pH stress than *A. nosocomialis* (RUH2624), *A. pittii* (SH024) and *A. calcoaceticus* (RUH2202). Interestingly, *A. baumannii* and *A. pittii* were unable to utilize most of the phosphorus sources despite both strains having the necessary genetic composition for phosphate metabolism. These two species were also able to survive in the mouse thigh muscle infection model, in contrast to *A. nosocomialis* and *A. calcoaceticus* **(chapter 5)**, suggesting that there may be a link between phosphate metabolism and virulence. Several studies have highlighted the key role of the Pho regulon in phosphate management, virulence and stress response [39].

Whether the inability of *A. baumannii* and *A. pittii* to utilize phosphorus is linked to expression of the Pho regulon remains a question that needs further evaluation.

The metabolic versatility of *A. baumannii* enables strains of this species to flourish in a variety of niches, from the hospital environment to the febrile patient.

Summary and main conclusions

The study of the pathogenesis of *A. baumannii* is still in its early phase. In the studies presented in this thesis, we compared possible virulence attributes among strains that differ in their behavior in the clinical setting, i.e., MDR *A. baumannii* strains known to be involved in outbreaks of infection, susceptible sporadic *A. baumannii* strains and strains of other, less virulent *Acinetobacter* species. We demonstrated that both *A. baumannii* and less virulent *Acinetobacter* species can adhere to surfaces and form a biofilm, albeit with a wide variation among strains of each species. These results first of all show that a single strain is not representative for the species. Secondly, the presence of many virulence attributes in both clinically relevant and less-relevant strains indicates that the clinical success of *A. baumannii* cannot be explained by these virulence factors alone. Moreover, our results suggest that the outcome of infection depends mainly on the host. In this respect, a specific host innate immune response induced by different *A. baumannii* strains was associated with the outcome of *A. baumannii* pneumonia. Thus, the ability of certain *A. baumannii* strains to induce specific immune responses in susceptible hosts in combination with their metabolic versatility and a MDR phenotype are likely to be important features associated with the clinical success of this pathogen.

To date, the genomes of different *Acinetobacter* strains have been sequenced. In this post-genomic era, studies have shifted towards gene expression and function [40]. Integrating the wealth of information from genomics, transcriptomics, proteomics and metabolomics with bacterial behavior and host responses will provide us more insight into the pathogenesis of *A. baumannii*. The work described in this thesis is a first step to unravel the factors that play a role in the pathogenesis of *A. baumannii*, which is critical in our efforts to develop improved diagnostic and therapeutic strategies against this pathogen.

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